HORIZON DISCOVERY

Application Note

The use of Horizon Discovery BRCA Reference Standards for the validation of EntroGen's BRCA Complete™, an inclusive NGS solution for detecting clinically relevant BRCA1/2 variants

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Introduction

Women with deleterious BRCA1/2 mutations are predisposed to a high lifetime risk of breast, ovarian, pancreatic, and possibly other cancers, while men with BRCA mutations have an increased risk of pancreatic, prostate, and breast cancer. The US Food and Drug Administration (FDA) and European Medicines Agency (EMA) have recently approved two poly ADP-ribose polymerase (PARP) inhibitors, Olaparib and Rubraca, for the treatment of ovarian cancer patients with BRCA1/2 mutations. This highlights the importance of a reliable assay that detects BRCA mutations for companion diagnostics and predicts cancer risk.

Targeted next-generation sequencing (NGS) technology has the capacity to simultaneously sequence BRCA1/2 exons in multiple samples at a reduced cost compared to whole genome and exome sequencing. NGS is predicted to replace Sanger sequencing as the gold standard in the clinical environment. However, implementing this powerful technology in a clinical laboratory presents a unique challenge due to the complex workflow, volume of data output, bioinformatics burden, and medicolegal implications of the generated data.

EntroGen BRCA Complete™ addresses these challenges by providing a complete sequencing solution from library preparation to data interpretation for detecting both germline and somatic mutations in BRCA1/2 exomes with full coverage and high specificity and sensitivity (Figure 1). BRCA Complete™ prevents allele dropouts and reduces off target reads through a multiplexed tiled amplicon PCR-approach with an error-free, high fidelity polymerase (Figures 2 & 3). Sample quality assessment assays available through EntroGen also increase the quality and reliability of NGS data. In addition, the panel includes user-friendly software designed to reduce the bioinformatics burden on the end-user. Horizon Discovery FFPE & genomic DNA reference standards with known alternate allele frequencies (AAF) for both germline and somatic BRCA1/2 mutations were used to validate the EntroGen BRCA Complete™ panel. The results of the analytical validation are described here.

Methods

BRCA Complete™ was evaluated with five different Horizon reference standards: HD753, HD789, HD793, HD794, and HD795. Libraries were prepared with BRCA Complete™ from 40 ng of the reference standards. Libraries were subsequently sequenced on Illumina® Miniseq™ using MiniSeq Mid Output Kit (300-cycles). 1.2 pM library was loaded with 5% PhiX control. A manifest-directed "amplicon workflow" was selected as the workflow for BRCA Complete™. The resulting variant call format (VCF) file was analyzed with EntroGen's proprietary software, VCFanalyzer. The uniformity matrix and depth of coverage for each amplicon was generated by the amplicon workflow.

Target specificity of the BRCA Complete™ panel was analyzed with the following workflow: the generated paired end FASTQ files were either analyzed with the resequencing workflow in MiniSeq™ or aligned with Bowtie/BWA against the whole human genome. The resulting BAM files were analyzed with a bio-conductor target enrichment quality control (TEQC) R-package. The genomic positions and % AAF of expected mutations in the Horizon Discovery reference standards were then compared with the results obtained with BRCA Complete™. At least three libraries for each reference sample were prepared and sequenced over a time period of 6 months.

Results

BRCA Complete™ covers all clinically relevant BRCA1/2 exon mutations including the deleterious BRCA1/2 missense alterations associated with PARP inhibitor sensitivity. BRCA Complete™ detected all clinically relevant BRCA1/2 exon mutations present in the Horizon Discovery reference standards, with a 0.2X and 0.15X mean uniformity of 96.47% and 100%, respectively. TEQC analysis suggests a highly specific target enrichment (99%) process. The AAF% determined reproducibly by BRCA Complete™ were similar to the expected and ddPCR-confirmed allelic frequencies in the Horizon Discovery Reference Standards. The data is summarized in Tables 1-4 below.

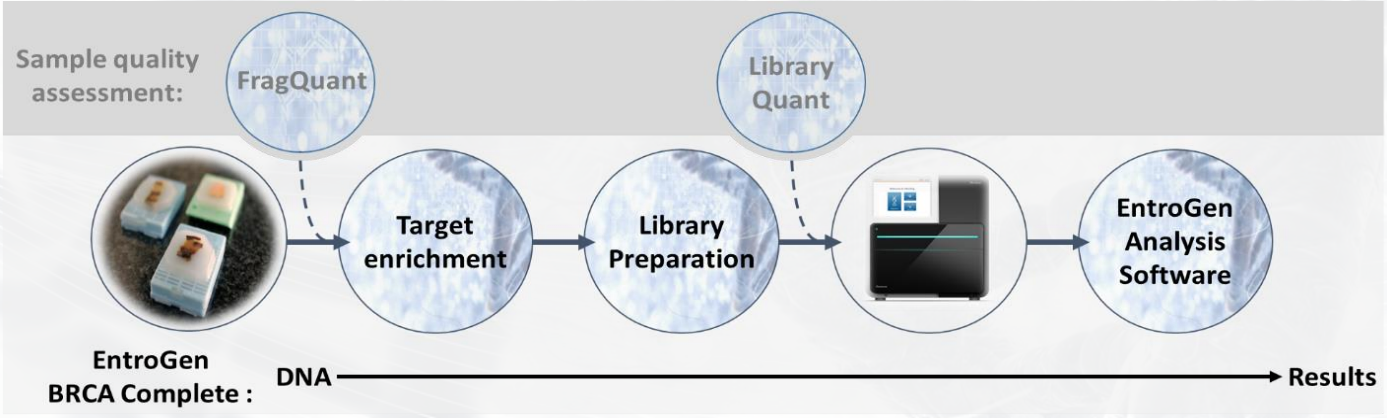

Figure 1. EntroGen BRCA Complete™ workflow. The panel offers all reagents necessary for target enrichment and library construction for Illumina® MiSeq and MiniSeq platforms from DNA isolated from patient samples. Sample quality assessment assays (DNA Fragmentation Quantification Assay and Library Quantification Kit for use in Illumina® workflows) are provided from EntroGen on demand. The complimentary software queries three independent databases to call clinically relevant variants based on Illumina® output reports.

Mutations Detected in Horizon Discovery Reference Standards by EntroGen BRCA Complete™

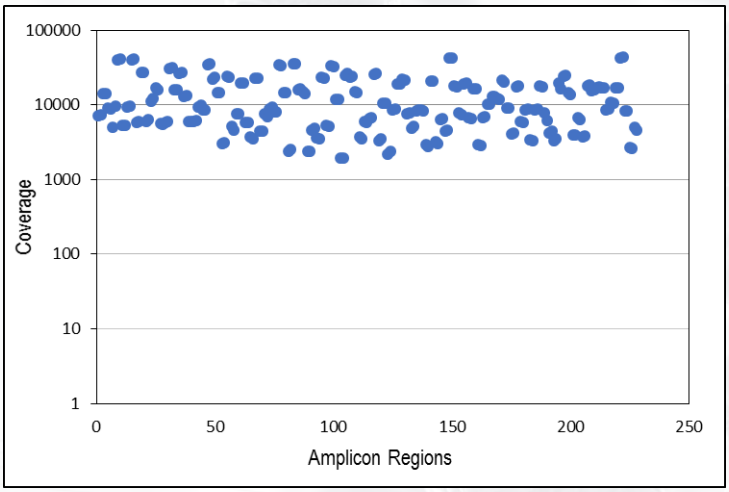

Figure 2. Uniformity of coverage of BRCA Complete™. A uniformity of 96.47% (0.2X mean) and 100% (0.15X mean) was obtained.

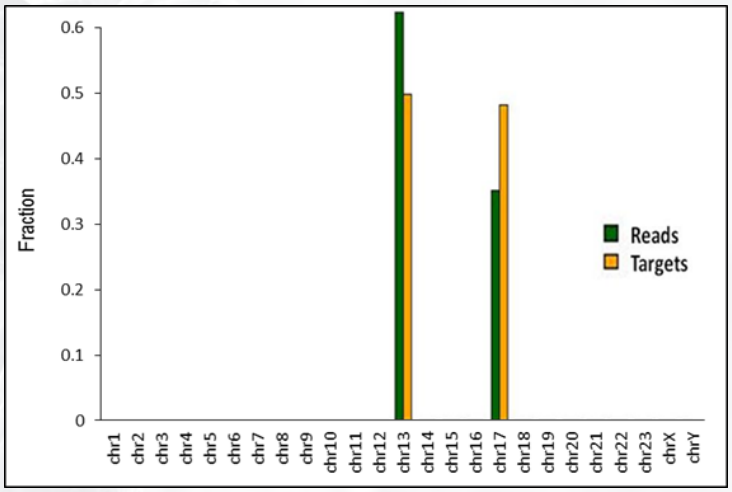

Figure 3. BRCA Complete™ demonstrates high target specificity. BRCA Complete™ demonstrates high target specificity with >99% reads on BRCA1 or BRCA2.

Conclusion

Horizon Discovery reference standards are a well characterised source of high quality, multi-variant material to facilitate the validation of NGS assays, such as BRCA Complete™. This assay offers an all inclusive solution to identify somatic and germline mutations in BRCA1 and BRCA2 genes with high accuracy and sensitivity, generating highly reproducible results (through multiple repeats). A manifest-directed amplicon workflow paired with complementary software allows clinically relevant mutations to be called directly from VCF files whilst reducing the bioinformatics burden on the end-user. Horizon Discovery BRCA reference standards, available in FFPE, gDNA, somatic and germline formats facilitate the standardization and establishment of analytical parameters when evaluating new NGS workflows.

"Horizon Discovery reference standards are a well characterised source of high quality, multi-variant material to facilitate the validation of NGS assays, such as BRCA Complete™."

Gary Potikyan - CSO, EntroGen

Table 1: BRCA Germline II Reference Standard (HD794, gDNA)

Gene	Coordinate (hg19)	Variant	Expected AF (%)	Horizon AF (%) Seq NGS	Horizon AF (%) ddPCR	Entrogen - 1 AF (%)	Entrogen - 2 AF (%)	Entrogen - 3 AF (%)
BRCA2	32929387	V2466A	100	100	100	**100**	**100**	**99.9**
BRCA2	32937354	I2675fs	50	60.94	50.2	**46.5**	**49.9**	**49.5**

Table 2: BRCA Germline I Reference Standard (HD793, gDNA)

Gene	Coordinate (hg19)	Variant	Expected AF (%)	Horizon AF (%) NGS	Horizon AF (%) ddPCR	Entrogen - 1 AF (%)	Entrogen - 2 AF (%)	Entrogen - 3 AF (%)
BRCA1	41223094	S1613G	50	56.48	49.7	**49.7**	**49.7**	**49.8**
BRCA1	41244000	K1183R	50	53.96	49.8	**49.8**	**50**	**49.3**
BRCA1	41245090	K820E	50	50.8	49.8	**49.1**	**50.7**	**49.6**
BRCA1	41246245	D435Y	50	45.35	50.2	**50.8**	**50.7**	**50.4**
BRCA1	41244936	P871L	100	100	100	**99.9**	**99.7**	**99.6**
BRCA2	32906480	N289H	50	61.29	49.7	**49.2**	**50.1**	**49.1**
BRCA2	32929387	V2466A	100	100	100	**99.9**	**100**	**100**
BRCA2	32911463	N991D	50	41.18	50	**49**	**48.9**	**49.3**
BRCA2	32913836	N1784fs	50	52	50.4	**47.8**	**46.7**	**46.4**

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Table 3: Structural Multiplex Reference Standard FFPE (HD789) and gDNA (HD753)

Sample	Gene	Coordinates (hg19)	Variant	Expected AF (%)	Entrogen - 1 AF (%)	Entrogen - 2 AF (%)	Entrogen - 3 AF (%)
*HD789	BRCA2	32913558	A1689fs	5.6	**5.4**	**5.5**	**6.1**
HD753	BRCA2	32913558	A1689fs	5.6	**5.8**	**5.2**	**6.6**

* HD789 - coming soon to the Horizon Discovery catalogue.

Table 4: BRCA Somatic Multiplex 1 Reference Standard HD795, (gDNA)

Gene	Coordinates (hg19)	Variant	Expected AF (%)	Horizon AF (%) Targeted NGS	Horizon AF (%) Exome NGS	Horizon AF (%) ddPCR	Entrogen - 1 AF (%)	Entrogen - 2 AF (%)	Entrogen - 3 AF (%)
BRCA1	41223094	S1613G	7.5	5.4	9.09	7.6	**8**	**8**	**7.7**
BRCA1	41244000	K1183R	7.5	7.1	NA	7.4	**7.3**	**6.6**	**6.6**
BRCA1	41245090	K820E	7.5	5	9.2	7.1	**6.6**	**7.4**	**7.2**
BRCA1	41234451	R1443STOP	32.5	23.5	23	32.4	**27**	**31**	**32**
BRCA1	41246245	D435Y	7.5	6.4	NA	7.6	**7.4**	**7.8**	**7.1**
BRCA1	41244936	P871L	15	13.6	14.36	14.9	**14.5**	**14.5**	**14.6**
BRCA2	32906480	N289H	7.5	6.4	NA	6.9	**9.6**	**7.6**	**7.9**
BRCA2	32929387	V2466A	100	100	100	100	**100**	**100**	**100**
BRCA2	32911463	N991D	7.5	4.7	NA	7.38	**7.3**	**6.6**	**7.5**
BRCA2	32913558	K1691fs	32.5	28.7	23.58	32.7	**32.1**	**32.5**	**32.5**
BRCA2	32913836	N1784fs	40	33.8	31.67	39.3	**37.7**	**38.9**	**38.6**
BRCA2	32912750	D1420Y	32.5	27.9	12	32.7	**29.3**	**29.0**	**28.8**
BRCA2	32937354	I2675fs	10	8.6	NA	10.4	**10.7**	**10.7**	**10.6**

BRCA Complete™ is available for research use in the US and for in-vitro diagnostic use in the EU.

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