Laboratory Verification of a BRCA1 and BRCA2 Massively Parallel Sequencing Assay from Wet Bench to Bioinformatics for Germline DNA Analysis

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Introduction

Hereditary breast and ovarian cancer (HBOC) is an autosomal dominant cancer syndrome frequently caused by germline pathogenic variants in the two DNA repair genes, BRCA1 and BRCA2. Due to high penetrance, approximately 50% of women with BRCA pathogenic variants will be diagnosed with breast cancer by age of 70 years.1 For ovarian cancer, these estimates were 40 and 18% of women with mutant BRCA1 and BRCA2 genes, respectively.1 Several poly ADP ribose polymerases (PARP) inhibitors have been approved for therapy in patients with HBOC syndrome with germline BRCA genetic testing.2

Abstract

Introduction A robust genetic test for BRCA1 and BRCA2 genes is necessary for the diagnosis, prognosis, and treatment of patients with hereditary breast and ovarian cancer. We evaluated a commercial amplicon-based massively parallel sequencing (MPS) assay, BRCA MASTR Plus on the MiSeq platform, for germline BRCA genetic testing.

Methods This study was performed on 31 DNA from cell lines and proficiency testing samples to establish the accuracy of the assay. A reference cell line DNA, NA12878 was used to determine the reproducibility of the assay. Discordant MPS result was resolved orthogonally by the current gold-standard Sanger sequencing method.

Results The analytical accuracy, sensitivity, and specificity for variant detection were 93.55, 92.86, and 100.00%, respectively. Both sequencing depth and variant allele frequencies were highly reproducible by comparing the NA12878 DNA tested in three separate runs. The single discordant result, later confirmed by Sanger sequencing was due to the inability of the MASTR Reporter software to identify a 40-bp deletion in BRCA1.

Conclusion The BRCA MASTR Plus assay on the MiSeq platform is accurate and reproducible for germline BRCA genetic testing, making it suitable for use in a clinical diagnostic laboratory. However, Sanger sequencing may still serve as a confirmatory method to improve diagnostic capability of the MPS assay.
frequency (VAF) of pathogenic BRCA variants from tumor profiling, genetic testing of germline variant is recommended. Genetic testing of the BRCA genes plays a vital role to allow identification of carriers of pathogenic variants and increased screening for early detection of breast and ovarian cancers in these individuals. A robust laboratory assay is crucial to enable genetic testing of HBOC syndrome. A wide spectrum of pathogenic variants is detectable throughout the coding and splice site regions of the BRCA genes. These genetic alterations are heterogeneous, including single nucleotide variants (SNVs), small insertions and deletions (indels) affecting a short stretch of nucleotides, large indels at exonic level and copy number variants (CNVs). With the advent of massively parallel sequencing (MPS), the laboratory accessibility to sequencing the two large BRCA genes is improved. A recent international survey revealed that 93% of the laboratories utilize MPS platforms for sequencing the BRCA genes. In this study, we evaluated a commercial amplicon-based MPS assay, BRCA MASTR Plus (Multiplicom, Niel, Belgium) on the MiSeq platform (Illumina; San Diego, California, United States), for germline BRCA1 and BRCA2 genetic testing.

Methods

DNA Samples

Samples tested in this study were cell line DNA from Coriell Institute for Medical Research (n = 10), namely, NA13714, NA14091, NA14624, NA14639, NA14788, NA14805, NA14623, NA14622, NA14170, and NA12878. Notably, NA12878 is a reference cell line characterized by the Genome in a Bottle (GIAB) Consortium hosted by National Institute of Standards and Technology (NIST). Twenty-one DNA samples accrued from College of American Pathologists/ American College of Medical Genetics (CAP/ACMG) BRCA1/2 Sequencing External Quality Assurance (EQA) Program were also tested in this study.

BRCA MASTR Plus Assay Library Preparation and Sequencing

Concentrations of DNA samples were measured using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Working DNA samples were diluted with nuclelease-free water (Invitrogen, Waltham, Massachusetts, United States) to a concentration of 10 ng/µL. Five µL of diluted DNA was subject to four multiplex polymerase chain reactions (PCRs) with reagents supplied in BRCA MASTR Plus kit (Multiplicom) according to manufacturer’s instructions. Five µL of PCR products from the multiplex PCR reactions were subject to electrophoresis using 2% (w/v) agarose gel in Tris-Borate-EDTA (TBE) buffer at 100 V for 30 minutes to verify the presence of PCR products with sizes exceeding 150 base pairs. Equal volumes of multiplex PCR products were pooled and purified with AMPure XP beads (Agencourt; Beverly, Massachusetts, United States). Universal PCR was performed on purified pooled PCR products with MID p7 and p5 adaptor and primer mixes supplied in drMID for Illumina NGS systems kit (Multiplicom). Five µL of the universal PCR products were subject to electrophoresis using 2% (w/v) agarose gel in TBE buffer at 100 V for 30 minutes to verify the presence of PCR products with sizes exceeding 200 base pairs. The universal PCR products purified with AMPure XP beads were diluted to 4 nM in TE buffer (Thermo Fisher Scientific) and pooled into a single library. The library was diluted to 12 pM and denatured using 0.2 N sodium hydroxide (NaOH). MPS was performed on the MiSeq system (Illumina) using MiSeq Reagent Micro Kit v2 (300 cycles).

Bioinformatics

The demultiplexed FASTQ sequence files were uploaded to MASTR Reporter v1.2.1, proprietary web-based software by Multiplicom. The application of BRCA MASTR Plus Dx Germline was selected to analyze the sequencing data in this study. For variant analysis, the minimum coverage depth and allele frequency were specified at 40× and 20%, respectively. Variants were classified according to the 2015 ACMG/AMP guidelines.

Sanger Confirmation

A set of primers was designed using Primer3 software to amplify exon 11 of the BRCA1 gene with expected PCR product size of 430 base pairs (bp). The forward and reverse primers were 5’cagaaactgcctgctgca3’ and 5’tgagggtcgcttcgtgac3’, respectively. PCR was performed using HotStarTaq Plus Master Mix Kit (QIAGEN; Hilden, Germany) on 50 ng of DNA input. PCR products were purified with GeneAll Expin Kit (GeneAll Biotechnology, Seoul, Korea) and subject to cycle sequencing reaction using the same forward and reverse PCR primers with BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific). Sanger sequencing was performed on cycle sequencing products purified with DyeEx 2.0 Spin kit (QIAGEN) on ABI 3130 Genetic Analyzer (Thermo Fisher Scientific). Sequence analysis was performed using ATF software (Conexio Genomics, Fremantle, Australia).

Statistical Analysis

Accuracy was calculated as the number of true positives and true negatives divided by the sum of true positives, true negatives, false positives, and false negatives using an online statistical software, MEDCALC.

Results

Sequencing Depth and Reproducibility of the Assay

With a pooling strategy with 10 samples per library, the minimum sequencing depth was above 400× and 350× for BRCA1 (69 amplicons) and BRCA2 (112 amplicons),
respectively (►Figs. 1 and 2). Comparing three batches of sequencing runs, the average sequencing depths per sample were 957 × (66; mean [standard deviation (SD)]) and 873 × (63) for the BRCA1 and BRCA2, genes respectively. DNA from NA12878 was tested in a pooled library in three separate runs. Nine heterozygous BRCA1 variants and three heterozygous and four homozygous BRCA2 variants were consistently identified with highly reproducible VAF (►Figs. 3 and 4). All BRCA variants identified by the current assay were in concordance with the variant datasets available from the Genetic Testing Reference Material (GeT-RM) browser at the National Center for Biotechnology Information (NCBI).

**Analytical Validity of the Assay**

In addition to NA12878, DNA samples from nine cell lines with known BRCA variants were evaluated (►Table 1). All results were concordant with the data on these cell lines available from the Coriell Institute for Medical Research. Twenty-one blinded DNA samples, accrued from the CAP/ACMG BRCA1/2 sequencing program, were tested.
From these samples, eight heterozygous frameshift variants, three heterozygous stop-gain variants, and three heterozygous SNVs were correctly identified in the BRCA genes. Notably, a 40-bp deletion variant in the BRCA1 gene was not conclusively reported by the MASTR Reporter; however, it was flagged as a long event (Fig. 5). Sanger sequencing further confirmed the long event as c.1175_1214del40 which was the expected variant according to CAP (Fig. 6). One EQA DNA sample which did not have any variants was correctly identified as being negative for BRCA variants. Altogether, for a total of 31 DNA samples, covering a range of different variants including SNVs,
| No. | DNA sample ID   | Gene          | Human Genome Variation Society (HGVS) nomenclature                  | MASTR Reporter coding level       | Protein level | Variant frequency (%) | VEP variant consequence/impact  | Zygosity     | Concordance | Variant classification |
|-----|----------------|---------------|-------------------------------------------------|----------------------------------|-----------------|-----------------------|--------------------------------|--------------|-------------|-----------------------|
| 1   | NA13714        | BRCA1         | c.5319dupC p.Asn1774Glnfs*16  | 48.84                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 2   | NA14091        | BRCA1         | c.5266dupC p.Gln1756Profs*74  | 49.17                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 3   | NA14624        | BRCA2         | c.572_572delCT p.Leu1908Argfs*2  | 47.81                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 4   | NA14639        | BRCA2         | c.6198,6199delTT p.Ser2067Thrfs*10  | 53.73                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 5   | NA14788        | BRCA2         | c.755_756delACAG p.Arg252Valfs*24  | 50.00                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 6   | NA14805        | BRCA2         | c.581G>A p.Trp194*  | 49.30                           | Stop gained/high | Heterozygous          | Yes                           | Pathogenic  |
| 7   | NA14623        | BRCA2         | c.125A>G p.Tyr42Gly  | 51.01                           | Missense/moderate | Heterozygous          | Yes                           | Benign       |
| 8   | NA14622        | BRCA2         | c.6275,6276delTT p.Leu2092Profs*7  | 48.92                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 9   | NA14770        | BRCA2         | c.5946delT p.Ser1982Argfs*22  | 50.30                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 10  | NA12878        | BRCA1 and BRCA2 | – | – | Refer to►Fig. 3 and►Fig. 4 for summaries | – | – | – | – |
| 11  | 2016A01        | BRCA1         | c.5266dupC p.Gln1756Profs*74  | 51.57                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 12  | 2016A02        | BRCA1         | c.4689C>G p.Tyr1563*  | 49.53                           | Stop gained/high | Heterozygous          | Yes                           | Pathogenic  |
| 13  | 2016A03        | BRCA2         | c.5946delT p.Ser1982Argfs*22  | 50.22                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 14  | 2016B04        | BRCA1         | c.1175_1214del40 p.Leu392Glnfs*20  | 51.90                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 15  | 2016B05        | BRCA1         | c.181T>G p.Cys61Gly  | 52.79                           | Missense/moderate | Heterozygous          | Yes                           | Pathogenic  |
| 16  | 2016B06        | BRCA1         | c.68,69delAG p.Glu23Valfs*17  | 51.62                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 17  | 2017A01        | BRCA1         | c.1175_1214del40 p.Leu392Glnfs*20  | 52.30                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 18  | 2017A02        | BRCA1         | c.2071delA p.Arg691Aspfs*24  | 50.60                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 19  | 2017A03        | BRCA1 and BRCA2 | – | – | Refer to►Fig. 3 and►Fig. 4 for summaries | – | – | – | – |
| 20  | 2017B04        | BRCA1         | c.581G>A p.Trp194*  | 50.74                           | Stop gained/high | Heterozygous          | Yes                           | Pathogenic  |
| 21  | 2017B05        | BRCA1         | c.3481_3491delGAAGATACTAG p.Glu1161Phefs*34  | 50.44                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 22  | 2017B06        | BRCA1         | c.7630G>A p.Glu2544Ser  | 49.30                           | Missense/moderate | Heterozygous          | Yes                           | Uncertain significance |
| 23  | 2018A01        | BRCA1         | c.1204delG p.Glu402Serfs*10  | 49.79                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 24  | 2018A02        | BRCA2         | c.437C>T p.Arg1443*  | 51.90                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 25  | 2018A03        | BRCA2         | c.437C>T p.Arg1443*  | 48.76                           | Stop gained/high | Heterozygous          | Yes                           | Pathogenic  |
| 26  | 2018B04        | BRCA1         | c.1175_1214del40 p.Leu392Glnfs*20  | 50.60                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 27  | 2018B05        | BRCA1         | c.181T>G p.Cys61Gly  | 47.8                            | Stop gained/high | Heterozygous          | Yes                           | Pathogenic  |
| 28  | 2018B06        | BRCA2         | c.581G>A p.Trp194*  | 49.30                           | Stop gained/high | Heterozygous          | Yes                           | Pathogenic  |
| 29  | 2019A01        | BRCA1         | c.68,69delAG p.Glu23Valfs*17  | 50.60                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 30  | 2019A02        | BRCA2         | c.6275,6276delTT p.Leu2092Profs*7  | 50.60                           | Frameshift/high  | Heterozygous          | Yes                           | Pathogenic  |
| 31  | 2019A03        | BRCA1         | c.4689C>G p.Tyr1563*  | 48.9                            | Stop gained/high | Heterozygous          | Yes                           | Pathogenic  |
deletions, and duplications in the BRCA genes, the analytical accuracy for variant detection was 93.55% (95% confidence interval [CI]: 78.58–99.21%). The analytical sensitivity and specificity were 92.86% (95% CI: 76.50–99.12%) and 100.00% (95% CI: 29.24–100.00%), respectively.

Discussion

Here we demonstrate the use of well-characterized cell line DNA and blinded proficiency testing samples for evaluation of a commercial MPS assay for the entire coding regions of
the BRCA1 and BRCA2 genes in germline samples. In the absence of clinical samples, for a clinical laboratory starting a new assay, EQA samples and cell line samples are good resources for evaluating analytical accuracy and precision. Using these samples, we demonstrated the accurate identification of 10 different frameshift variants, 3 different stop gain variants, and 3 different SNVs in the BRCA1 and BRCA2 genes.

All samples showed concordance with the expected variants except for two EQA samples which harbor the same 40-bp deletion, c.1175_1214del40, in the BRCA1 gene. First reported in 1994,7,8 this deletion is not an uncommon pathogenic variant in HBOC patients (ClinVar, accessed on January 10, 2021). The inability to determine insertions and deletions spanning more than 30-bp is a declared limitation of the assay. Hence the laboratory may need to supplement the MPS assay with Sanger sequencing or use an alternative bioinformatics pipeline to analyze the sequencing data to confirm the exact deletion or insertion.

Conclusion

In conclusion, we have shown high reproducibility and accuracy of the BRCA MASTR Plus assay on the MiSeq platform. The simple bench workflow in combination with rapid automated data analysis by the MASTR Reporter software make it suitable for use for germline BRCA1 and BRCA2 genetic testing in a clinical diagnostic laboratory. However, Sanger sequencing may still serve as a confirmatory method to improve diagnostic capability of the MPS assay.

Funding

None.

Conflict of Interests

None declared.

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