

# Characterisation of a novel, patient-like cfDNA reference material for molecular diagnostic liquid biopsy assays

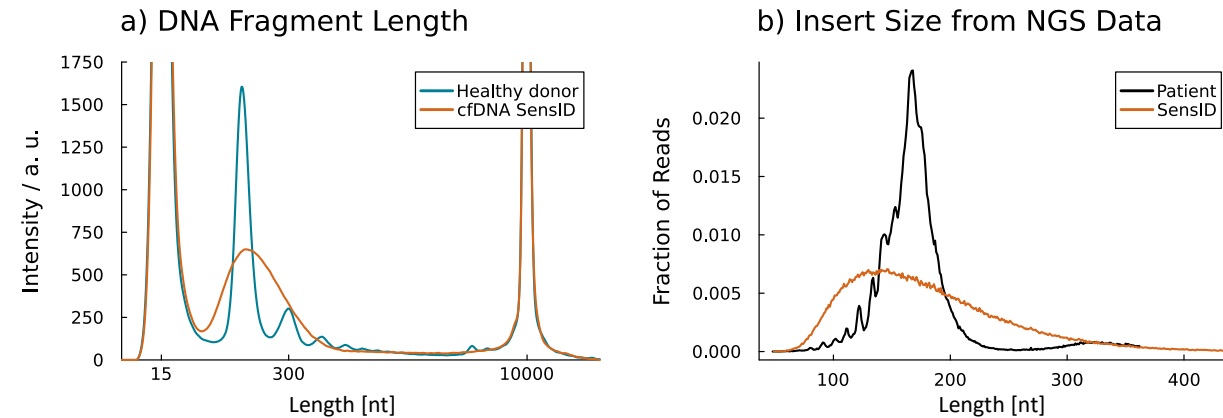
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## Background and Introduction

Molecular diagnostic analysis of cfDNA purified after liquid biopsy is rapidly gaining importance and acceptance for clinical diagnostics. A wide variety of pre-analytical and analytical methods are being combined resulting in different lab-specific workflows. However, seemingly simple differences e.g. in choice of sample collection tubes can lead to strong workflow-specific differences in limits of detection for one and the same assay (1, 2). The need for precise and reliable detection of actionable molecular alterations from cfDNA for precision medicine is therefore leading to an increased need in reference materials (RM) e.g. for ring trials to enable interlaboratory comparison (3, 4). A common way for generation of cfDNA RM is the combination of randomly sheared genomic DNA with spike-in variants in adjustable allele frequencies for molecular alterations of interest. This enables reproducible and scalable manufacturing of RM with a fragment size peak in the range of 160 – 170 bp similar to „natural“ cfDNA (Fig 1).

## Objective

Both users and important regulatory bodies such as the Food and Drug Administration of the USA (FDA) are asking for truly patient-like material e.g. derived from cell cultures if contrived samples with rare molecular markers are required in clinical studies (5). In response to such demands a novel approach was developed to produce „nucleosomal cfDNA“ (n-cfDNA) from cell lines.



**Fig 1: Fragment size profile of sheared cfDNA reference material**  
Size distribution of reference material as used e.g. in (2) and (4) analysed by different methods.

- Size profile as determined using the Agilent TapeStation. For comparison, the size distribution of natural cfDNA from a healthy donor is shown in blue.
- Size profile as determined by NGS duplex sequencing as described in (7). The size distribution of a patient cfDNA is shown in black.

## Material and Methods

A proprietary method was developed for standardised fragmentation of chromosomal DNA based on nucleosomal protection. The method was developed in an attempt to mimick the commonly accepted way of in-vivo generation of natural cfDNA (6). Cell culture, lysis & purification, fragmentation of gDNA, spike-ins are based on proprietary and/or patent-pending procedures. Manufacturing processes are according to DIN EN ISO13485. Functional testing of RM was performed by ddPCR on the Bio-Rad QX200™ system. NGS and data analysis for duplex sequencing of the sheared cfDNA RM is described in (7).



## Results & Discussion

The size profile of the nucleosomal cfDNA resulting from this process is indistinguishable from that of natural cfDNA from a self-declared healthy donor (Fig 3 a). The process can also be used for non-lymphoblastoid cell lines such as cancer cell lines of choice (Fig 3 b) and such n-cfDNA populations can be mixed to create patient-like blends of n-cfDNA populations. The wildtype cell line is healthy-donor lymphocyte-derived and can therefore be considered to be a good equivalent to natural occurring, healthy, wildtype background cfDNA, which originates mostly from blood-cells (6).

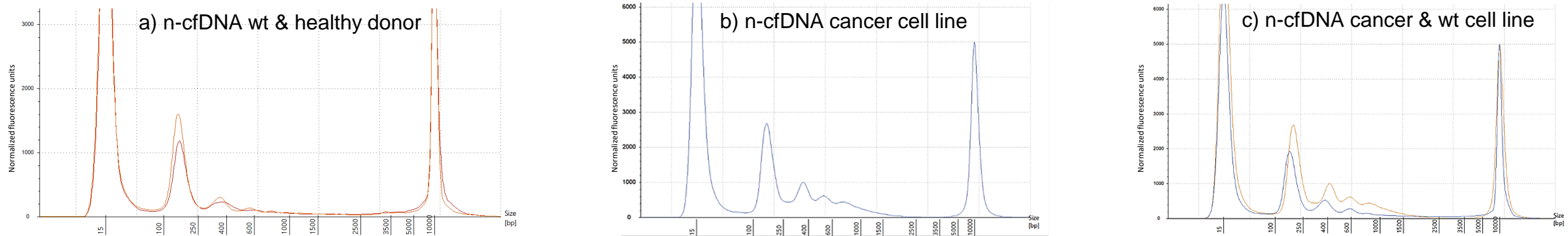
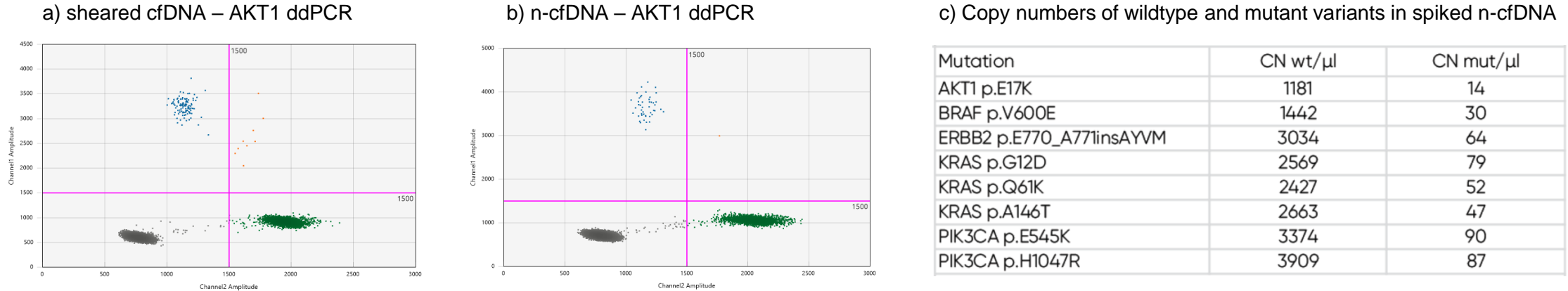


Fig 3: **Fragment size profiles of novel nucleosomal cfDNA**, analysis by Agilent TapeStation

- a) n-cfDNA derived from a wildtype, lymphoblastoid cell line (BDXXP4), for comparison, native cfDNA from a healthy donor is shown in red
- b) n-cfDNA derived from a cancer cell line harboring multiple mutations (SID-CL006)
- c) n-cfDNA from wt (yellow) and cancer cell line (blue); note the difference in the fragment size peak, showing the smaller peak position to mimick circulating tumor DNA (ctDNA)

## Results & Discussion

The functional comparability of the randomly sheared and nucleosomal cfDNA was tested. The n-cfDNA of BDXXP4 was used as full genomic wildtype background to spike in synthetic dsDNA sequences covering various different actionable mutations. For this purpose the mutation panel of the well-established „5-Gene-Multiplex“ standard reference material was used (2). This 5-Gene-Multiplex n-cfDNA was analysed by ddPCR using the Bio-Rad QX200™ System (Fig 4). The n-cfDNA can be used for manufacturing of reference material in the same way as the previously established 5-Gene-Multiplex standard reference material based on sheared cfDNA. Various different lots of wt n-cfDNA were also tested for compatibility with NGS workflows (including methylation analysis) and resulted in good library and NGS data generation (confidential personal communications, data not shown).



**Fig 4: Compatibility of n-cfDNA with ddPCR and synthetic spike-in sequences**

Standard sheared cfDNA and n-cfDNA were spiked with a standard mixture of AKT1/BRAF/ERBB2/KRAS and PIK3CA variants to achieve 1 - 3 % AF. Aliquots were tested by ddPCR using the Bio-Rad QX200™ System.

- AKT1 ddPCR results of sheared cfDNA spiked with a standard 5-Gene-Multiplex panel (2)
- AKT1 ddPCR results of n-cfDNA spiked with the 5-Gene-Multiplex panel
- Copy numbers (CN) of wildtype (wt) and multiple different spiked mutant alleles (mut) in n-cfDNA as determined by ddPCR

## Conclusions

- A process has been developed that enables manufacturing of cfDNA with a nucleosomal fragmentation profile for use as reference material:
- The novel, patient-like nucleosomal cfDNA is a very close mimick to and virtually indistinguishable from healthy donor-derived cfDNA
- It is compatible with ddPCR- and NGS-based assays.
- The novel n-cfDNA RM may increase acceptance of regulatory bodies such as the FDA and other stakeholders for use as contrived samples e.g. in clinical studies and for analytical performance evaluation.

## Acknowledgements

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### References:

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