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Next-Generation Sequencing of the *BRCA1* and *BRCA2* Genes for the Genetic Diagnostics of Hereditary Breast and/or Ovarian Cancer

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Genetic testing for hereditary breast and/or ovarian cancer mostly relies on laborious molecular tools that use Sanger sequencing to scan for mutations in the *BRCA1* and *BRCA2* genes. We explored a more efficient genetic screening strategy based on next-generation sequencing of the *BRCA1* and *BRCA2* genes in 210 hereditary breast and/or ovarian cancer patients. We first validated this approach in a cohort of 115 samples with previously known *BRCA1* and *BRCA2* mutations and polymorphisms. Genomic DNA was amplified using the Ion AmpliSeq *BRCA1* and *BRCA2* panel. The DNA Libraries were pooled, barcoded, and sequenced using an Ion Torrent Personal Genome Machine sequencer. The combination of different robust bioinformatics tools allowed detection of all previously known pathogenic mutations and polymorphisms in the 115 samples, without detecting spurious pathogenic calls. We then used the same assay in a discovery cohort of 95 uncharacterized hereditary breast and/or ovarian cancer patients for *BRCA1* and *BRCA2*. In addition, we describe the allelic frequencies across 210 hereditary breast and/or ovarian cancer patients of 74 unique definitely and likely pathogenic and uncertain *BRCA1* and *BRCA2* variants, some of which have not been previously annotated in the public databases. Targeted next-generation sequencing is ready to substitute classic molecular methods to perform genetic testing on the *BRCA1* and *BRCA2* genes and provides a greater opportunity for more comprehensive testing of at-risk patients. *(J Mol Diagn 2015, 17: 162–170; http://dx.doi.org/10.1016/j.jmoldx.2014.11.004)*

The presence of germline mutations in the highly penetrant breast cancer susceptibility genes *BRCA1* (17q21; Online Mendelian Inheritance of Man no. 113705, *http://www.ncbi.nlm.nih.gov/omim*) and *BRCA2* (13q12.3; Online Mendelian Inheritance of Man no. 600185, *http://www.ncbi.nlm.nih.gov/omim*) predispose women to fivefold or greater elevations in the risk of hereditary breast and/or ovarian cancer (HBOC).[1–6] The *BRCA1* and *BRCA2* genes are the most clinically important genetic predictors of HBOC susceptibility and account for 5% to 15% of these two cancers, especially in families with several affected patients over multiple generations.[7,8] Conventional genetic testing of the *BRCA1* and *BRCA2* genes has become an integral part of clinical practice, which consists of the highly sensitive but labor-intensive screening of PCR-amplified individual exons by denaturing gradient-gel electrophoresis,[9] denaturing high-pressure liquid chromatography,[10] single-stranded conformational polymorphism high-resolution melting,[11] and/or direct Sanger sequencing of the PCR products.[12–14] Large structural variants, such as exonic deletions and duplications, are tested using multiplex ligation-dependent probe amplification[15] or quantitative PCR.[16]

In addition, the size of the *BRCA1* and *BRCA2* genes (5592 bp and 10,257 bp, respectively) and their high-allelic

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heterogeneity (Breast Cancer Information Core Database, registration required, *http://research.nhgri.nih.gov/bic/*, last accessed May 5, 2014), together with the lack of mutation hot spots, make genetic testing of *BRCA1* and *BRCA2* technically challenging. Most mutations are private variants, with a total of 1706 *BRCA1* and 1446 *BRCA2* mutations reported to date in The Human Gene Mutation Database (HGMD, registration required, *http://www.hgmd.cf.ac.uk*, last accessed May 5, 2014). On top of that, the prohibitively laborious and cost-intensive nature of the conventional gene-by-gene screening methods described above restricts their availability to only high-risk patients with significant family history of HBOC and hinders the establishment of widespread *BRCA1* and *BRCA2* genetic screening programs for the personalized risk assessment of HBOC to an at-risk but yet symptomless female population. Such screening programs would play a crucial role in the early HBOC detection and prevention because approximately 50% of the clinical cases carrying a mutation have been estimated to remain undetected because of the current restrictive access to *BRCA1* and *BRCA2* genetic testing.[17]

Therefore, there is a need for more high-throughput and cost-effective screening approaches that could reduce the turnaround time for routine *BRCA1* and *BRCA2* genetic screening, especially now with the coming personalized pharmacogenetic therapies using chemical inhibitors of poly (ADP-ribose) polymerases (PARPs) with selective toxicity for tumors derived from germline carriers of mutations in *BRCA1* and *BRCA2*.[18–23] Next-generation sequencing (NGS) technologies have emerged as a powerful tool for the discovery of causative mutations and novel disease genes and are rapidly affecting genetic diagnostics delivering fast, inexpensive, and detailed genetic information. We sought to validate a semiconductor benchtop NGS platform called the Personal Genome Machine (PGM; Life Technologies, Carlsbad, CA) in combination with the ready-to-use Ion AmpliSeq *BRCA1* and *BRCA2* Panel (Life Technologies) as an accurate, comprehensive, and cost-effective alternative to conventional *BRCA1* and *BRCA2* genetic testing in a medical diagnostics setup. We first assessed the sensitivity and specificity of this assay in a validation cohort of 115 patients, in whom we detected all the previously known mutations, and then used it in a discovery cohort of 95 patients with unknown *BRCA1* and *BRCA2* mutations.

Materials and Methods

Patients

In total, 210 patients with a mean age ± SD of 43 ± 14 years were recruited into the study between November 2010 and January 2014. The validation cohort included 115 patients who had previously undergone conventional genetic diagnosis by Sanger sequencing of all *BRCA1* and *BRCA2* exons, and if results were negative, multiplex ligation-dependent probe amplification was also applied. The discovery cohort consisted of 95 consecutive patients received for genetic diagnosis for which no mutations were known. Family history or diagnosis of HBOC was documented for 68% of the studied individuals. On the basis of the recommendation of their physicians, all participants requested genetic testing for the *BRCA1* and *BRCA2* genes. All samples were anonymized and blindly sequenced and analyzed (D.T.). The study was approved by the Ethical Commission of the Faculty of Medicine of the University of Rostock (registry no. A 2014-0072), and informed consent was signed by all contributing participants before the *BRCA1* and *BRCA2* genetic testing.

DNA Extraction

DNA was isolated from EDTA blood using two automated procedures. The spin column−based extraction was performed on a QIAcube instrument with QIAamp DNA Blood Mini QIAcube Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Alternatively, the QIAsymphony DSP DNA Mini Kit (Qiagen) on the QIAsymphony instrument was used to purify the DNA from blood. After extraction, all DNA samples were stored at −20°C. Before the analysis, the DNA quality and concentration were determined photometrically (OD_{260}/OD_{280}, 1.8 to 2.0).

Amplicon Library Construction

The target regions in the *BRCA1* and *BRCA2* genes were amplified using the Ion AmpliSeq *BRCA1* and *BRCA2* Panel (Life Technologies). The panel consists of three primer pools (167 amplicons) that target the entire coding region, including 10 to 20 bp of intronic flanking sequences around all coding exons, of both genes. To amplify each library, 4 μL of 5× Ion AmpliSeq HiFi master mix, 10 μL of 2× Ion AmpliSeq primer pool (three of them in separate wells for each sample), 10 ng of genomic DNA per reaction (2 μL of 5 ng/μL of stock), and 4 μL of nuclease free water were mixed together. The following temperature profile was applied to the final 20 μL of the PCR mixture: 99°C for 2 minutes, 99°C for 15 seconds, and 60°C for 4 minutes (19 cycles), with a final hold at 10°C. Then primer sequences were partially digested, and adapters and barcodes ligated to the amplicons as described in Ion AmpliSeq library preparation manual. Each library were marked with a unique adapter provided in the Ion Xpress Barcode Adapters 1 to 16 Kit (Life Technologies). Purified libraries were quantified with the Qubit version 2.0 fluorometer (Life Technologies) using the Qubit dsDNA HS assay kit, diluted to approximately 100 pmol/L and combined in equimolar proportion. Freshly prepared library stock dilutions were used on the same day for the preparation of enriched, template-positive ion sphere particles. Automated protocols were run on the Ion OneTouch 2 System and the Ion OneTouch ES Instrument (Life Technologies) according to the version of the user guide and using the 200-bp chemistry kits.

Table 1 Mean Sequencing Quality Control and Coverage Statistics of *BRCA1* and *BRCA2* in the Validation and Discovery Cohorts

	Validation (n = 115)		Discovery (n = 95)	
Cohort	Mean	SD	Mean	SD
Total reads	384,135	139,612	310,700	103,773
Mapped reads	381,546	139,281	308,192	103,588
On target, %	95.01	4.31	95.47	2.78
Uniformity, %	97.29	2.95	96.10	5.61
BRCA1 mean coverage (X)	2331.23	873.61	1882.16	675.49
% *BRCA1* target bases covered = 0X	0.00	0.00	0.00	0.00
% *BRCA1* target bases covered ≥1X	100.00	0.00	100.00	0.00
% *BRCA1* target bases covered ≥5X	100.00	0.00	99.98	0.15
% *BRCA1* target bases covered ≥10X	100.00	0.00	99.98	0.15
% *BRCA1* target bases covered ≥20X	99.99	0.14	99.97	0.23
% *BRCA1* target bases covered ≥50X	99.93	0.33	99.83	0.49
% *BRCA1* target bases covered ≥80X	99.77	0.64	99.63	0.58
% *BRCA1* target bases covered ≥100X	99.66	1.20	99.51	0.82
% *BRCA1* target bases covered ≥200X	98.85	3.44	98.37	2.27
% *BRCA1* target bases covered ≥300X	97.54	5.15	96.33	5.37
% *BRCA1* target bases covered ≥400X	95.66	7.87	93.91	7.75
% *BRCA1* target bases covered ≥500X	93.95	10.19	91.07	10.16
% *BRCA1* target bases covered ≥1000X	84.55	18.61	75.95	20.48
BRCA2 mean coverage (X)	2092.45	758.96	1668.12	621.19
% *BRCA2* target bases covered = 0X	0.00	0.00	0.00	0.00
% *BRCA2* target bases covered ≥1X	100.00	0.00	100.00	0.00
% *BRCA2* target bases covered ≥5X	99.99	0.10	100.00	0.00
% *BRCA2* target bases covered ≥10X	99.97	0.16	99.99	0.11
% *BRCA2* target bases covered ≥20X	99.89	0.33	99.91	0.27
% *BRCA2* target bases covered ≥50X	99.66	0.50	99.59	0.54
% *BRCA2* target bases covered ≥80X	99.09	0.75	99.11	0.86
% *BRCA2* target bases covered ≥100X	98.97	1.03	98.91	1.03
% *BRCA2* target bases covered ≥200X	97.80	3.64	97.08	3.83
% *BRCA2* target bases covered ≥300X	96.37	6.74	94.42	7.49
% *BRCA2* target bases covered ≥400X	94.96	9.58	91.38	10.61
% *BRCA2* target bases covered ≥500X	93.31	12.17	87.79	13.97
% *BRCA2* target bases covered ≥1000X	82.04	21.46	69.37	24.70

Sequencing on the Ion Torrent Platform

All barcoded samples were sequenced on the PGM (Life Technologies), with 318 chips taking 16 samples on a single chip per sequencing run. The chip-loading procedure was performed twice according to the user's guide for the on Ion PGM sequencing 200 kit version 2 (Life Technologies).

Data Analysis

Raw sequence data analysis, including base calling, demultiplexing, alignment to the hg19 human reference genome (Genome Reference Consortium GRCh37), and variant calling, was performed using the Torrent Suite software version 4.0.2 (Life Technologies). For the variantCaller plugin, we used the optimized parameters for the *BRCA1* and *BRCA2* panel. Variants were annotated using Annovar[24] and in-house ad hoc bioinformatics tools. Alignments were visually verified with the Integrative Genomics Viewer version 2.1[25] and Alamut version 2.2 (Interactive Biosoftware, Rouen, France).

Variant analysis was performed without bias with a cascade of filtering steps previously described.[26] The reference sequences used were NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2*. All candidate variants were required on both sequenced DNA strands and to account for ≥20% of total reads at that site with a minimum depth of coverage of 80×. Common polymorphisms (≥5% in the general population) were discarded by comparison with dbSNP138, the 1000G (*http://www.1000genomes.org*, last accessed May 5, 2014), the Exome Variant Server (*http://evs.gs.washington.edu*, last accessed May 5, 2014), and an in-house exome variant database to filter out both common benign variants and recurrent artifact variant calls. However, because these databases also contain known disease-associated mutations, all detected variants were compared with our internal mutation database (CentoMD) and HGMD to directly identify and annotate changes previously described in the literature as definitely and likely pathogenic, uncertain, and neutral variants.

The 95% CIs were calculated by statistical inference using the SD.[27,28] In instances where there were no false-positive

results (SD = 0), the 95% CIs were produced with the Wilson score method.[29]

Evaluation of the Pathogenicity of the Variants

Evaluation of the pathogenicity of the variants not previously described in the literature and absent in the CentoMD and HGMD databases was performed with the following criteria. Mutations predicted to result in a premature truncated protein, including nonsense, frameshift mutations, large genomic rearrangements, and canonical splice site mutations, were classified as definitely pathogenic. Missense variants were considered *a priori* unclassified sequence variants, and their potential pathogenicity was evaluated taking into consideration the biophysical and biochemical difference between wild-type and mutant amino acid, the evolutionary conservation of the amino acid residue in orthologs,[30] a number of *in silico* predictors (Sift, Polyphen, Mutation taster, and Condel), and population data. Then unclassified sequence variants were classified into three groups: likely pathogenic, neutral, and variants of uncertain significance when previously conflicting information has been published about their functionality. Noncanonical splicing variants were analyzed using Alamut version 2.2 (Interactive Biosoftware), a software package that uses different splice site prediction programs to compare the normal and variant sequences for differences in potential regulatory signals. All candidate pathogenic variants not previously identified were confirmed by conventional PCR amplification and Sanger sequencing. Segregation of these changes with the disease was assessed for all available family members.

Results

Sequencing Statistics

The Ion AmpliSeq *BRCA1* and *BRCA2* Panel (Life Technologies) generates 167 amplicons of 200 bp on average, which cover all targeted coding exons and exon-intron boundaries (including 10 to 20 bases of flanking sequences around all targeted coding exons) of the *BRCA1* and *BRCA2* genes. It has been designed to yield sequence coverage redundancy with overlapping amplicons across exons. Sequencing of the *BRCA1* and *BRCA2* genes in the 210 patients generated a mean of 350,915 reads per patient. On average, 95.22% of these reads mapped to the targeted regions of *BRCA1* and *BRCA2*. In the validation cohort, an evenly distributed mean depth of coverage of 2331X and 2092X for *BRCA1* and *BRCA2* was achieved, respectively, on average across the 115 samples (Table 1). Ninety-nine percent of the targeted base pairs of *BRCA1* and 98% of *BRCA2* were covered by >100 reads. To determine whether coverage was substantially lower for any region, we calculated the proportion of base pairs that were captured by <80 reads, which is the minimum that we required to perform variant calling. The proportion of these poorly covered regions accounted for 0.23% of *BRCA1* and 0.91% *BRCA2* targeted base pairs, with all of them randomly spread over intronic regions at the ends the amplicons and sequencing reads. The mean coverage achieved in the discovery cohort was of 1882X and 1668X for *BRCA1* and *BRCA2*, respectively, across the 95 samples (Table 1). No faulty NGS amplicons with an average coverage <80X were detected in any of the samples of the two cohorts. Therefore, no Sanger repeats were required for gap filling in the NGS data. However, in the hypothetic case that in future analyses this problem happens, the faulty NGS amplicons would always be backup with Sanger repeats.

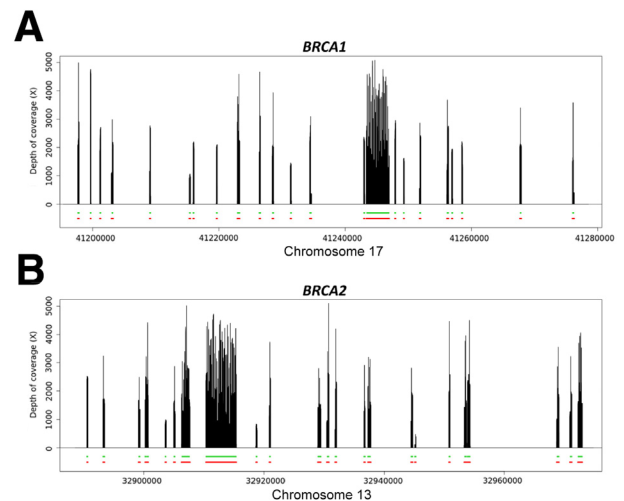

Figure 1 Representation of the mean depth of coverage of *BRCA1* (**A**) and *BRCA2* (**B**) in the validation and discovery cohorts. **Green lines** represent the exons of the genes, **red lines,** the amplicons of the assay.

From these data, we can conclude that all samples were uniformly covered at depths that in all cases exceed by far the minimum coverage required for reliable variant calling (Figure 1). The minor differences among samples were neutralized by the excessive overall coverage achieved by the assay. The sequence quality metrics of this data warrant a confident detection of variants in all patients.

Detection of *BRCA1* and *BRCA2* Variants in the Validation Cohort

The selection of the samples for this study was made with the idea of including as many different types of *BRCA1* and *BRCA2* variants as possible (inside and outside homopolymer regions), to simulate a real-world diagnostics scenario, so that we could test the performance of the NGS assay for different types of genetic variation. To assess the sensitivity of the assay, we blindly inspected all mapped sequence reads from the 115 samples with previously defined mutations of the validation cohort already analyzed with the conventional diagnostic workflow (D.T. and M.E.R.W.).

In the validation cohort, we identified all previously known mutations and variants, including single nucleotide variants and insertions and deletions, achieving a sensitivity of 100% (95% CI, 99.71%−100%). In summary, a total of 1311 (590 in *BRCA1* and 721 in *BRCA2*) variants in their

Table 2 *BRCA1* and *BRCA2* Mutations Identified in the 115 Samples of the Validation Cohort

Gene*	Location	cDNA change	Protein change	dbSNP138	HGMD	Variant type	Validation cohort	Discovery cohort	Homozygous/heterozygous of total
BRCA1	Ex. 07	c.503A>C	p.(K168T)	rs273901743	—	Uncertain	1	0	0/1 of 210
BRCA1	Ex. 07	c.536A>G	p.(Y179C)	rs56187033	CM030786	Uncertain	1	4	0/5 of 210
BRCA1	Ex. 10	c.1456T>C	p.(F486L)	rs55906931	—	Uncertain	1	4	0/5 of 210
BRCA1	Ex. 10	c.1648A>C	p.(N550H)	rs56012641	CM025218	Uncertain	1	4	0/5 of 210
BRCA1	Ex. 10	c.2071del	p.(R691fs)	rs80357688	CD982486	Definitely pathogenic	1	0	0/1 of 210
BRCA1	Ex. 10	c.3569C>T	p.(P1190L)	—	—	Likely pathogenic	1	0	0/1 of 210
BRCA1	Ex. 12	c.4236del	p.(A1412fs)	—	—	Likely pathogenic	1	0	0/1 of 210
BRCA1	Ex. 15	c.4535G>T	p.(S1512I)	rs1800744	CM960183	Uncertain	1	0	0/1 of 210
BRCA1	Ex. 16	c.4787C>A	p.(S1596*)	—	—	Likely pathogenic	2	3	0/5 of 210
BRCA1	Int. 16	c.4986+2T>A	p.(?)	—	—	Likely pathogenic	1	0	0/1 of 210
BRCA1	Ex. 17	c.5062G>T	p.(V1688F)	—	—	Likely pathogenic	1	0	0/1 of 210
BRCA1	Ex. 18	c.5096G>A	p.(R1699Q)	rs41293459	CM034007	Likely pathogenic	1	0	0/1 of 210
BRCA1	Ex. 19	c.5177_5180del	p.(1726_1727del)	rs80357975	CD972067	Definitely pathogenic	1	0	0/1 of 210
BRCA1	Ex. 20	c.5266dup	p.(Q1756fs)	rs80357906	CI941841	Definitely pathogenic	1	2	0/3 of 210
BRCA1	Int. 23	c.5468-10C>A	p.(?)	rs8176316	CS086718	Uncertain	1	0	0/1 of 210
BRCA2	Ex. 03	c.122C>T	p.(P41L)	—	—	Likely pathogenic	1	1	0/2 of 210
BRCA2	Ex. 05	c.467_468insT	p.(D156fs)	—	CI020251	Definitely pathogenic	1	0	0/1 of 210
BRCA2	Ex. 10	c.965_968del	p.(322_323del)	—	—	Likely pathogenic	1	0	0/1 of 210
BRCA2	Ex. 10	c.1151C>T	p.(S384F)	rs41293475	CM065036	Uncertain	1	0	0/1 of 210
BRCA2	Ex. 10	c.1550A>G	p.(N517S)	rs80358439	—	Uncertain	1	0	0/1 of 210
BRCA2	Ex. 10	c.1792A>G	p.(T598A)	rs28897710	CM035689	Uncertain	1	1	0/2 of 210
BRCA2	Ex. 10	c.1813dup	p.(I605fs)	rs80359308	CI972557	Definitely pathogenic	1	0	0/1 of 210
BRCA2	Ex. 11	c.2803G>A	p.(D935N)	rs28897716	CM994285	Uncertain	1	0	0/1 of 210
BRCA2	Ex. 11	c.3318C>G	p.(S1106R)	—	—	Likely pathogenic	1	0	0/1 of 210
BRCA2	Ex. 11	c.3503T>C	p.(M1168T)	—	—	Likely pathogenic	1	0	0/1 of 210
BRCA2	Ex. 11	c.4258G>T	p.(D1420Y)	rs28897727	CM003133	Uncertain	1	0	0/1 of 210
BRCA2	Ex. 12	c.6935A>T	p.(D2312V)	rs80358916	CS119639	Likely pathogenic	1	0	0/1 of 210
BRCA2	Int. 13	c.7008-62A>G	p.(?)	rs76584943	CS014426	Uncertain	1	1	0/2 of 210
BRCA2	Ex. 14	c.7068_7069del	p.(2356_2357del)	—	—	Likely pathogenic	1	0	0/1 of 210
BRCA2	Ex. 15	c.7544C>T	p.(T2515I)	rs28897744	CM994287	Uncertain	1	0	0/1 of 210
BRCA2	Ex. 18	c.8187G>T	p.(K2729N)	rs80359065	CM021957	Uncertain	1	0	0/1 of 210
BRCA2	Ex. 22	c.8851G>A	p.(A2951T)	rs11571769	CM970186	Uncertain	1	1	0/2 of 210
BRCA2	Int. 22	c.8954-3C>G	p.(?)	rs81002844	CS124767	Likely pathogenic	1	0	0/1 of 210
BRCA2	Ex. 23	c.9097_9098insT	p.(T3033fs)	—	—	Likely pathogenic	1	0	0/1 of 210

*NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2*.
Ex., exon; HGMD, Human Gene Mutation Database; Int., intron.

correct zygosity status were identified across the validation cohort. Notably, 150 (11.44%) of these variants were spread along different homopolymer stretches ($n \geq 4$) of the *BRCA1* and *BRCA2* genes, highlighting the robustness of the NGS assay even in complex genomic regions. No spurious pathogenic mutations were found in any of the samples of the validation cohort. A summary of the definitely pathogenic mutations, likely pathogenic mutations, and uncertain variants in these samples, as detected by conventional Sanger sequencing, is listed in Table 2. An overview of all neutral variants identified in this study is given in Supplemental Table S1.

Thirty-three of the eighty-two unique variants included in the validation cohort were present in two or more patients. This finding reveals a consistent detection of sequence variants between two or more samples. Because most of the samples bearing similar variants were multiplexed in independent sample pools and were analyzed in different sequencing runs, we conclude that this NGS strategy offers great robustness for the detection of *BRCA1* and *BRCA2* sequence variants.

To assess the specificity of the assay across the targeted bases of the *BRCA1* and *BRCA2* genes, we evaluated all sequenced positions previously screened by Sanger sequencing in the validation cohort. Genotype data were available across the 115 samples of the validation cohort for a total of 968,415 and 1,614,715 sites within the targeted regions of *BRCA1* and *BRCA2*, respectively. Specificity of detecting non-variant sites from the reference genome was 99.99% (967,814/967,825; 95% CI, 99.99%−100%) for *BRCA1* and 99.99% (1,613,878/1,613,994; 95% CI, 99.99%−100%) for *BRCA2*.

The positive predictive value of the assay, calculated as (number of true positives)/(number of true positives + number of false positives), was 91.17% [(1311)/(1311+127); 95% CI, 89.72%−92.62%]; 98.17% [(590)/(590+11); 95% CI,

Table 3 *BRCA1* and *BRCA2* Mutations Identified in the 95 Samples of the Discovery Cohort

Gene*	Location	cDNA change	Protein change	dbSNP138	HGMD	Variant type	Validation cohort	Discovery cohort	Homozygous/heterozygous of total
BRCA1	Ex. 02	c.66_67del	p.(22_23del)	rs199805151	—	Likely pathogenic	0	1	0/1 of 210
BRCA1	Int. 02	c.81-2del	p.(?)	rs273902791	CD041902	Definitely pathogenic	0	1	0/1 of 210
BRCA1	Ex. 07	c.536A>G	p.(Y179C)	rs56187033	CM030786	Uncertain	1	4	0/5 of 210
BRCA1	Int. 07	c.547+2T>A	p.(?)	rs80358047	CS973719	Definitely pathogenic	0	1	0/1 of 210
BRCA1	Ex. 08	c.557C>A	p.(S186Y)	rs55688530	CS118470	Uncertain	0	1	0/1 of 210
BRCA1	Ex. 08	c.591C>T	p.(C197C)	rs1799965	CS1211734	Uncertain	0	1	0/1 of 210
BRCA1	Ex. 10	c.736T>G	p.(L246V)	rs28897675	CS042532	Definitely pathogenic	0	1	0/1 of 210
BRCA1	Ex. 10	c.798_799del	p.(266_267del)	rs80357724	CD951604	Definitely pathogenic	0	1	0/1 of 210
BRCA1	Ex. 10	c.1088A>G	p.(N363S)	—	—	Likely pathogenic	0	1	0/1 of 210
BRCA1	Ex. 10	c.1175_1214del	p.(392_405del)	—	—	Likely pathogenic	0	1	0/1 of 210
BRCA1	Ex. 10	c.1456T>C	p.(F486L)	rs55906931	—	Likely pathogenic	1	4	0/5 of 210
BRCA1	Ex. 10	c.1648A>C	p.(N550H)	rs56012641	CM025218	Uncertain	1	4	0/5 of 210
BRCA1	Ex. 10	c.2157dup	p.(E720fs)	rs80357715	CI962222	Definitely pathogenic	0	1	0/1 of 210
BRCA1	Ex. 10	c.2281G>T	p.(E761*)	—	—	Likely pathogenic	0	5	0/5 of 210
BRCA1	Ex. 10	c.2315T>C	p.(V772A)	rs80357467	CM940174	Uncertain	0	1	0/1 of 210
BRCA1	Ex. 10	c.2521C>T	p.(R841W)	rs1800709	CM004236	Uncertain	0	1	0/1 of 210
BRCA1	Ex. 10	c.2668G>A	p.(G890R)	rs80357200	—	Uncertain	0	1	0/1 of 210
BRCA1	Ex. 10	c.3748G>T	p.(E1250*)	rs28897686	CM940177	Definitely pathogenic	0	1	0/1 of 210
BRCA1	Ex. 10	c.3756_3759del	p.(1252_1253del)	rs80357868	CD941618	Definitely pathogenic	0	2	0/2 of 210
BRCA1	Ex. 10	c.4039A>G	p.(R1347G)	rs28897689	CM960181	Uncertain	0	1	0/1 of 210
BRCA1	Ex. 11	c.4136_4137del	p.(1379_1379del)	—	CD065671	Definitely pathogenic	0	2	0/2 of 210
BRCA1	Ex. 12	c.4327C>G	p.(R1443G)	rs41293455	CM940179	Uncertain	0	1	0/1 of 210
BRCA1	Ex. 15	c.4636G>A	p.(D1546N)	rs28897691	—	Uncertain	0	1	0/1 of 210
BRCA1	Ex. 16	c.4689C>G	p.(Y1563*)	rs80357433	CM960185	Definitely pathogenic	0	1	0/1 of 210
BRCA1	Ex. 16	c.4787C>A	p.(S1596*)	—	—	Likely pathogenic	2	3	0/5 of 210
BRCA1	Int. 17	c.5074+2T>C	p.(?)	rs80358089	—	Likely pathogenic	0	1	0/1 of 210
BRCA1	Int. 17	c.5075-2A>C	p.(?)	rs80358066	CS982089	Definitely pathogenic	0	1	0/1 of 210
BRCA1	Ex. 18	c.5138T>C	p.(V1713A)	rs80357132	CM950154	Definitely pathogenic	0	2	0/2 of 210
BRCA1	Ex. 20	c.5266dup	p.(Q1756fs)	rs80357906	CI941841	Definitely pathogenic	1	2	0/3 of 210
BRCA2	Ex. 03	c.122C>T	p.(P41L)	—	—	Likely pathogenic	1	1	0/2 of 210
BRCA2	Ex. 03	c.231T>G	p.(T77T)	rs114446594	CS118469	Uncertain	0	1	0/1 of 210
BRCA2	Ex. 10	c.1792A>G	p.(T598A)	rs28897710	CM035689	Uncertain	1	1	0/2 of 210
BRCA2	Ex. 11	c.1964C>G	p.(P655R)	rs28897712	—	Uncertain	0	1	0/1 of 210
BRCA2	Ex. 11	c.2138A>T	p.(Q713L)	rs55816687	CM057493	Uncertain	0	1	0/1 of 210
BRCA2	Ex. 11	c.3367A>G	p.(S1123G)	rs80358581	—	Uncertain	0	1	0/1 of 210
BRCA2	Ex. 11	c.5198C>T	p.(S1733F)	rs55639415	—	Uncertain	0	1	0/1 of 210
BRCA2	Ex. 11	c.5216A>C	p.(Y1739S)	—	—	Likely pathogenic	0	1	0/1 of 210
BRCA2	Ex. 11	c.5312G>A	p.(G1771D)	rs80358755	CM041731	Uncertain	0	1	0/1 of 210
BRCA2	Ex. 11	c.5718_5719del	p.(1906_1907del)	rs80359530	—	Likely pathogenic	0	1	0/1 of 210
BRCA2	Ex. 11	c.5946del	p.(S1982fs)	rs80359550	CD961857	Definitely pathogenic	0	2	0/2 of 210
BRCA2	Ex. 13	c.7007G>A	p.(R2336H)	rs28897743	CM053138	Definitely pathogenic	0	1	1/0 of 210
BRCA2	Int. 13	c.7008-62A>G	p.(?)	rs76584943	CS014426	Uncertain	1	1	0/2 of 210
BRCA2	Ex. 18	c.8215G>A	p.(V2739I)	rs80359069	—	Uncertain	0	1	0/1 of 210
BRCA2	Ex. 22	c.8830A>T	p.(I2944F)	rs4987047	CM050183	Uncertain	0	1	0/1 of 210
BRCA2	Ex. 22	c.8851G>A	p.(A2951T)	rs11571769	CM970186	Uncertain	1	1	0/2 of 210
BRCA2	Ex. 23	c.9117G>A	p.(P3039P)	rs28897756	CS004014	Definitely pathogenic	0	1	0/1 of 210
BRCA2	Ex. 24	c.9154C>T	p.(R3052W)	rs45580035	CM082511	Definitely pathogenic	0	1	0/1 of 210
BRCA2	Ex. 26	c.9586A>G	p.(K3196E)	rs80359228	CM003134	Uncertain	0	2	0/2 of 210
BRCA2	Ex. 26	c.9634G>C	p.(G3212R)	rs55775473	—	Uncertain	0	1	0/1 of 210

*NM_007294.3 for *BRCA1* and NM_000059.3 for *BRCA2*.

Ex., exon; HGMD, Human Gene Mutation Database; Int., intron.

97.12%−99.22%] for *BRCA1*, and 86.14% [(721)/(721+116); 95% CI, 83.81%−88.47%] for *BRCA2*. Notably, none of these 127 (26 unique) false-positive variants were predicted to have a pathological outcome (ie, spurious mutation calls), and the difference between *BRCA1* and *BRCA2* might in part be explained by the larger size of the latter. The negative predictive value of the assay, calculated as (number of true negatives)/(number of true negatives + number of false negatives), was 100% [(2,581,692)/(2,581,692+0); 95% CI, 100%−100%], 100% [(967,803)/(967,803+0); 95% CI, 100%−100%] for *BRCA1*, and 100% [(1,613,878)/(1,613,878+0); 95% CI, 100%−100%] for *BRCA2*.

Identification of *BRCA1* and *BRCA2* Mutations in the Discovery Cohort

In the discovery cohort, 95 samples not previously tested were blindly sequenced (ie, no prior information was available about their genotype neither to the NGS sequencing team nor to the bioinformatics group; J.S., J.K., and T.Z.) with the NGS approach tested here. We identified 48 (29 unique) and 22 (20 unique) definitely pathogenic, likely pathogenic and uncertain variants in the *BRCA1* and *BRCA2* genes, respectively. These variants were identified in 49 of the 95 patients in the discovery cohort, and all of them were confirmed with Sanger sequencing (Table 3).

Discussion

After the tremendous impact of NGS technologies on the discovery of disease-causing genes during the last 4 years, we are now witnessing the introduction of these technologies for diagnostic applications. The aim is to rapidly revolutionize the field of genetic diagnostics, making it much more cost- and time effective and accurate. During the last three decades, Sanger sequencing of individual DNA fragments has been the gold standard for the identification of clinically relevant mutations in the terms of routine diagnostics, including the *BRCA1* and *BRCA2* genes. However, this rather costly, stepwise, and time-consuming technology will be gradually replaced by NGS technologies, which offer higher throughput and scalability and, as a corollary, reduced costs per sequenced nucleotide and a shorter turnaround time.[27]

Our aim was to evaluate and establish an NGS workflow based on the low-cost, moderate-throughput Ion Torrent PGM benchtop next-generation sequencer (Life Technologies) as a routine method for the genetic screening of *BRCA1* and *BRCA2* for HBOC diagnostics. We validated the assay in a cohort of 115 patients who had previously undergone full *BRCA1* and *BRCA2* Sanger sequencing. After mapping the sequencing reads to the reference genome and performing blind variant calling and filtering (D.T. and M.E.R.W.), our bioinformatics pipeline successfully retrieved all known sequence variants in their correct zygosity status. With this approach, we were able to identify a heterogeneous panel of *BRCA1* and *BRCA2* variants, including single nucleotide variants and insertions and deletions, even in homopolymer sequences. Then, we analyzed a discovery cohort of uncharacterized patients and reached a diagnostic rate of 51.57% (49 of 95 patients), allowing test reporting 7 days after receiving the DNA samples. Our results indicate the suitability of this NGS configuration for the routine clinical diagnosis of HBOC predisposition.

Although the Ion AmpliSeq *BRCA1* and *BRCA2* Panel (Life Technologies) includes 10 to 20 bp of intronic flanking sequences around all coding exons of both genes, in some cases it can detect intronic variants, which are >60 nucleotides away from the splice junction sites (Supplemental Table S1), such as c.441+64T>G, because of the design constraints of some amplicons. The problem with variants detected in these distant positions is that they are not always called with both the Sanger and NGS assays because the amplicons of the two assays do not perfectly overlap in these deep intronic regions. For instance, the c.441+64T>G variant is specific of the NGS assay because the Sanger primers we had did not cover that position. In addition, because this (and most of the) intronic variant(s) has no predicted potential pathogenicity, confirmation is not necessary. Therefore, it was neither tested with Sanger, nor considered in the genotype comparisons of the validation cohort. However, there are other examples of such variants (eg, c.8755-66T>C) in overlapping NGS and Sanger amplicons that were called with both the Sanger and NGS assays and that were considered for the validation study.

Although our study does not include intra- or interassay precision assessment as well as carryover or specimen stability assessment, we believe that the comprehensive NGS versus Sanger genotype comparison performed in the validation cohort provides very robust statistics about the sensitivity and specificity of the NGS assay.

Recently, different NGS platforms and genomic enrichment strategies have been tested for the identification of sequence variants in *BRCA1* and *BRCA2* for HBOC diagnostics.[26,27,31−38] In our case, we decided to adopt in our HBOC diagnostics workflow the PGM in combination with the Ion AmpliSeq *BRCA1* and *BRCA2* Panel (Life Technologies) because it delivers fast turnaround time coupled with throughput flexibility, enabling rapid time to results in processing either a small or large number of samples. In addition, it offers fast library construction for affordable targeted sequencing of the *BRCA1* and *BRCA2* genes based on ultrahigh-multiplex PCR, requiring as low as 30 ng of input DNA. All these arguments make it a convenient NGS configuration easily adaptable by diagnostic laboratories and an accurate, economical, and easy-to-implement end-to-end solution.

We foresee that the transition during the next years of NGS technologies from basic research to the routine detection of mutations in genetic loci with well-documented diagnostic value will take advantage not only of the new benchtop NGS platforms, which can be much more easily incorporated in the daily clinical practice, but also of automated workflows and simplified bioinformatics analyses able to generate medical report-like outputs adapted to clinical laboratories. Currently, our ability to discover genetic variation in a patient genome is running far ahead of our ability to interpret that variation. The success of NGS for medical genetics hinges on the accuracy in distinguishing causal from benign alleles, which is the key challenge for interpreting DNA sequence data for diagnostics. We are still in the process of defining the methods and guidelines for the application of NGS to clinical genetic diagnostics, mainly by defining the variant calling and exclusion metrics based on sequence quality and depth of coverage. The almost perfect sensitivity and

specificity and the absence of false-positive and false-negative results indicate the unnecessary redundant Sanger confirmation of the *BRCA1* and *BRCA2* mutations detected using NGS. However, in this initial phase, we still recommend that novel mutations are validated by Sanger sequencing before informing the patient, although the feasibility of targeted NGS as a standalone diagnostics test has already been suggested.[39]

Clinical diagnostic tools must meet very stringent sensitivity and specificity parameters, while keeping their cost- and time effectiveness. We estimate that this high-throughput NGS assay represents a minimum of twofold cost savings per sample and makes the whole diagnostic process at least 4 times faster when compared with the techniques conventionally used for the genetic testing of the *BRCA1* and *BRCA2* genes. These numbers are consistent with a previous study assessing cost and turnaround time of a similar NGS configuration for HBOC diagnostics.[27] In addition, this strategy offers a complete definition of the two genes without the need, anymore, for stepwise testing and choosing which gene or exon to sequence first. The challenge for shortening the time to give results will allow the implementation of high-throughput sequencing in a new era for widespread *BRCA1* and *BRCA2* screening, avoiding the time-consuming conventional approaches and making possible faster reporting times of HBOC genetic predisposition for the early implementation of personalized pharmacogenetic treatments using PARPs a few days after DNA withdrawal for genetic testing.

In conclusion, this study reveals the feasibility and readiness of comprehensive genetic testing of the *BRCA1* and *BRCA2* genes in a benchtop NGS platform for HBOC diagnostics. It is cost- and time effective and meets the sensitivity and specificity requirements demanded for genetic diagnostics, providing NGS and bioinformatics approaches ready to substitute classic molecular tools in routine genetic diagnostics setups. Therefore, we have incorporated this assay in our HBOC diagnostics workflow.

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Supplemental Data

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