TECHNICAL NOTES

Multiplex ctDNA Reference Standards for Oncology Liquid Biopsy and Companion Diagnostics

Indoctrination

Cancer is frequently diagnosed at advanced stages when treatment tends to have limited efficacy. With the advent and clinical application of various sequencing technologies, liquid biopsy was introduced as a novel diagnostic concept in 2010 (Ignatiadis, et al, 2021). In comparison to traditional invasive tissue biopsy or clinical imaging, liquid biopsy offers an alternative option, as a minimally invasive method for cancer molecular profiling and precision medicine.

Liquid biopsy focus the cell-free DNA (cfDNA) and circulating tumour DNA (ctDNA) present in human body fluids, particularly in whole blood or plasma. In conjunction with Next Generation Sequencing (NGS) based assays, the genetic profile and molecular biomarkers of several cancers can be readily obtained (Chen & Zhao, 2019). Furthermore, this high resolution approach is also capable to detect the minimal residual diseases (MRD) following medical treatment or radiotherapy to monitor recurrence (Pantel & Alix, 2019). As the number of cancer biomarkers and related companion diagnostic panels continues to grow, there is also an increasing need for high quality, well-characterised reference standards to contribute to and validate the accuracy of assays. GeneWell has developed a variety of clinically relevant, multiplex ctDNA reference standards, which are derived from cell lines to maximally mimic patient samples. These standards contains genetically identified pathogenic variants and can be employed as quality controls for the whole workflow of assays to contribute to the accuracy, consistency, and reliability of results.

Clinical Background

In 2016, the Food and Drug Administration (FDA) approved the first ctDNA liquid biopsy test to detect epidermal growth factor receptor (EGFR) gene mutations in patients with non-small-cell lung cancer (NSCLC) as a companion diagnostic for molecular targeted drug of EGFR-tyrosine kinase inhibitor (TKI, EGFR-TKI) (FDA, 2024). More recently, multi-gene panel assays of liquid biopsy have been approved as companion diagnostics and have been used in routine clinical settings, mainly correlated with lung and breast cancers.

NSCLC and related biomarkers

Lung cancer is the leading cause of cancer death worldwide, with non-small cell lung cancer (NSCLC) accounting for the majority of cases. It is the most frequent cancer both in terms of incidence as well as with respect to mortality, approximately 1.2 million deaths per year, which is equivalent to 17.8% of the world total (Bertoli, et al, 2023). In the aspect of liquid biopsy, various mutations are identified related with the progress of NSCLC, such as EGFR, KRAS mutations and ALK rearrangement. Besides the role in early diagnosis, these biomarkers can also be used to predict the personal efficiency of specific medicines among individuals. Table1 lists the FDA approved panels and their targeted variants.

Breast Cancer and related biomarkers

Breast cancer (BC) is the most frequently diagnosed malignancy and the leading cause of cancer-related deaths in women worldwide. In 2020, more than 2.2 million new cases were diagnosed and 684 996 deaths were reported globally (Tay &Tan, 2021). Difference with NSCLC, ctDNA can be detected in the early stage of several subtypes of breast cancer, which highlights the advantage of liquid biopsy in early diagnosis. Mutations in TP53, PIK3CA and ERBB2 are identified as crucial biomarkers in both pathogenesis and treatments (Table 1).

Manufactures	Assays/Panels (Indications)	Medicines	Variants
Resolution Bioscience	Agilent Resolution ctDx FIRST assay (NSCLC)	Krazati (adagrasib)	KRAS G12C
Roche Molecular Systems	cobas EGFR Mutation Test v2 (NSCLC)	Iressa (gefitinib)	EGFR Exon19del, L858R, T790M
Foundation Medicine	FoundationOne Liquid CDx (NSCLC)	Iressa (gefitinib)	EGFR Exon19del, L858R, T790M
		BRAFTOVI (encorafenib)	BRAF V600E
		Tabrecta (capmatinib)	MET exon 14 skipping
		Alecensa (alectinib)	ALK rearrangements
		Rozlytrek (entrectinib)	ROS1 fusions
Guardant Health	Guardant360 CDx (NSCLC)	Tagrisso (osimertinib)	EGFR exon 19 deletions, EGFR exon 21 L858R, and T790M
		Rybrevant (amivantamb)	EGFR exon 20 insertions
		Lumakras (sotorasib)	KRAS G12C
		ENHERTU (fam-trastuzumab deruxtecan-nxki)	ERBB2 Activating Mutations (SNVs And Exon 20 Insertions)
Guardant Health	Guardant360 CDx (Breast Cancer)	Orserdu (elacestrant)	ESR1 missense mutations between codons 310 and 547
Qiagen Manchester, Ltd.	Therascreen PIK3CA RGQ PCR Kit (Breast Cancer)	Piqray (alpelisib)	PIK3CA C420R, E542K, E545A, E545D [1635G>T only], E545G, E545K, Q546E, Q546R, H1047L, H1047R, and H1047Y
Foundation Medicine	FoundationOne Liquid CDx (Breast Cancer)	Piqray (alpelisib)	

Table1 FDA approved liquid biopsy assays for NSCLC and breast cancer companion diagnostics.

Product Overview

In light of the aforementioned research, GeneWell has developed multiplex ctDNA reference standards encompassing single nucleotide variants (SNV), insertion, deletion, fusion, and copy number variants (CNV) among EGFR, KRAS, NRAS, PIK3CA, ROS1, ALK, MET and ERBB2 genes (Table 2). These variants have been demonstrated to be pathogenic or clinically relevant to NSCLC and breast cancer.

These standards are all cell line derived and fragmented to a length of approximately 160bp to maximally mimic clinical samples. The diagram in Figure 1 illustrates the consistency in fragment distribution between real patient sample and the GeneWell reference standards. In Onco Structural Multiplex 5% ctDNA Reference Standard, the allelic frequencies are uniformly set at 5% for the purpose of performance validation and limit of detection (LoD) determination. The inclusion of a variety of mutation types renders it an optimal reference standard to evaluate the

performance of detecting low AF variants in multiplex NGS panels. Moreover, the AFs for the variants in Onco SNV Multiplex ctDNA Reference Standard Set are also designed to be 0.1% and 1%, in accordance with the major LoD of commercial MRD NGS panels. Together with the genetically matched wild-type (0%) standard, the set can be used for the verification of detection accuracy in an ultra-low AF level to avoid false negative results. In addition, GeneWell has also developed the Onco MRD ctDNA reference standard with a gradient AF from 0.005% to 0.5%, which will better contribute to the development and routine quality control of MRD panels.

Product Code	Product Name		Specifications
IB-GW-OCTM001	Onco Structural Multiplex 5% ctDNA Reference Standard		20ng/μl, 0.5μg/tube
IB-GW-OCTM005	Onco Wildtype ctDNA Reference Standard		20ng/μl, 0.5μg/tube
IB-GW-OCTM009	Onco SNV Multiplex ctDNA Reference Standard Set		20ng/μl, 0.5μg/tube*3
Variants	**Mutation Type**	**Expected AFs**	
		IB-GW-OCTM001	IB-GW-OCTM009	IB-GW-OCTM005
EGFR G719S	SNV	//	0%	//
PIK3CA H1047R	SNV	//	0%	//
KRAS G13D	SNV	//	0%	//
BRAF V600E	SNV	//	0%	//
AKT1 E17K	SNV	5%	0%	//
ROS1 CD74(6)-ROS1(34)	Fusion	5%	0%	//
ALK EML4(6)-ALK(20)	Fusion	5%	0%	//
MET Amplification	CNV	3.5 copies	2 copies	//
ERBB2 Amplification	CNV	7.0 copies	2 copies	//
PIK3CA E545K	SNV	5%	0%	0%, 0.1%, 1%
EGFR E746_A750del	Deletion	5%	0%	0%, 0.1%, 1%
EGFR A767_V769dup	Insertion	5%	0%	0%, 0.1%, 1%
EGFR L858R	SNV	//	0%	0%, 0.1%, 1%
EGFR T790M	SNV	//	0%	0%, 0.1%, 1%
KRAS G12D	SNV	//	0%	0%, 0.1%, 1%
KRAS A146T	SNV	//	0%	0%, 0.1%, 1%
NRAS Q61K	SNV	//	0%	0%, 0.1%, 1%

Table 2 Variants list of GeneWell ctDNA reference standards and corresponding allelic frequencies.

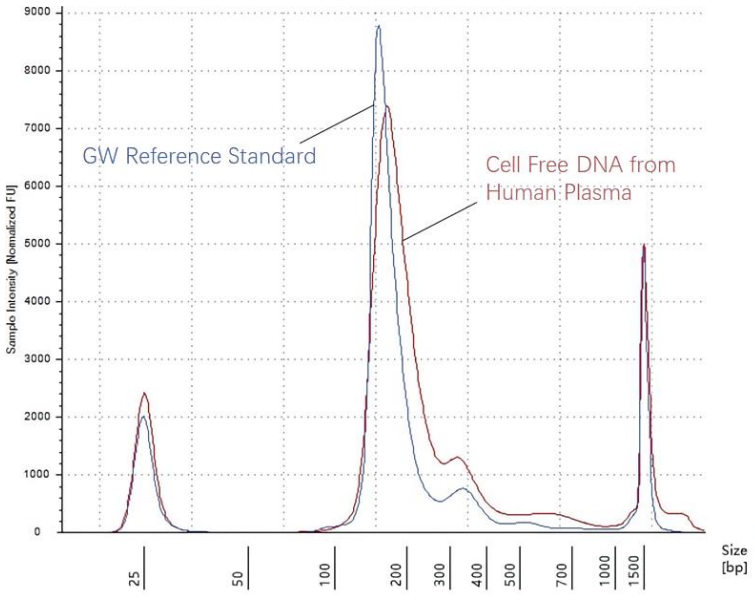

Figure 1 Comparison in fragment size between real human plasma and the GeneWell ctDNA Reference Standard.

The red lines stands for cell free DNA (cfDNA) extracted from human plasma, and the blue lines stands for GeneWell ctDNA reference standards. The two peak at the left and right of the diagram are the lowest and highest ladder. The overlapping peaks in the middle indicates the similarity between two samples.

Production validation

Immortalised cell lines were selected according to the somatic pathogenic mutations. The genetic background of those cell lines are verified by Whole Genome Sequencing(WGS) or Whole Exome Sequencing (WES) to ensure a consistent background of our standards. Relevant mutations are then confirmed by both Sanger Sequencing and digital droplet PCR (ddPCR, Roche). The reference standards underwent validation at distinct stages during the development and manufacturing process as per ISO9001 to ensure the accuracy, consistency and traceability of GeneWell ctDNA Reference Standards.

Genomic DNA were extracted from cell lines and blended in defined ratio, then fragmented to specific length following our unique protocols. The reference standards are precisely verified for their concentration, fragment size, and allelic frequencies in our internal validation process. The Figure 2 shows a example result of ddPCR and Agilent TapeStation D1000.

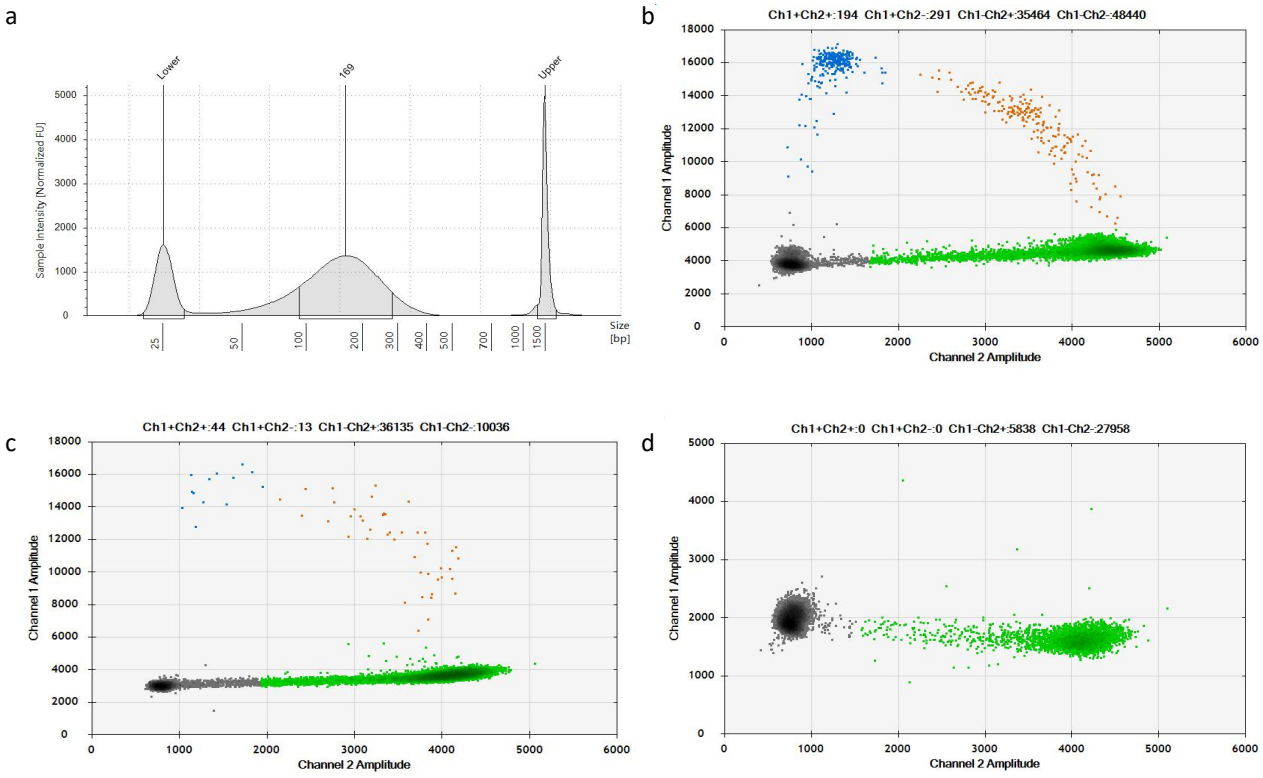

Figure2 The example results of internal validation of Genewell Onco SNV Multiplex ctDNA Reference Standard Set.
a) ctDNA analysis for size distribution by TapeStation D1000 system, represents batches of 1% allelic frequencies standards in the set of IB-GW-OCTM009 within the acceptance criteria for an average size of approximately 160bp. b,c,d) Allelic frequencies of claimed variants assessed by quad-ruplicate Droplet Digital PCR analysis using specific probes on the BioRad QX200 ddPCR platform. Using NRAS Q61K as an exmaple, the scatterplots above indicate clear classifications on all allelic frequency gradients (1%-b, 0.1%-c, WT-d) and the ddPCR assays on ctDNA standards confirmed all the claimed variants are at expected allelic frequencies with high reproducibility.

Applications

GeneWell ctDNA reference standards can be treated as clinical samples and incorporated into the assay process from its inception, in conjunction with testing samples. These standards are able to measure the efficiency of extraction process, verifying library quality, and assisting with the validation of bioinformatics analysis procedure. Moreover, as reference materials for routine QC, these standards can also be run on specific platforms periodically to ensure consistent performance, or on different platforms for parallel comparison. With regards to the assay development, these standards can facilitate the determination of LoD and assist with the registration process as external quality materials.

Summary

In this application note, we show how we have developed and validated the GeneWell ctDNA Reference Standards as a valuable control for achieving confidence at all the stages of development and validation of liquid biopsy assays. These reference standard can be used to validate and control the detection assays of genetic variants, and recommend to be used as an routine quality control material to bring reliability and consistency to the assays.

References

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