Impact Of Reference Material On Limit Of Detection In Analytical Performance Evaluation Of Liquid Biopsy NGS Assays

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INTRODUCTION

Molecular diagnostic analysis of cfDNA from liquid biopsy by Next Generation Sequencing (NGS) is rapidly gaining importance and acceptance for clinical diagnostics. Different NGS approaches are used in different workflows in the ongoing quest to achieve the highest possible sensitivity. The ultimate goal is the highly sensitive identification of clinically actionable sequence variants to provide crucial guidance for therapy e. g. to support early treatment decisions in recurring cancer after initial therapy. Since there are no validated tests commercially available for many clinical applications, laboratories are forced to develop their own NGS assays as laboratory developed tests (LDTs).

NGS allows to analyze relatively large genomic regions compared to other methods. This presents a challenge in the analytical performance evaluation which is required e. g. by the In-Vitro Diagnostic Regulation (EU) 2017/746 (IVDR) Art. 5 even for LDTs. It is crucial to demonstrate that meaningful signals can be reliably differentiated from background noise.

Suitable reference materials are an indispensable tool for such NGS assay validation, in particular with respect to the accurate determination of the limit of blank (LoB) and the limit of detection (LoD). Here we present data on the impact of the quality of reference material for optimizing LoB and LoD in an NGS duplex sequencing assay.

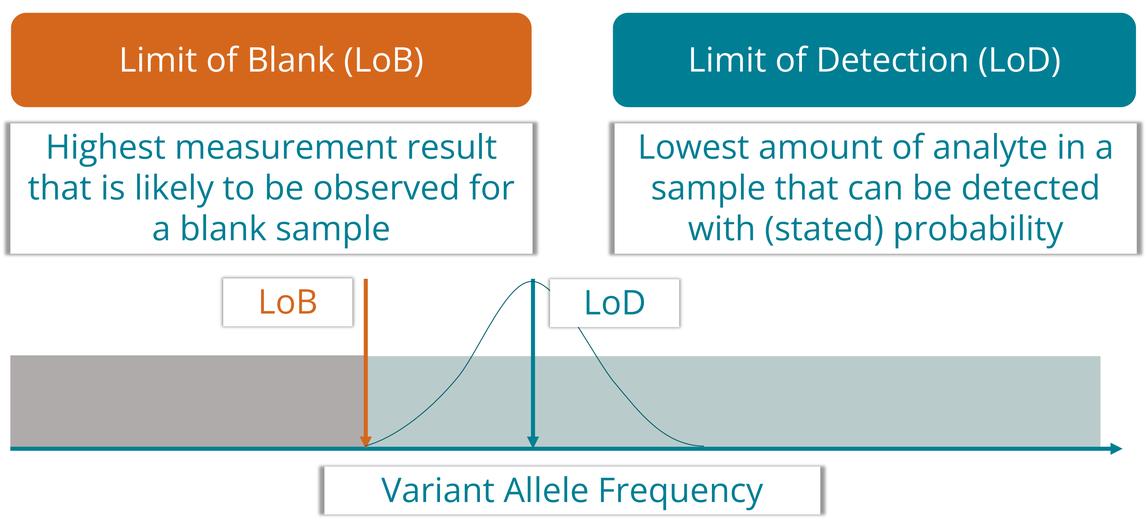


Fig. 1: Schematic display of LoB and LoD. The measurement of the variant allele frequency (VAF) of a genomic variant is always error-prone and may deviate from the expected value. The LoB represents a threshold below which all results are considered false-positive.

Since the LoD is always higher than the LoB, only an accurate measurement of the LoB allows to systematically explore the lowest possible LoD. In addition, there is no orthogonal method available at reasonable cost to confirm NGS liquid biopsy assay results. This means that the LoB must be determined in such a way that the precision of the assay is close to 100%.

METHODS

The target region of the NGS panel with duplex unique molecular barcodes (UMI) comprises the coding region +/- 15 bp at exon-intron borders of 9 complete genes and hotspot regions of 10 additional genes associated with cancer. Library preparation of cfDNA samples was performed using IDT´s xGen™ cfDNA & FFPE DNA Library Preparation Kit. Paired-end sequencing with 2x151 bp reads was performed on a NovaSeq 6000 aiming a sequencing depth of 50.000x.

The NGS data analysis was performed as described in [2]. Raw data from the sequencing instrument was uploaded to the varvis® platform and aligned against the hg38 reference genome followed by variant calling using the varvis® bioinformatics pipeline with the manufacturer's validated settings. A minimum of two reads were used to construct a strand-specific consensus read. Strand-specific consensus reads were then combined to create a final consensus read. An average depth of coverage (consensus reads) of more than 1000x was achieved.

In the case of reference materials, the resulting variant calls are compared to the expected variants as specified by the manufacturer and published data (GiaB 4.2.1).

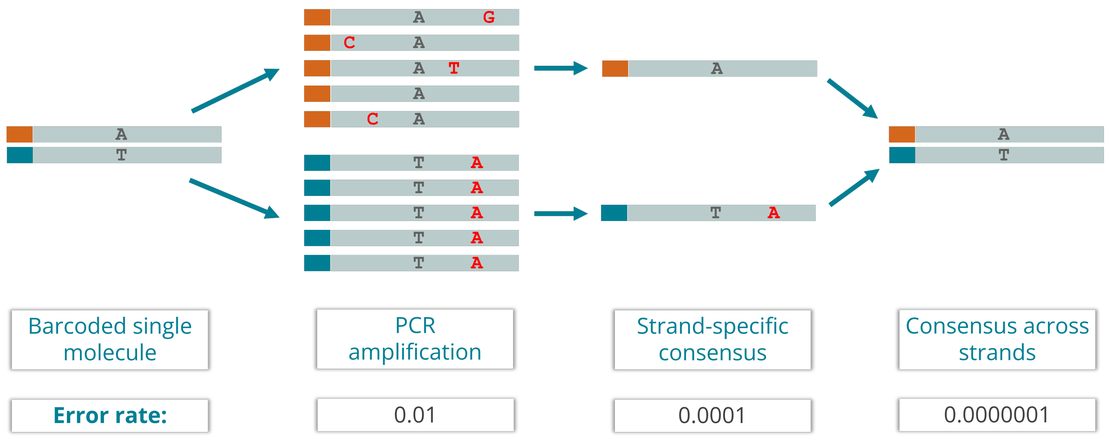


Fig 2: Principle of analysis of duplex sequencing barcodes

Manufacturing of cfDNA reference material at SensID is performed by purifying genomic DNA from lymphoblastoid cell lines. Lysis & purification, shearing of genomic DNA as well as design & manufacturing of spike-in sequences are based on proprietary and patent-pending DIN EN ISO 13485 compliant standard operation procedures of SensID.

SensID reference material, patient-derived cfDNA as well as cfDNA reference material from another manufacturer was sequenced using the same protocol and achieving equivalent quality metrics (> 1000x depth of consensus coverage).

RESULTS

The use of sheared genomic DNA as cfDNA reference material enables reproducible and scalable manufacturing of standard and customized reference material with a fragment size peak in the range of 160 – 170 bp similar to „natural" cfDNA [3, 4]. The size profile based on read length of sequences generated by NGS provides a good match to patient-derived cfDNA (Fig 3).

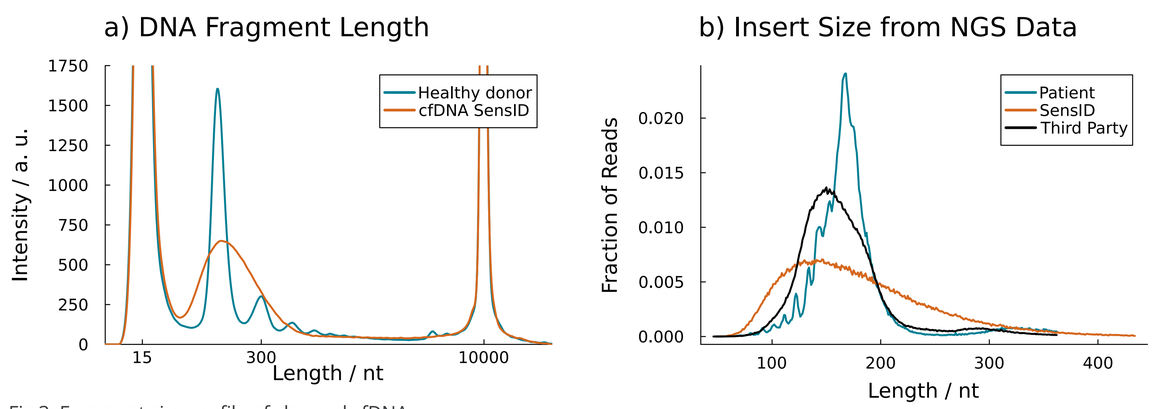


Fig 3: Fragment size profile of sheared cfDNA
a) Typical size distribution of SensID sheared cfDNA reference material as analyzed by Agilent TapeStation (red). For comparison, the size distribution of natural cfDNA from a healthy donor is shown in blue.
b) Size distribution of SensID sheared cfDNA RM (red), patient-derived cfDNA (blue) and a third-party reference material determined from NGS data as described in [2].

Variant calling by varvis® shows large differences in the number of false-positive variant calls in the third-party reference material compared to SensID material (Fig 4). False positive variants in this context are variants that are not expected according to manufacturer's information. In the allele frequency range below 0.5% VAF, in particular, the noise detected in the third-party's material rises steeply, even overlapping spike-in variants at 0.5% VAF.

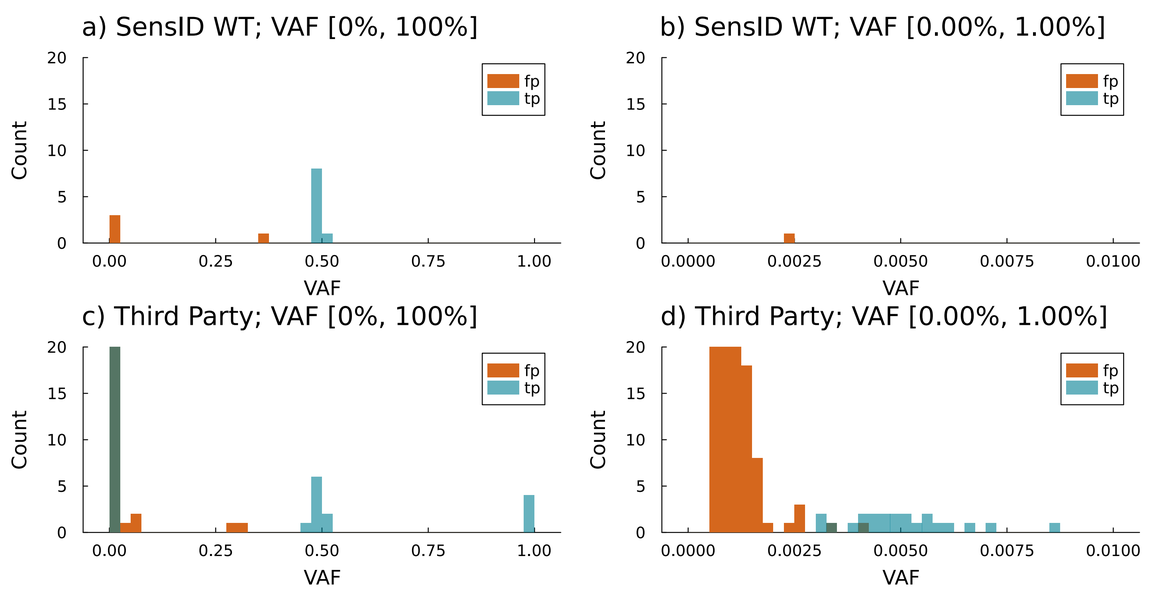
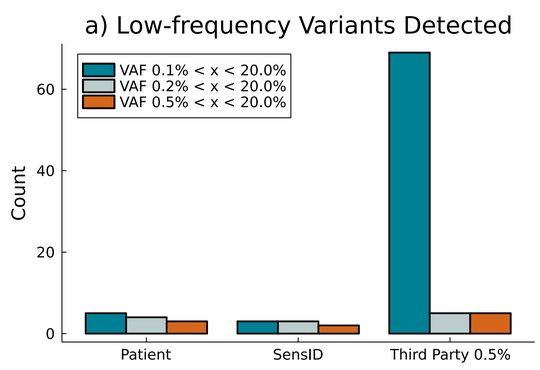


Fig 4: Low-frequency variants detected in SensID and a third-party's reference material. a) SensID wild-type (WT) material, VAF range [0%, 100%], shows the expected germline background and few false-positive variants in regions that are difficult to analyze with NGS (repetitive regions). b) SensID WT material with x-axis zoomed to VAF range [0.00%, 1.00]. c) Third-party's reference material with 0.5% spike-in variants, VAF range [0%, 100%]. In addition to the expected germline background, a large number of variants is detected with VAF less than 0.5%. d) Third-party reference material with 0.5% spike-in with x-axis zoomed to VAF range [0.00%, 1.00].



b)

VAF Interval	Third Party	SensID	Patient
0.1% < x < 20.0%	120	3	5
0.2% < x < 20.0%	3	3	4
0.5% < x < 20.0%	3	2	3

Fig 5: Total number of low-frequency variants detected in various VAF intervals relevant for performance evaluation. a) Comparison of patient-derived material with SensID and third-party. b) Tabular representation of the data shown in a).

SensID reference material provides a low level of noise while third-party's material contains more than 100 variants in the VAF range between 0.1% and 20.0%.

CONCLUSION

Sheared genomic DNA with spike-in sequences is suitable as reference material for duplex sequencing-based NGS methods. The accurate determination of the LoB, however, requires reference material that exhibits a very low level of noise in the low-frequency range. This comparison of different reference materials and patient-derived cfDNA shows that not all reference materials are suitable to evaluate the performance of NGS assays below a VAF of 1%. A performance evaluation that uses reference material not suitable for the task may significantly underestimate the analytical performance of the assay. The lower noise level of SensID reference material enables a lower LoB and LoD for performance evaluation of NGS duplex sequencing assay in a clinical diagnostic environment.

References:
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