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Improving Genetic Testing for Hereditary Cancers: A Multi-Site Assessment Using a Multiplexed Reference Material

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INTRODUCTION

Inherited cancers are caused by germline mutations increasing the risk to develop malignancies such as Lynch syndrome, Hereditary Breast and Ovarian Cancer (HBOC) and many others.

As about 5-10% of cancers are linked to an inherited pathogenic variant, comprehensive genetic testing is important to implement surveillance and risk-reduction strategies, detect cancer at an early, more treatable stage, and provide tailored treatment and therapies.

Inherited cancer assays are developing rapidly, with some panels screening for mutations in hundreds of genes. To perform validation and assess assay performance, it is usually necessary to source multiple samples with mutations in each gene which is difficult and costly. Here we describe the development and evaluation of a prototype biosynthetic reference material designed to address these validation challenges for variants relevant in many inherited cancers.

MATERIALS AND METHODS

LGC Clinical Diagnostics designed a highly multiplexed reference material containing 61 pathogenic variants in 55 cancer-predisposing genes.

Biosynthetic DNA constructs bearing the variants were blended with genomic DNA from the GM24385 cell line at a target 50% variant allele frequency (VAF). The presence of large deletions was mimicked by constructs with the sequences spanning the deletion breakpoints. In case of the MSH2 Boland inversion, the 5' and 3' breakpoints were present on 2 constructs.

The VAFs of variants in a prototype lot were determined by digital PCR (Bio-Rad QX200™) and orthogonally tested by several laboratories using targeted hybrid capture NGS assays.

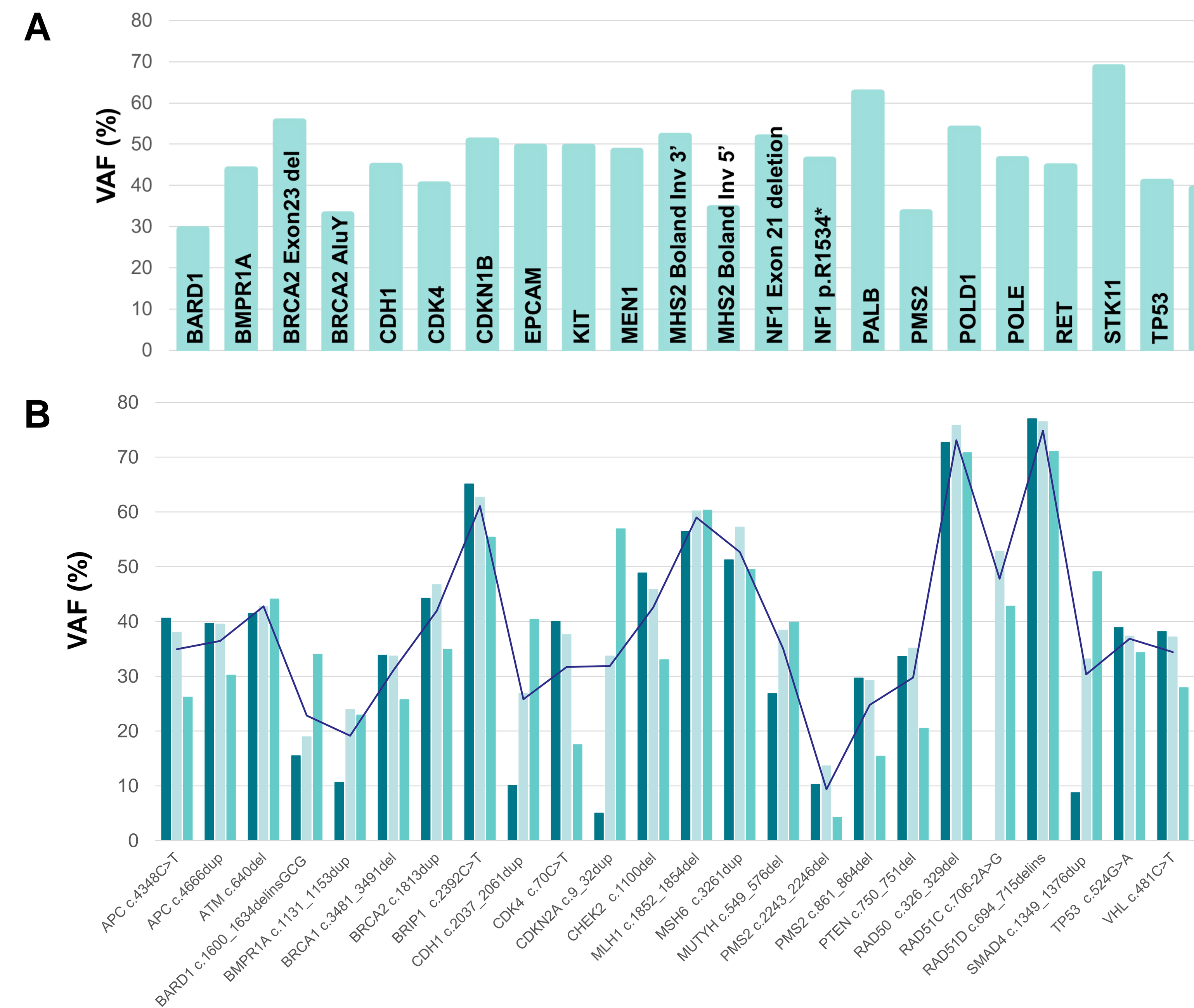
Testing Site	NGS platform	Variant calling
ViennaLab® Hereditary Cancer Assay 31 genes, coding exons 50 ng input, triplicate	Illumina MiSeq™	GENOVESA software; SNV + INDEL; SV (deletions, duplications, inversions) by paired end signatures; CNV calling by read depth
Lab# 2 Custom Twist panel 90 genes, exons + intronic variants 100 ng input, 5 replicates	Element Biosciences AVITII™	Genoox Franklin pipeline; SNV + INDELs, SVs; CNV (≥2 exons for heterozygous deletion/insertion calls)
nonacus GALEAS™ Hereditary Plus assay 146 genes 100 ng input, duplicate	Illumina NextSeq™ 2000	GALEAS™ pipeline; SNV + INDEL; SV (deletions, duplications, inversions) by paired end signatures; CNV, MEI (mobile element insertion)

RESULTS

Gene	Nucleotide change	Protein change / description	Vienna Lab	HALO	Nonacus
APC	c.4348C>T	p.R1450*	40,6	38,0	26,2
	c.4666dup	p.T1556fs	39,6	39,5	30,2
ATM	c.640del	p.S214fs	41,5	42,7	44,1
AXIN2	c.1994dup	p.N666fs	N/A	36,9	35,8
BAP1	c.1433_1449dup	p.P484fs	N/A	31,1	33,8
BARD1	c.1600_1634delinsGCG	p.T546fs	15.5	18,9	34,0
BMPR1A	c.1131_1153dup	p.V385fs	10,6	23,9	22,9
BRCA1	c.3481_3491del	p.E1161fs	33,9	33,7	25,7
BRCA2	c.1813dup	p.I605fs	44,2	46,7	34,9
	c.8954-8_9136del	Exon 23 deletion	N/A	56,4	31,6
	c.9342_9343ins	AluYb9 insertion	ND*	53	11,1 ¶
BRIP1	c.2392C>T	p.R798*	65,1	62,7	55,4
CDH1	c.2037_2061dup	p.C688delinsHLRGQRV*	10,1	26,9	40,4
CDK4	c.70C>T	p.R24C	40,0	37,6	17,5
CDKN1B	c.59_77dup	p.S27fs	N/A	ND	51,7
CDKN2A	c.9_32dup	p.A4_P11dup	5,0	33,7	56,9
CHEK2	c.1100del	p.T367fs	48,8	45,9	33,0
DICER1	c.682_724dup	p.V242fs	N/A	45,8	69,9
EPCAM	c.859-1462_*1999del	3' deletion affecting MSH2	ND	ND*	ND*
FH	c.37_92del	p.P13fs	N/A	21,9	38,3
FLCN	c.1285dup	p.H429fs	N/A	45,9	42,9
HOXB13	c.844_845del	p.A282fs	N/A	42,6	38,0
KIT	c.1676T>C	p.V559A	N/A	48,9	33,3
MAX	c.183_195del	p.Q62fs	N/A	64,5	64,3
MEN1	c.1382_1404dup	p.E469fs	N/A	15,7	28,0
MET	c.3281A>G	p.H1094R	N/A	48,2	38,6
MITF	c.773_785dup	p.D263fs	N/A	56,0	60,7
MLH1	c.1852_1854del	p.K618del	56,5	60,2	60,3
MSH2	c.942+3A>T	p.?	36,8	N/A	25,0
	c.-125-9509096_1277-3165inv	Boland Inversion 3'	N/A	N/A	26,7 ¶
	c.-125-9509096_1277-3165inv	Boland Inversion 5'			
MSH6	c.3261dup	p.F1088fs	51,3	57,2	49,5
MUTYH	c.549_576del	p.L184fs	26,9	38,4	39,9
NBN	c.667_668ins	p.K223delinsIYIFII*	ND*	39,2	36,0
NF1	c.2410-110_2850+65delinsAAAA	Exon 21 deletion	N/A	55,6	ND
NF1	c.4600C>T	p.R1534*	N/A	46,4	30,7
NTHL1	c.417_436dup	p.L146fs	N/A	44,1	53,3
PALB2	c.3114-1_3201+2del	Exon 11 deletion	N/A	ND*	ND
PDGFRA	c.2537A>T	p.D846V	N/A	71,7	76,2
PMS2	c.2243_2246del	p.K748fs	10,3	13,6	4,2 ¶
	c.861_864del	p.R287fs	29,7	29,2	15,4
POLD1	c.1433G>A	p.S478N	N/A	51,8	48,6
POLE	c.1270C>G	p.L424V	N/A	43,5	32,2
PTCH1	c.202-16_227del	Deep splice site	N/A	50,8	67,0
PTEN	c.750_751del	p.C250fs	33,6	35,1	20,5
RAD50	c.326_329del	p.T109fs	72,7	75,8	70,8
RAD51C	c.706-2A>G	p. ?	76,7	52,8	42,8
RAD51D	c.694_715delins	p.R232*	77,0**	76,4	71,0
RET	c.2753T>C	p.M918T	N/A	40,8	70,3
SDHA	c.1785delins	p.E595fs	N/A	65,3	33,9
SDHAF2	c.267del	p.F89fs	N/A	73,9	61,5
SDHB	c.42_43ins	p.A15delinsHSP*	N/A	40,2	56,2 ¶
SDHC	c.250_251del	p.L84fs	N/A	68,1	63,2
SDHD	c.383_386dup	p.L129fs	N/A	65,7	57,3
SMAD4	c.1349_1376dup	p.A460fs	8,8	33,2	49,1
SMARCA4	c.917_941del	p.Q306fs	N/A	47,3	51,3
STK11	c.291-10_922del	Exon 2-7 deletion	83,4	ND*	80,4 ¶
TMEM127	c.24_48del	p.L9fs	N/A	59,2	62,0
TP53	c.524G>A	p.R175H	38,9	37,3	34,3
TSC1	c.850_881delins	p.R284fs	N/A	70,4	54,9
TSC2	c.2640-1G>A	p.?	N/A	42,2	43,6
VHL	c.481C>T	p.R161*	38,2	37,2	27,9

Table 1. List of biosynthetic variants present in the Seraseq Inherited Cancer DNA Mix v2 and average VAF (%) detected by the different NGS assays. *evidence of the variant present in the data despite not being detected by the variant caller. **individual SNVs contributing to this variant were detected separately. ¶variant was detected after modification of the default analysis pipeline. N/A gene or variant were not covered by the assay design.

Fig 1. A) Average VAFs of variants measured by ddPCR (3 measurements). The detected VAFs ranged between 29,4% and 68,6%, reflecting the early prototype blending of the reference material.
B) Average VAFs of variants detected by NGS at each test site, overall average shown as dark blue line. The reported VAFs ranged between 4% and 77%.



26 out of 29 expected variants were detected by the ViennaLab Hereditary Cancer assay. Some exon deletions were not detected, as regions outside exons could not be called as CNVs via read depth/split read analysis. 56 out of 60 expected variants were detected by the HALO Precision Diagnostics™ assay (in development) and reported VAFs were in the range of 12-75%. Most variants were detected with high confidence. 58 out of 61 expected variants were detected by the Nonacus GALEAS Hereditary Plus v2 assay (in development) and reported VAFs were in the range of 4-80%. Most variants were detected with high confidence. Detection of Boland inversion and AluY insertion required implementing additional variant callers.

Fig 2. Detection of the MSH2 Boland inversion by split-read analysis (GALEAS Hereditary Plus design and v2 pipeline module).

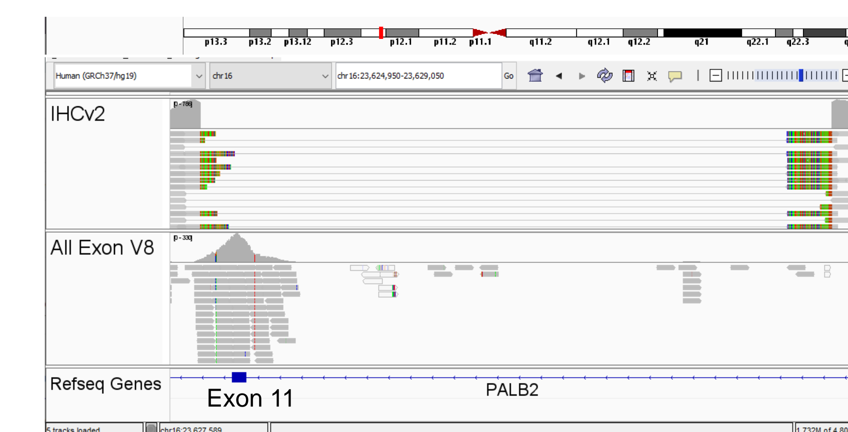


Fig 3. Expected detection of the PALB2 deletion by WES (synthetic data vs Agilent All Exon v8 on GM24385 cell line). Capture of the breakpoints requires extending probes outside of exon 11.

DISCUSSION

Identifying some variants was challenging and required modifications to alignment, variant calling, and annotation settings. For example, RAD51D c.694_715delins is detected as two separate c.694C>T and c.715C>T variants (e.g. at ViennaLab), unless the variant caller is set to merge.

None of the NGS assays were able to detect the PALB2 and EPCAM deletions with intronic breakpoints (Table 2 and Fig. 3). As targeted and WES panel designs often improve on-target rate metrics by moving capture probes into coding exons, this prevents the capture intronic deletion breakpoints when whole exons are deleted. Variants such as the MSH2 Boland inversion involve intronic breakpoints with no reduction in exon coverage and are undetectable unless sequenced.

This reference material is not designed for assays calling exon deletions with CNV tools based on reduced coverage. (EPCAM and STK11 deletion breakpoints were detected at HaloDx, but not not picked up by the CNV read-depth variant caller). Nevertheless, it shows broad compatibility across assays and sequencing platforms and the complex variants can be used to test and improve analysis pipelines.

At Nonacus, the Boland inversion (Fig. 2), and BRCA2 and STK11 large deletions were detected by a combination of short-range and long-range paired-end mapping and split-read analysis. An Alu-specific module using repeat masking allowed successful calling of the BRCA2 AluY insertion; both increased detection accuracy from 85,5% to 95,1%. These updates are to be released as a part of the GALEAS pipeline.

CONCLUSIONS

This study shows the benefits of using a highly multiplexed reference material to challenge assay design and bioinformatics pipeline in the detection of clinically relevant variants including complex and challenging deletions, insertions and inversions. It can support the needs of assay development, optimization and validation, and since it is based on a GIAB cell line, it can also help labs comply with quality requirements for routine QC performance monitoring.

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