

Allelic Frequency Measurement of Multiplex I cfDNA Reference Standard Set using Droplet Digital™ PCR, Ion Torrent™ and MiSeq™.

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Introduction

A major clinical goal, especially for oncology, is to achieve non-invasive disease assessment and progression monitoring. The analysis of circulating, cell free DNA (cfDNA) is currently the most promising technical advances to provide an alternative to tumor biopsy. Translating the application of cfDNA detection into the clinic requires stringent procedures to enable both accurate and precise measurements. Meanwhile cfDNA detection methodologies face new challenges due to the low quantity of highly-fragmented material available from biofluids. Furthermore, analysis of cfDNA is often intended to identify very low allele frequency mutations, including 0.1% and lower, that push the detection limits of current technologies. Until now, the availability of reference standards to assess cfDNA analytical workflows has broadly been limited to variable clinical specimens.

In response to this need, Horizon has developed the cfDNA Reference Standard range to support the innovation and standardization of this valuable technology. Derived from engineered human cell lines, the reference standards are provided as fragmented human genomic DNA (average size 160 bp) resembling cfDNA derived from human plasma (see Figure 1). The reference standards are provided at allelic frequencies down to 0.1% across a range of variants with a matched wild type. Copy number concentrations and expected allelic frequencies of each product are provided to allow establishment of standard curves. These reference standards will help to drive forward the development, validation and demonstration of the performance of cfDNA assays and platforms on a routine basis.

The aim of this study is to demonstrate the utility of Horizon's Reference Standard across a range of representative analytical platforms. This study was performed in collaboration with Horizon's commercial and academic international partners using Multiplex I cfDNA Reference Standard Set to challenge the limit of detection (LOD) of the participating partners' workflows.

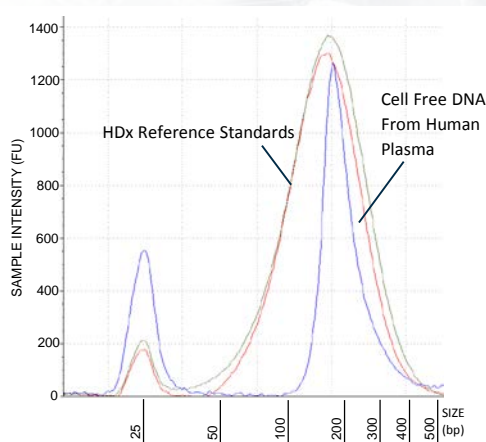


Figure 1. Example trace of the fragment sizes collected by D1000 DNA ScreenTape assay, comparing cfDNA HDx Reference Standards (Red and Green traces) to cfDNA extracted from human plasma (Blue trace). cfDNA from human plasma was provided by CareDx, Inc. Leftmost peaks - internal marker for the assay. Rightmost peaks - fragmented materials.

Method

The Droplet Digital PCR characterization was performed in house (Horizon, QX100™ ddPCR™ platform), and NGS experiments were performed by Horizon's partners following standard protocol and workflows established in the respective partners. The concentration of each Multiplex I cfDNA Reference Standard was determined using the respective partners' preferred DNA concentration measurement method prior start of any assay analysis. For the purpose of this study, LOD is defined as the lowest variant allelic frequency that can be called on a given workflow.

Multiplex I cfDNA Reference Standard Set (Catalogue #: HD780) was sent to Horizon's participating partners. For the components of the set, see Table 1. The expected allelic frequencies and list of variants in the cfDNA reference standard are summarised in Table 2.

Table 3 summarises the assay panels, assay designs, platforms, and reference standard assessed in participating laboratories.

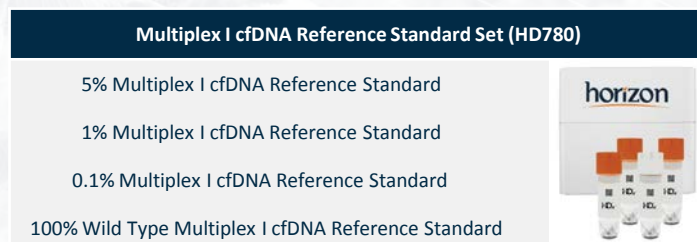


Table 1. List of products in Multiplex I cfDNA Reference Standard Set.

Gene	Variant	Expected Allelic Frequency (AF%)			
		5% Multiplex I	1% Multiplex I	0.1% Multiplex I	100% WT Multiplex I
EGFR	L858R	5.0	1.0	0.10	0.00
EGFR	ΔE746 - A750	5.0	1.0	0.10	0.00
EGFR	T790M	5.0	1.0	0.10	0.00
EGFR	V769 - D770insASV	5.0	1.0	0.10	0.00
KRAS	G12D	6.3	1.3	0.13	0.00
NRAS	Q61K	6.3	1.3	0.13	0.00
NRAS	A59T	6.3	1.3	0.13	0.00
PIK3CA	E545K	6.3	1.3	0.13	0.00

Table 2. Expected allelic frequency of 5%, 1%, 0.1% and 100% Wild Type Multiplex I cfDNA Reference Standards called by Droplet Digital PCR at Horizon. Please refer to Multiplex I cfDNA Reference Standard Product Insert (HD780) for more information. This version contains an amendment to the AF% for EGFR V769 - D770insASV.

* For Research Use Only. Not for Use in Diagnostic Procedures.

	Droplet Digital PCR	Ion Torrent	MiSeq
Assay Panel	Horizon's custom probe	Ion AmpliSeq™ Colon and Lung Cancer Panel v2	Accel-Amplicon™ 56G Oncology Panel
DNA Input/Assay	100ng	10ng	10ng
Average Coverage	NA	8000x	5000x
5% Multiplex I	+	+	+
1% Multiplex I	+	+	+
0.1% Multiplex I	+	+	-
100% WT Multiplex I	+	+	+

Table 3. Multiplex I cfDNA Reference Standards assessed by various platforms using respective assay panel and design. Note: + sign represent product was tested, - sign represents product was not tested.

Results

The expected allelic frequency of all listed variants in the 5% and 1% Multiplex I cfDNA Reference Standard were called within reasonable precision by Droplet Digital PCR, Ion Torrent and MiSeq platforms (see Figure 2A and 2B).

The listed variants in the 0.1% Multiplex I cfDNA Reference Standard were assessed on the Droplet Digital PCR and Ion Torrent platforms only (see Figure 2C). The expected allelic frequency of EGFR L858R, EGFR V769 - D770insASV and NRAS Q61K variants were called, whereas other variants were only able to be detected on Droplet Digital PCR. Examining the calls from Ion Torrent, the expected allelic frequency of the variants were not called. The partner providing data on the MiSeq platform opted out of testing the 0.1% Multiplex I cfDNA Reference Standard due to experience with the LOD of their workflow.

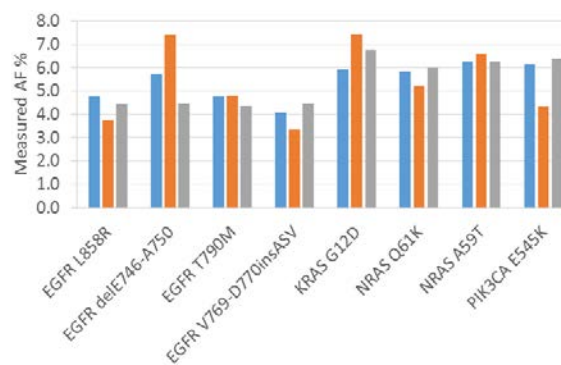
Low levels of assay background were observed in the 100% Wild Type Multiplex I cfDNA Reference Standard on Droplet Digital PCR and Ion Torrent platforms (see Figure 2D). A key factor in determining assay performance is the LOD. By using the 100% Wild Type Multiplex I cfDNA Reference Standard the LOD was established with confidence. In this study, low assay background of up to 0.02% allelic frequency was observed for all variants except EGFR T790M (0.07%) on Droplet Digital PCR by Horizon. To support this, no single positive droplets were observed, demonstrating that the 100% Wild Type Multiplex I cfDNA Reference Standard is truly wild type for the assessed variants. Examining the data from the MiSeq platform, the partner's workflow did not generate a comparable assay background (see Figure 2D). The combination of these observations demonstrate that the 100% Wild Type Multiplex I cfDNA Reference Standard has strong utility in applications such as the assessment of assay sensitivity and specificity of cfDNA assays and platforms.

Summary/Conclusion

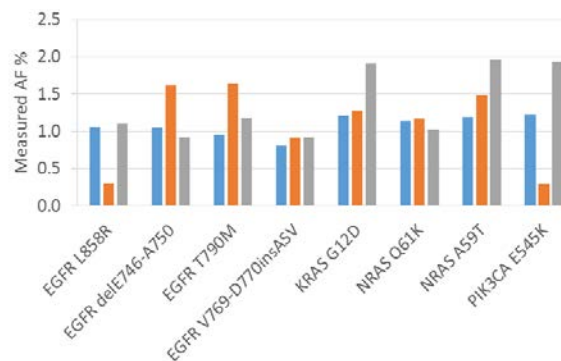
This study demonstrates the utility of Horizon's renewable, quantitative and genetically defined Reference Standards in the assessment of cfDNA workflows. Applications include assay development, optimization, and validation of workflows including those involving amplicon-based assays when used on platforms such as the Ion Torrent and MiSeq. Further studies on Horizon's cfDNA Reference Standards are taking place and data will be published.

Horizon is proud to provide cfDNA Reference Standards to drive forward the development and standardization of non-invasive oncology disease assessment and progression monitoring workflows.

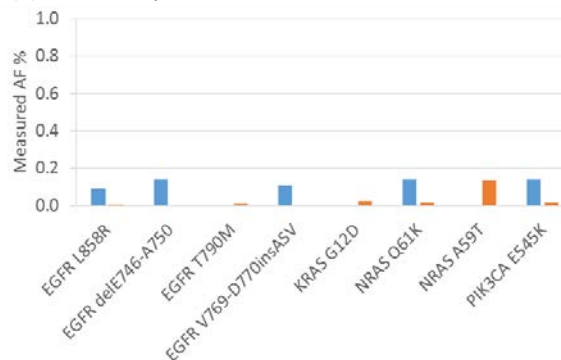
(A) 5% Multiplex I cfDNA Reference Standard



(B) 1% Multiplex I cfDNA Reference Standard



(C) 0.1% Multiplex I cfDNA Reference Standard



(D) 100% WT Multiplex I cfDNA Reference Standard

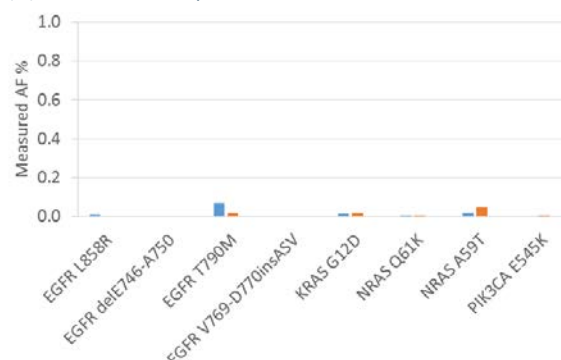


Figure 2. A-D. Measured allelic frequencies (AF%) of 8 variants present in Multiplex I cfDNA Reference Standard. (A) 5%, (B) 1% and (D) 100% WT Multiplex I cfDNA Reference Standard were assessed using Droplet Digital PCR (blue), Ion Torrent (orange) and MiSeq (grey). (C) 0.1% Multiplex I cfDNA Reference Standard was measured using Droplet Digital PCR (blue) and Ion Torrent (orange).

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