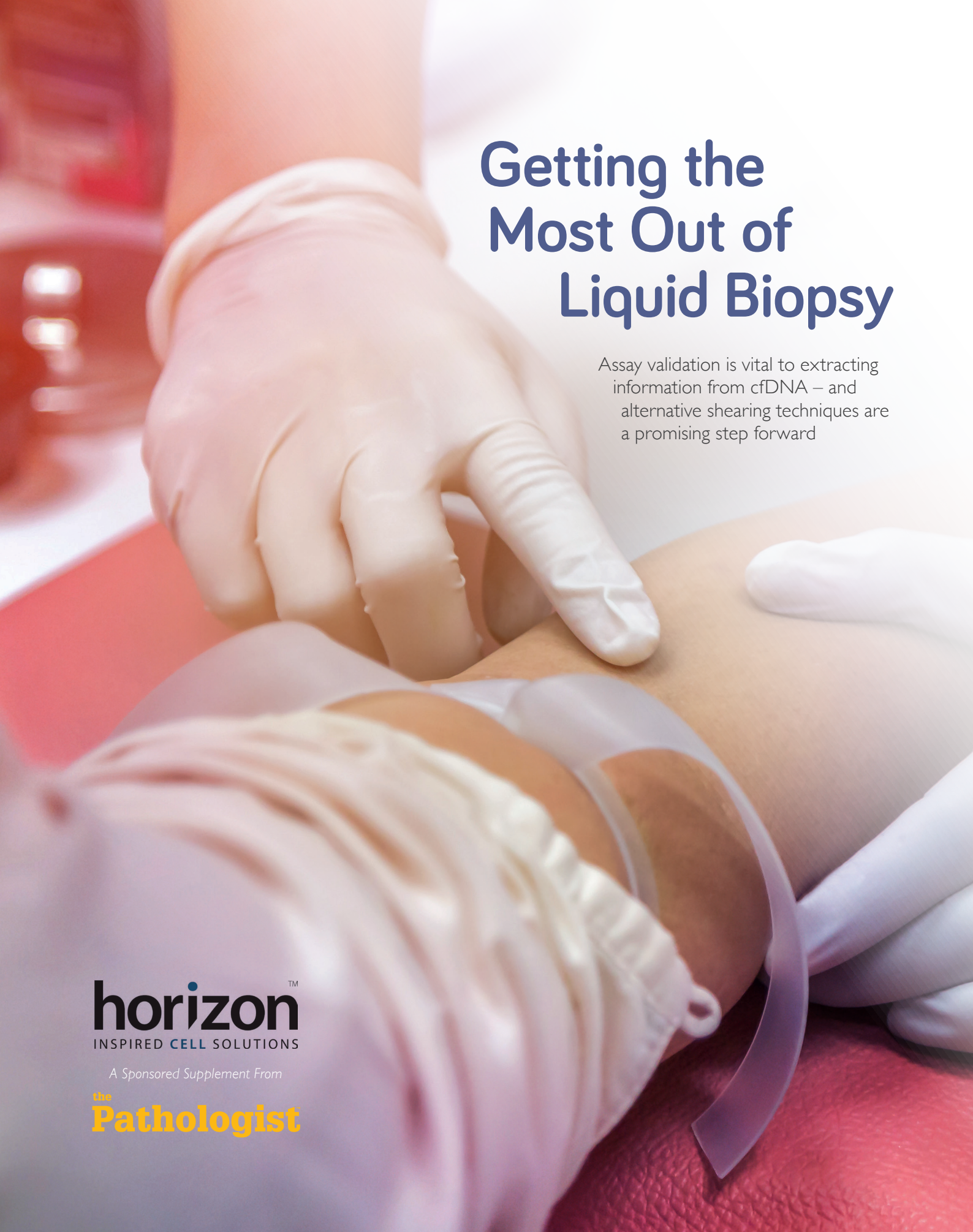

Getting the Most Out of Liquid Biopsy

Assay validation is vital to extracting information from cfDNA – and alternative shearing techniques are a promising step forward

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Building a Better Biopsy

Combating the challenges of liquid biopsy with cfDNA synthetic plasma reference standards

By Lisa M. Wright, PhD

Medical professionals in the cancer sphere are all familiar with solid tumor testing for patients. And although valuable, these procedures are also painful, invasive, and costly in multiple ways. Liquid biopsy – the approach of examining fluid samples, usually blood, for biomarkers – holds many advantages over solid tumor testing. It is less invasive for the patient and has improved levels of sensitivity to detect low-frequency somatic driver mutations. In oncology, pathologists often examine circulating free DNA (cfDNA) for markers that indicate the presence of cancer, its molecular characteristics, and the tumor's susceptibility to treatment.

The industry is pushing to make liquid biopsy the go-to method of collecting clinical DNA samples for oncology genotyping. Liquid biopsies can be taken at the point of diagnosis for routine monitoring during treatment, enabling practitioners to rapidly detect the appearance of resistance mutations that might indicate the need for a change of therapy. One day, liquid biopsy could even be used for preventative cancer screening in the general population. The ultimate goal is to facilitate earlier diagnosis and better treatment outcomes.

As with any new technology, using cfDNA for diagnosis via liquid biopsy has its challenges. Common technical hurdles include:

1. *Sample handling*
   Liquid biopsy workflows involve additional sample handling steps. For example, clinical labs that have been handling robust FFPE blocks for many decades are now faced with processing blood samples, which have shorter shelf lives and require multiple extraction steps. Each step must be properly validated to ensure it does not introduce errors into the final results.
2. *Reliability of results*
   Liquid biopsy assays must operate at much lower limits of detection than previous FFPE-based sequencing. As a result, the technology needs to be rigorously tested to ensure it can accurately call variants down to between 0.1–5 percent allele frequency without calling false positives.
3. *Sample variability*
   Human plasma naturally displays high lot-to-lot variability, making it difficult to control and implement a consistent protocol for your diagnostic assay. Inconsistencies in the clinical blood draw and immediate blood storage process, which can vary between phlebotomists and hospitals, can introduce further sample variation. Controlling for this variation and introducing a consistent protocol is essential for the success of wide-scale liquid biopsy adoption.

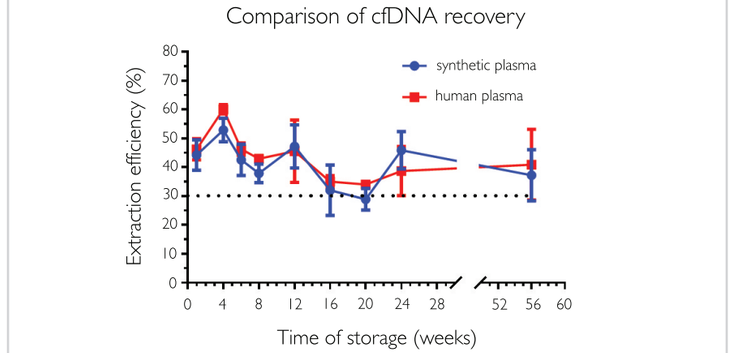
Gene	*Variant*	*Allelic Frequency*			
		5%	1%	0.1%	0% (WT)
EGFR	L858R	5.0	1.0	ND	ND
EGFR	ΔE746-A750	4.9	0.9	ND	ND
EGFR	T790M	4.9	1.1	ND	ND
EGFR	V769-D770ins	5.0	1.0	ND	ND
KRAS	G12D	5.1	1.0	ND	ND
NRAS	Q61K	4.9	0.9	ND	ND
NRAS	A59T	5.2	1.1	0.7	0.7
PIKC3A	E545K	5.0	1.0	ND	ND

Human plasma	*Horizon's synthetic plasma*
Variable quantity and concentrations	Defined volume and concentrations
Lot-to-lot variability	Lot-to-lot stability
Irregular supply	Reliable supply
Contamination with other analytes and/or genomic DNA	No interfering analytes or genomic DNA
cfDNA degradation: time-limited storage	Long-term cfDNA stability: over 24 months

Table 1. Comparing human and synthetic plasma as reference standards for cfDNA assays.

The two big challenges

1. *Limit of detection and false positive error rates*
   A key challenge in using cfDNA to detect cancers early is the extremely low quantities of cfDNA in patients' blood. So how can we be confident in our lower limit of detection and ensure that we're not seeing false positives? The answer: an appropriate reference standard. Using a reference standard with a range of precisely defined allelic frequencies can help determine a true limit of detection and reduce the risk of false positives



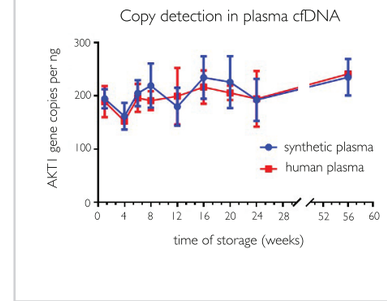


Figure 1. cfDNA recovery (left) and *ALK1* gene copy number deletion (right) in Horizon's synthetic plasma reference standard.

In this example dataset, the reference standard informs the user that i) the reliable limit of detection for this cfDNA assay is 1 percent allelic frequency, and ii) they are calling a false positive for *NRAS* A59T. When you run a reference standard before a patient sample, you can be sure of the limit of detection for your assay. It also allows pipeline optimization; you can recalibrate and amend your workflow to counter any false results, which gives you confidence when handling real patient samples.

2. *The variability and instability of human plasma*
   Using human plasma as a control for your cfDNA assay comes with numerous challenges (see Table 1). Yes, human plasma matches your patient sample behaviors, but this does not always outweigh the challenges that come with using it as a reliable control for diagnosis.

Our approach to testing:

1. 400 ng of cfDNA was spiked into 1 mL of human or synthetic plasma and stored at -80°C.
2. cfDNA was extracted using a Circulating Nucleic Acid kit (Qiagen); extraction efficiency was measured with Qubit BR Reagents (Molecular Probes).
3. Total *AKT1* gene copies were quantified by ddPCR (Biorad).

Take control of your workflow

Having well-characterized cell line-derived reference standards that closely mimic real patient samples, with clinically relevant variants defined by a gold standard mechanism like droplet digital PCR (ddPCR), allows new liquid biopsy assays to be properly validated. Users can:

- check that their workflows accurately detect all of the variants in the control material at the correct allele frequencies without calling false positives
- validate and control for the introduction of errors during the DNA extraction procedure
- ensure that the design of their liquid biopsy sequencing assay functions effectively with no amplicon dropout (liquid biopsy assays need to sequence from smaller fragments of DNA than was previously required in fresh tissue or FFPE assays)

Horizon has developed a range of cell line-derived cfDNA reference standards to help develop, optimize, monitor, and control the accuracy of new patient tests. These materials contain a range of actionable variants in key cancer genes at well-characterized allele frequencies as determined by ddPCR. The variants are located within genomic DNA and have an average fragment size of 160 bp.

Find out more at tp.txp.to/horizon/cfDNA

Our cfDNA material in synthetic plasma helps users to monitor the entire liquid biopsy workflow from DNA extraction to interpretation of results, giving labs confidence in the accuracy of their test.
Find out more at tp.txp.to/synthplasma

What's next?

The ability to examine and support cancer patients using liquid biopsy is hugely exciting. It promises to make genetic analysis more accessible with only a simple blood draw, and it encourages more frequent testing in all aspects of cancer management – pre-disease preventative monitoring, diagnosis, treatment, tumor evolution, resistance management, and long-term remission surveillance and check-up. For both laboratory professionals and the patients they serve, liquid biopsy with appropriate reference standards is the way to a brighter future.

Dr. Wright is Diagnostics Business Unit Leader at Horizon Discovery plc.

Comparison of cfDNA Reference Material Prepared using Enzymatic Fragmentation or Sonication for the Validation of Liquid Biopsy Assays

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Introduction

Liquid biopsies hold great promise to revolutionize the field of clinical oncology testing. Cell-free DNA (cfDNA) can be extracted from a routine patient blood sample and used to determine the genetic profile of a solid tumor located elsewhere within the body. This facilitates more informative disease management for the clinician, without the need for invasive surgery for the patient. With new cfDNA NGS assays being able to detect variants from as little as 2–10 ng DNA, assay validation to ensure sufficient accuracy has never been so critical. Reference materials that closely mimic real cfDNA samples are essential to support this effort. Here, we present results from a comparative study of DNA fragmentation methods applied during the production of cfDNA reference standards. We show a comparison between enzymatic fragmentation and mechanical shearing (sonication), and the benefit of including a size selection step for data accuracy and performance of NGS gene panel workflow.

Methods

DNA extracted from engineered cancer cell lines, representing the Multiplex I blend at 5 percent or 0.1 percent, was fragmented by mechanical or enzymatic shearing. In addition, a size selection step was included to obtain a fragment size distribution profile that closely mimics real cfDNA samples. The allele frequency of specific variants was confirmed by ddPCR. The eight-sample cfDNA material experimental set was externally tested on the Illumina TruSight Tumor15 (TST-15) panel and the Oncomine Breast cfDNA Assay v2 (OBA v2) to assess library preparation and variant calling performance. NGS was performed on the MiSeqDx system in RUO mode and the Ion S5 for the TST-15 and OBA v2 assays respectively. MiSeqDx system filter settings for analysis with Variant Studio (and automatic analysis) were: Read depth >500 and MAF >2 percent.

Results

Tapestation analysis confirmed that both sonication and enzymatic fragmentation produced cfDNA with a peak size in line with real clinical samples (Fig.1). Proof of principle variant detection by ddPCR confirmed the presence of three of the eight mutations across two of four genes (EGFR, KRAS, NRAS and PIK3CA) at either 0.1 percent or 5 percent variant allele frequency (Fig. 2). Library preparation using both Illumina TST-15 gene panel and OBA v2 showed good library yield across all eight samples (Fig. 3). NGS sequencing results showed good and comparable variant calling ability between both sonicated and enzymatically sheared samples (Fig. 4). Although enzymatically sheared cfDNA did not show a significant increase in NGS library yield, it did display slightly more accurate variant calling on the TST-15 assay, in addition to a more defined tapestation profile – centred around 168 bp, which was further enhanced when combined with a size selection step (Fig. 1A yellow trace, Fig 3 and (Fig. 4). In addition, due to a lower limit of detection, the OBA v2 was able to detect many of the variants at 0.1 percent allele frequency (Fig. 4).

TruSight Tumor 15		ng/µL
1	5% sonic. + 31097	27.6
2	5% sonic. + SS _ 31168	49.3
3	5% enzymatic _ 31116	44.7
4	5% enzymatic + SS _ 31119	38.5
5	0.1% sonic. _ 29637	50.5
6	0.1% sonic. + SS _ 31169	49.7
7	0.1% enzymatic _ 31117	42.7
8	0.1% enzymatic + SS _ 31120	57.2
Oncomine Breast cfDNA Assay v2		nM
1	5% sonic. + 31097	11.3
2	5% sonic. + SS _ 31168	6.8
3	5% enzymatic _ 31116	11.5
4	5% enzymatic + SS _ 31119	10.0
5	0.1% sonic. _ 29637	7.7
6	0.1% sonic. + SS _ 31169	7.9
7	0.1% enzymatic _ 31117	10.4
8	0.1% enzymatic + SS _ 31120	12.2

Figure 3: NGS Library Yield

Conclusion

Results show good performance of both sonicated and enzymatically sheared cfDNA material, where all variants present above the LOD could be detected on the Illumina TST-15 assay.

Although enzymatically sheared cfDNA did not show any increase in NGS library yield, it did display slightly more accurate variant calling, in addition to a more defined tapestation profile – centred around 168 bp, which was further enhanced when combined with a size selection step (Fig. 1A yellow trace). This highlights the potential of these alternative techniques to produce cfDNA that is highly commutable to patient samples and suitable for the validation of ddPCR and NGS liquid biopsy assays.

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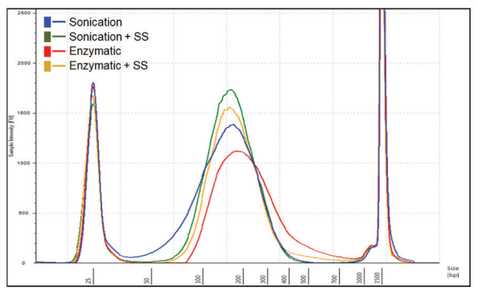
A)



B)

Sample	Peak (bp)	Region [35-950bp] Average Fragment Length (bp)	% of Total
Sonication	178	170	95.08
Sonication + SS	171	180	96.54
Enzymatic	187	243	94.95
Enzymatic + SS	168	196	95.58

Figure 1: Size Distribution
(A) TapeStation analysis of size distribution of all sample conditions (B) Table listing peak size and average fragment length

	Mutation	5% AF samples Expected AF (%)	5% AF samples Acceptance Criteria (%)	5% AF samples Measured AF (%)	0.1% AF samples Expected AF (%)	0.1% AF samples Acceptance Criteria (%)	0.1% AF samples Measured AF (%)
Sonication	EGFR L858R	5	3.5 - 6.5	4.5	0.10	0.05-0.15	0.13
	EGFR V769-D770insASV	5	3.5 - 6.5	4.4	0.10	0.07 - 0.20	0.11
	NRAS Q61K	6.3	4.4 - 8.2	6.3	0.13	0.07-0.20	0.14
Sonication + SS	EGFR L858R	5	3.5 - 6.5	4.6	0.10	0.05-0.15	0.13
	EGFR V769-D770insASV	5	3.5 - 6.5	4.2	0.10	0.07 - 0.20	0.10
	NRAS Q61K	6.3	4.4 - 8.2	6.4	0.13	0.07-0.20	0.12
Enzymatic	EGFR L858R	5	3.5 - 6.5	4.7	0.10	0.05-0.15	0.12
	EGFR V769-D770insASV	5	3.5 - 6.5	4.5	0.10	0.07 - 0.20	0.10
	NRAS Q61K	6.3	4.4 - 8.2	6.6	0.13	0.07-0.20	0.13
Enzymatic + SS	EGFR L858R	5	3.5 - 6.5	4.7	0.10	0.05-0.15	0.13
	EGFR V769-D770insASV	5	3.5 - 6.5	4.3	0.10	0.07 - 0.20	0.10
	NRAS Q61K	6.3	4.4 - 8.2	6.5	0.13	0.07-0.20	0.14

Figure 2: Representative ddPCR QC analysis on three of the eight mutations at either 0.1 percent or 5 percent variant AF

Expected Variant AF (%) (ddPCR)	Gene	Variant	Type	Protein Position	AA Change	Exon	Consequence	Sonication (31097) TST15	Sonication (31097) Oncomine	Sonication + Size Selection (31168) TST15	Sonication + Size Selection (31168) Oncomine	Enzymatic (31116) TST15	Enzymatic (31116) Oncomine	Enzymatic + Size Selection (31119) TST15	Enzymatic + Size Selection (31119) Oncomine
5.0	EGFR	ΔE746 - A750*	deletion	0	KELREA/K	19/28	inframe-deletion	7.1	-	8.2	-	4.7	-	5.5	-
5.0	EGFR	L858R	snv	858	L/R	21/28	missense	4.4	3.9	2.7	4.7	5.9	4.4	6.1	5.2
5.0	EGFR	T790M*	snv	790	T/M	20/28	missense	5.6	-	3.6	-	5.9	-	4.2	-
5.0	EGFR	V769 - D770insASV*	insertion	766	M/MASV	20/28	inframe-insertion	5.9	-	4.0	-	4.3	-	3.8	-
6.3	KRAS	G12D	snv	12	G/D	2/6	missense	7.7	6.5	7.9	6.6	6.5	7.2	5.8	7.0
6.3	NRAS	Q61K*	snv	61	Q/K	3/7	missense	9.2	-	5.2	-	6.5	-	6.8	-
6.3	NRAS	A59T*	snv	59	A/T	3/7	missense	7.0	-	8.3	-	6.2	-	6.9	-
6.3	PIK3CA	E545K	snv	545	E/K	10/21	missense	5.3	6.7	6.3	5.9	6.5	5.6	6.7	7.0

Expected Variant AF (ddPCR)	Gene	Variant	Type	Protein Position	AA Change	Exon	Consequence	Sonication (31097) TST15	Sonication (31097) Oncomine	Sonication + Size Selection (31168) TST15	Sonication + Size Selection (31168) Oncomine	Enzymatic (31116) TST15	Enzymatic (31116) Oncomine	Enzymatic + Size Selection (31119) TST15	Enzymatic + Size Selection (31119) Oncomine
0.1%	EGFR	ΔE746 - A750*	deletion	0	KELREA/K	19/28	inframe-deletion	Not detected (below LOD)	-	Not detected (below LOD)	-	Not detected (below LOD)	-	Not detected (below LOD)	-
	EGFR	L858R	snv	858	L/R	21/28	missense		0.11		-		-		-
	EGFR	T790M*	snv	790	T/M	20/28	missense		-		-		-		-
	EGFR	V769 - D770insASV*	insertion	766	M/MASV	20/28	inframe-insertion		-		-		-		-
	KRAS	G12D	snv	12	G/D	2/6	missense		0.31		0.12		0.12		0.13
	NRAS	Q61K*	snv	61	Q/K	3/7	missense		-		-		-		-
	NRAS	A59T*	snv	59	A/T	3/7	missense		-		-		-		-
	PIK3CA	E545K	snv	545	E/K	10/21	missense		0.19		0.23		0.13		0.17

** Variants not present on Oncomine Breast cfDNA Assay v2*

Figure 4: NGS results from 5 percent and 0.1 percent AF cfDNA test samples run on both the Illumina TST-15 assay and Oncomine Breast cfDNA Assay v2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