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Benchmarking of Amplicon-Based Next-Generation Sequencing Panels Combined with Bioinformatics Solutions for Germline *BRCA1* and *BRCA2* Alteration Detection

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The recent deployment of next-generation sequencing approaches in routine laboratory analysis has considerably modified the landscape of *BRCA1* and *BRCA2* germline alteration detection in patients with a high risk of developing breast and/or ovarian cancer. Several commercial multiplex amplicon-based panels and bioinformatics solutions are currently available. In this study, we evaluated the combinations of several *BRCA* testing assays and bioinformatics solutions for the identification of single-nucleotide variants, insertion/deletion variants, and copy number variations (CNVs). Four assays (*BRCA* Tumor, *BRCA* HC, Ion AmpliSeq *BRCA*, and Access Array *BRCA*) and two commercial bioinformatics solutions (SeqNext software version 4.3.1 and Sophia DDM version 5.0.13) were tested on a set of 28 previously genotyped samples. All solutions exhibited accurate detection of single-nucleotide variants and insertion/deletion variants, except for Ion AmpliSeq *BRCA*, which exhibited a decrease in coverage. Of interest, for CNV analysis, the best accuracy was observed with the Sophia DDM platform regardless of the *BRCA* kit used. Finally, the performance of the most relevant combination (*BRCA* Tumor and Sophia DDM) was blindly validated on an independent set of 152 samples. Altogether, our results emphasize the need to accurately compare and control both molecular next-generation sequencing approaches and bioinformatics pipelines to limit the number of discrepant alterations and to provide a powerful tool for reliable detection of genetic alterations in *BRCA1* and *BRCA2*, notably CNVs. *(J Mol Diagn 2018, 20: 754–764; https://doi.org/10.1016/j.jmoldx.2018.06.003)*

Since their discovery in the 1990s, the *BRCA1* and *BRCA2* genes have been widely reported as the main penetrant genes causing a hereditary predisposition to breast and ovarian cancer syndrome.[1,2] Germline mutations in *BRCA1* and *BRCA2* confer a cumulative risk of breast cancer at the age of 70 years, estimated at 57% to 65% and 45% to 55%, respectively, and a risk of ovarian cancer, estimated at 39% to 44% and 11% to 18%, respectively.[3–5] The identification of a deleterious *BRCA1* or *BRCA2* alteration within a family has important consequences for patients' medical care, including enhanced screening and often prophylactic (risk-reducing) surgery.[6,7] Recently, the poly(adenosine diphosphate–ribose) polymerase inhibitor olaparib (Lynparza, Astrazeneca, Cambridge, UK) has been approved by the US Food and Drug Administration as a maintenance

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treatment for patients with a high-grade serous ovarian carcinoma, carrying *BRCA1* or *BRCA2* mutations at the germline or somatic level.[8,9] In 2017, olaparib has also been shown to have promising activity in patients with *BRCA*-associated metastatic breast cancer and may represent a new therapeutic option for these women in the next few years.[10] For all of these reasons, genetic testing for germline mutations in *BRCA* has become crucial for both prevention and therapeutic aims.

A broad range of pathogenic variations distributed throughout the *BRCA1* and *BRCA2* genes have been reported in the literature. These aberrations predominantly include single-nucleotide variations (SNVs) and short insertion/deletion variations (indels) located in the coding regions or near intron-exon boundaries that affect exon splicing. Another kind of deleterious mutation involved in hereditary predispositions to breast and ovarian cancer syndrome is large genomic rearrangements with the deletion or duplication of one or several exons, known as a copy number variation (CNV).

Until the past few years, Sanger sequencing and multiplex ligand probe-dependent amplification (MLPA) remained the gold standards for the detection of SNVs/indels and CNVs, respectively. However, the use of these approaches for the genetic testing of *BRCA* genes is particularly complex, time consuming, and expensive, and requires extensive technical labor. The emergence of the next-generation sequencing (NGS) technologies has considerably modified the genetic testing workflow in molecular diagnostic laboratories. In particular, NGS allowed laboratories to increase throughput by sample multiplexing and to simultaneously test *BRCA1/2*, thus reducing the cost and the time required to deliver genetic testing results.[11] Amplicon-based panel approaches are currently used routinely in laboratories for *BRCA* gene testing.[12] These multiplex PCR-based methods are suited for sequencing a small number of genes to detect SNVs and indels. However, one of the main difficulties still encountered by PCR enrichment methods is the specific and accurate detection of CNVs.

Several multiplex PCR kits are currently commercially available or ready for use. To the best of our knowledge, a direct and systematic comparison of these methods has so far never been performed. In the attempt to fill this gap, four amplicon-based kits for library preparation and two bioinformatics software programs were benchmarked on a set of 28 samples and a blind validation of the most relevant solution was performed (J.A.V. and M.L.) on an independent cohort of 152 samples to determine the best approach for the genetic testing of *BRCA* genes.

Materials and Methods

Patients and DNA Samples

All patients included in the study underwent pretest counseling, during which they were informed about the significance of molecular screening and signed a written informed consent form. A total of 180 genomic DNA (gDNA) samples from unrelated individuals clinically diagnosed with a cancer predisposition syndrome and previously characterized by conventional approaches (Sanger sequencing and/or MLPA) were selected and divided into two groups: a first set (set 1, $n = 28$), composed of samples harboring commonly analyzed genetic alterations, on which three or four different amplicon-based NGS panels were tested; and a second set (set 2, $n = 152$) to blindly evaluate the most appropriate approach on a larger cohort.

Blood samples were exclusively collected in EDTA tubes, and gDNA was extracted from blood lymphocytes by using the MagNA Pure Compact Nucleic Acid Isolation Kit I—Large Volume (Roche Diagnostics, Meylan, France) on the MagNA Pure Compact instrument (Roche Diagnostics), according to the manufacturer's recommendations. Extracted DNA was quantified by using the Qubit dsDNA Broad Range Assay Kit in combination with a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA).

Ion AmpliSeq *BRCA1* and *BRCA2* Panel Experiment

The *BRCA1* and *BRCA2* genes were amplified with the Ion AmpliSeq *BRCA1* and *BRCA2* Community Panel (Thermo Fisher Scientific), which consists of three primer pools covering 167 amplicons. gDNA (10 ng) was used to prepare libraries, according to the manufacturer's recommendations. Briefly, target regions from DNA were amplified by using the AmpliSeq *BRCA1* and *BRCA2* primer pools. The primer sequences were then partially digested with FuPa reagent, and adapters and barcodes were ligated with DNA ligase. The libraries were purified by using Agencourt AMPure XP (Beckman Coulter, Nyon, Switzerland); amplified by PCR, as described in the user guide; purified again; and quantified on a Qubit 2.0 fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). On the basis of the calculated library concentrations, the libraries were pooled to equimolar concentration. Emulsion PCR and chip loading were then performed with an Ion Chef in combination with the Ion PGM Hi-Q Chef Kit and the Ion 318 Chip Kit v2, according to the manufacturer's recommendations (Thermo Fisher Scientific). Finally, sequencing was performed on an Ion PGM sequencer with the Ion PGM Hi-Q Sequencing Kit and analyzed with the use of the Ion Reporter software version 4.4 (Thermo Fisher Scientific).

Multiplicom BRCA MASTR Panel Assays

Two amplicon-based panels from Multiplicom (Niel, Belgium) were tested, following the manufacturer's instructions: the *BRCA* MASTR Plus Dx (*BRCA* Tumor) and the *BRCA* Hereditary Cancer MASTR Plus (*BRCA* HC), which consist of four and five primer pools, respectively. Briefly, 30 ng of gDNA was used per target-specific multiplex PCR run. A second PCR round was then performed to incorporate molecular barcodes and sequencing adaptors. The PCR products were then purified with Agencourt AMPure XP, quantified on a

Qubit instrument, and pooled to equimolar concentrations. The libraries were paired-end sequenced (2 × 250 cycles) on a MiSeq instrument (Illumina, San Diego, CA). The *BRCA* HC panel allowed the detection of alterations in 23 additional genes, but only results for *BRCA1* and *BRCA2* genes were considered in the present study.

Access Array *BRCA1/BRCA2* Target-Specific Panel Assay

gDNA (50 ng) was used for library preparation. Regions of interest were amplified by using the Access Array *BRCA1/BRCA2* Target-Specific Panel (Fluidigm, San Francisco, CA) in combination with a 48.48 Fluidigm Access Array System. Libraries were then collected, indexed, pooled, and quantified by using D1000 ScreenTapes and a 4200 TapeStation instrument (Agilent Technologies, Santa Clara, CA). Pair-end sequencing (2 × 150 cycles) was finally performed with a MiSeq instrument (Illumina).

Bioinformatics NGS Data Analysis

FASTQ files were analyzed with the use of two bioinformatics software programs: Sophia DDM version 5.0.13 (SOPHiA GENETICS, Saint Sulpice, Switzerland) and SeqNext version 4.3.1 (JSI Medical Systems, Ettenheim, Germany). The *BRCA1* (NM_007294.2) and *BRCA2* (NM_000059.3) sequences from the National Center for Biotechnology Information database were used as references.

The Sophia DDM platform relies on patented advanced technologies that combined three algorithms for alteration detection: PEPPER is used for accurate SNP and indel detection, MUSKAT is used for CNV identification, and MOKA is used for annotation. The SeqNext software is based on different algorithms. In this software, filters were specifically defined for the detection of germline alterations. Thus, reads for which 50% of the bases have a quality score <20 (representing an error rate of 1 in 100, with a corresponding call accuracy of 99%) and amplicons with a read depth <50× were excluded from the analysis. Moreover, whatever the bioinformatics analysis used, variants with a variant allele frequency (VAF) <20% were filtered out, because they were considered artifacts in the context of germline alteration detection.

For CNV detection, calling was performed for each multiplex PCR by comparing the relative coverage of one sample with the other samples within the same experiment. For this purpose, the composition of the primer plexes must be known. A CNV is reported when all the amplicons that target a specific exon are deleted or duplicated.

All variants were reported according to the Human Genome Variation Society guidelines and classified according to the Universal Mutation Database for *BRCA*,[13] ClinVar, the Breast International Consortium database, or the Leiden Open Variation Database.[14]

Results

Comparison of *BRCA* Panel Specifications

Four commercially available amplicon-based kits for *BRCA* testing were selected for benchmarking: i) the *BRCA* MASTR Plus Dx Panel (*BRCA* Tumor; Multiplicom), ii) the *BRCA* Hereditary Cancer MASTR Plus Panel (*BRCA* HC;

Table 1 Specifications of the Amplicon-Based Panels Evaluated in the Study

Variable	*BRCA* Tumor	*BRCA* HC*	Access Array *BRCA*	Ion AmpliSeq *BRCA*
Target region size, nt	20,620	24,824	20,055	22,419
Number of amplicons	181	97 (561 Amplicons in total)	184	167
Minimum amplicon length, bp	122	240	149	126
Maximum amplicon length, bp	229	390	209	298
Minimum overlap between amplicons, nt	3	19	0	1
Maximum overlap between amplicons, nt	106	184	107	107
Median overlap between amplicons, nt	24	68	33	6
Minimum intronic region targeted, nt†	−15; +5	−12; +5	−10; +1	−7; +20
Maximum intronic region targeted, nt†	−80; +78	−220; +201	−110; +110	−156; +158
Median intronic region targeted, nt†	−52; +47	−79; +68	−37; +34	−69; +55
Target region in the 5′-UTR of *BRCA1*‡	Full exon 2 with 37 nt in intron 1	5 nt of the 5′-UTR are missing (exon 1)	5 nt upstream the start codon	Full exon 2 with 116 nt in intron 1
Target region in the 3′-UTR of *BRCA1*	66 nt downstream the stop codon	133 nt downstream the stop codon	95 nt downstream the stop codon	93 nt downstream the stop codon
Target region in the 5′-UTR of *BRCA2*‡	36 nt upstream the start codon	32 nt upstream the start codon	Full exon 2 with 60 nt in intron 1	Full exon 2 with 15 nt in intron 1
Target region in the 3′-UTR of *BRCA2*	50 nt downstream the stop codon	146 nt downstream the stop codon	54 nt downstream the stop codon	72 nt downstream the stop codon

*Specifications presented are only related to *BRCA1* and *BRCA2* genes.
†Upstream or downstream intronic region targeted (−nt; +nt).
‡The start codon is located in the exon 2 of *BRCA1* and *BRCA2*.
nt, nucleotides; UTR, untranslated region.

Multiplicom), iii) the Ion AmpliSeq *BRCA1* and *BRCA2* Panel (Ion AmpliSeq *BRCA*; Thermo Fisher Scientific), and iv) the Access Array *BRCA1*/*BRCA2* Target-Specific Panel (Access Array *BRCA*; Fluidigm).

The specifications of each panel were first analyzed, such as the target region size, number and size of amplicons, overlapping of amplicons, and coverage of the intron/exon boundary regions (Table 1). The *BRCA* HC Panel covered the largest sequence of *BRCA1* and *BRCA2* genes [24,824 nucleotides (nt)], with important coverage of the noncoding sequences (intron, 5′-untranslated region and 3′-untranslated region). However, this panel included the lowest number of amplicons ($n = 97$), which was compensated for by the length of the amplicons (from 240 to 390 bp). Indeed, the Ion AmpliSeq *BRCA* Panel (from 126 to 390 bp) and the *BRCA* HC Panel displayed the longest amplicons, making them unsuitable for fragmented or low-quality DNA.

One limitation of amplicon-based panels could be the level of overlap between amplicons. Indeed, the presence of a variant (polymorphisms, single-nucleotide variants, or short indels) in the primer binding site may prevent primer hybridization, leading to an allele bias amplification of the amplicon. Thus, a deleterious mutation present on the same allele will not be detected and, consequently, would lead to false-negative (FN) results.[15–17] One solution to overcome this issue is to increase amplicon overlapping, as previously shown by Chong et al[17] for *BRCA1* and *BRCA2* genetic alteration detection. In the panels tested, the minimum overlap between different amplicons was zero, one, or three nucleotides for the Access Array *BRCA* Panel, Ion AmpliSeq *BRCA* Panel, and *BRCA* Tumor Panel, respectively (Table 1). For the Access Array Panel, this low-overlap issue could be easily overcome, because this particular technology allowed the complete customization of the panel by the addition/removal of primers at any time. Furthermore, for this panel and the *BRCA* Tumor Panel, low-level tiling is limited to small numbers of amplicons, with a median overlap between amplicons of 33 and 24 nt, respectively. However, this concern could be more problematic with the Ion AmpliSeq *BRCA* Panel, because the median overlap between amplicons is only 6 nt.

Alterations at splicing-donor and splicing-acceptor sites may also have deleterious effects because of affecting the splicing process. Mutations at canonical AG/CT splice sites (−2; +2) have always been reported to disrupt splicing. In addition, alterations located outside these regions can also induce aberrant exon skipping of *BRCA1* and *BRCA2*.[18–21] Except for the Access Array *BRCA* Panel, all the panels covered at least the canonical splicing sites.

Experimental Design and Workflow Setup

A first set (set 1) of 28 samples previously characterized by Sanger and MLPA was used for the benchmarking study. Libraries were sequenced by using a MiSeq or a PGM sequencing system, according to the kits used (Figure 1). The performances of two bioinformatics approaches, the SeqNext software and the Sophia DDM platform, were also evaluated (Figure 1). To adequately challenge the different methods, samples affected by genetic alterations, which are commonly analyzed in hereditary predispositions to breast and ovarian cancer syndrome, were specifically selected. Thus, 16 samples were affected by deleterious SNVs or indels (nine in *BRCA1* and seven in *BRCA2*), seven samples had variants of unknown significance, five samples exhibited CNVs involving *BRCA1*, and two samples had polymorphisms (neutral variants) (Table 2). Among the indels, seven samples harbored a deletion of 1 to 10 nt, five samples had an insertion or a duplication of 1 to 28 nt, and one sample had an insertion-deletion. The CNVs consisted of heterozygous deletions in *BRCA1* of exons 1 to 2, 1 to 22, 16 to 17, and 21 to 24 as well as a duplication of exon 13. For the comparison study, 17 samples were analyzed by each of the four approaches (*BRCA* Tumor, *BRCA* HC, Ion AmpliSeq *BRCA*, and Access Array *BRCA*) and 11 samples

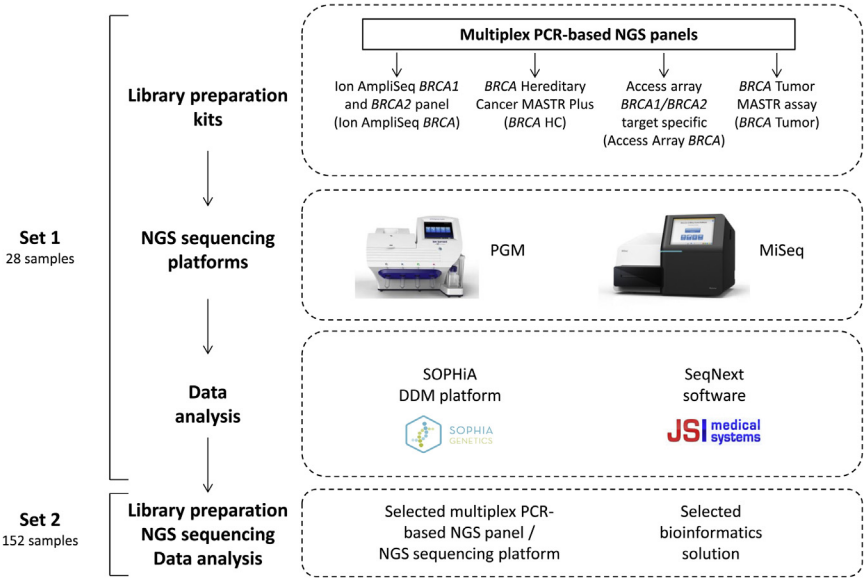

Figure 1 Design of benchmarking experiments. Four PCR-based kits and two bioinformatics software solutions were assessed on a set of 28 samples previously characterized for the detection of *BRCA1* and *BRCA2* alterations. An independent cohort of 152 samples was used for validation. NGS, next-generation sequencing.

Table 2 *BRCA1* and *BRCA2* Alterations Harbored by the Samples Present in Set 1

Sample	Alteration type	Gene	Location	HGVS cDNA*	HGVS protein†	Clinical relevance‡
S01	CNV	*BRCA1*	Del exon 1 to 22	c.1-?_5406+?del	p.?	Deleterious
S02	CNV	*BRCA1*	Del exon 16 to 17	c.4676-?_5074+?del	p.?	Deleterious
S03	SNV	*BRCA2*	Exon 10	c.887A>G	p.Tyr296Cys	VUS
S03	Indel	*BRCA2*	Exon 16	c.7638_7647del	p.Lys2547*	Deleterious
S04	Indel	*BRCA2*	Exon 11	c.6596del	p.Thr2199Ilefs*7	Deleterious
S05	Indel	*BRCA1*	Exon 20	c.5266dup	p.Gln1756Profs*74	Deleterious
S06	Indel	*BRCA1*	Exon 11	c.798_799del	p.Ser267Lysfs*19	Deleterious
S07	SNV	*BRCA2*	Exon 11	c.2830A>T	p.Lys944*	Deleterious
S08	Indel	*BRCA2*	Exon 23	c.9026_9030del	p.Tyr3009Serfs*7	Deleterious
S09	Indel	*BRCA1*	Exon 11	c.927_928insGAAAACC	p.Gln310Glufs*12	Deleterious
S10	Indel	*BRCA1*	Exon 11	c.2709_2710del	p.Cys903*	Deleterious
S11	SNV	*BRCA2*	Exon 11	c.5986G>A	p.Ala1996Thr	VUS
S12	NA	NA	NA	NA	NA	NA
S13	NA	NA	NA	NA	NA	NA
S14	SNV	*BRCA2*	Exon 11	c.5682C>G	p.Tyr1894*	Deleterious
S15	Indel	*BRCA2*	Exon 11	c.6024dup	p.Gln2009Alafs*9	Deleterious
S16	SNV	*BRCA2*	Exon 11	c.3419G>A	p.Ser1140Asn	VUS
	SNV	*BRCA2*	Exon 25	c.9382C>T	p.Arg3128*	Deleterious
S17	Indel	*BRCA1*	Exon 11	c.3477_3480del	p.Ile1159Metfs*50	Deleterious
S18	Indel	*BRCA1*	Exon 11	c.3839_3844delinsAGGC	p.Ser1280*	Deleterious
S19	Indel	*BRCA1*	Exon 11	c.1953dup	p.Lys652Glufs*21	Deleterious
S20	SNV	*BRCA1*	Exon 18	c.5100A>G	p.=	VUS
S21	SNV	*BRCA2*	Exon 11	c.5635G>A	p.Glu1879Lys	VUS
S22	SNV	*BRCA2*	Exon 15	c.7448G>A	p.Ser2483Asn	VUS
S23	SNV	*BRCA1*	Exon 13	c.4213A>G	p.Ile1405Val	VUS
S24	Indel	*BRCA1*	Exon 11	c.3949_3976dup	p.His1326Leufs*13	Deleterious
S25	Indel	*BRCA1*	Exon 17	c.5030_5033del	p.Thr1677Ilefs*2	Deleterious
S26	CNV	*BRCA1*	Dup exon 13	c.4186-?_4357+?dup	p.?	Deleterious
S27	CNV	*BRCA1*	Del exon 21 to 24	c.5278-?_5592+?del	p.?	Deleterious
S28	CNV	*BRCA1*	Del exon 1 to 2	c.-232-?_80+?del	p.?	Deleterious

*Nomenclature was numbered on the basis of the transcripts NM_007294 for *BRCA1* and NM_000059 for *BRCA2* (*https://www.ncbi.nlm.nih.gov/nuccore*). Alterations followed the HGVS nomenclature guideline, with +1 corresponding to the A of the ATG of the translation initiation codon.

†Expected consequence at the protein level following the HGVS nomenclature guideline.

‡Only variants of unknown significance or reported as deleterious are presented in this table.

CNV, copy number variation; Del, deletion; Dup, duplication; Indel, insertion/deletion; HGVS, Human Genome Variation Society; NA, no alteration; SNV, single-nucleotide variation; VUS, variant of unknown significance.

were analyzed by three methods (*BRCA* Tumor, Ion AmpliSeq *BRCA*, and Access Array *BRCA*). A second set (set 2) of 152 independent samples was then used to validate the combined process, determined according to set 1 (Figure 1).

Assessment of the *BRCA* Kit Sequencing Performances

To comply with the exhaustive analyses needed in routine diagnostics, the total number of reads, the median depth of coverage, and the coverage uniformity were computed for both *BRCA1* and *BRCA2* (Figure 2). In these experiments, the *BRCA* HC Kit revealed the highest homogeneity between samples in terms of the number of reads and median coverage, whereas the Access Array *BRCA* Kit had the lowest homogeneity (Figure 2, A and B). However, the *BRCA* HC Kit exhibited the lowest coverage uniformity between amplicons (89.7%) of all the kits (Figure 2C). This observation was validated by the representation of the normalized cumulative coverage per kit (Figure 2D). Indeed, when the median coverage per amplicon was computed and the amplicons were sorted according to their average depth of coverage, the *BRCA* HC Kit curve was the farthest from the theoretical line representing perfectly uniform coverage (Figure 2D).

For each sample sequenced, the depth and uniformity of coverage were more specifically analyzed across the regions targeted for *BRCA1* and *BRCA2* by the different panels (Figures 3 and 4). As previously reported, a minimum depth of 30× to 50× and 50× to 130× is required to reliably detect SNVs and indels, respectively.[22,23] Because the aim of this study was to identify diverse types of alterations (SNVs, indels, and CNVs), experiments were designed to obtain an average depth of coverage of 200× with a minimum of 50×. The *BRCA* Tumor Panel displayed the best depth of coverage, resulting in a minimum coverage >200× for all samples, as well as the highest coverage uniformity among amplicons (Figures 3 and 4).

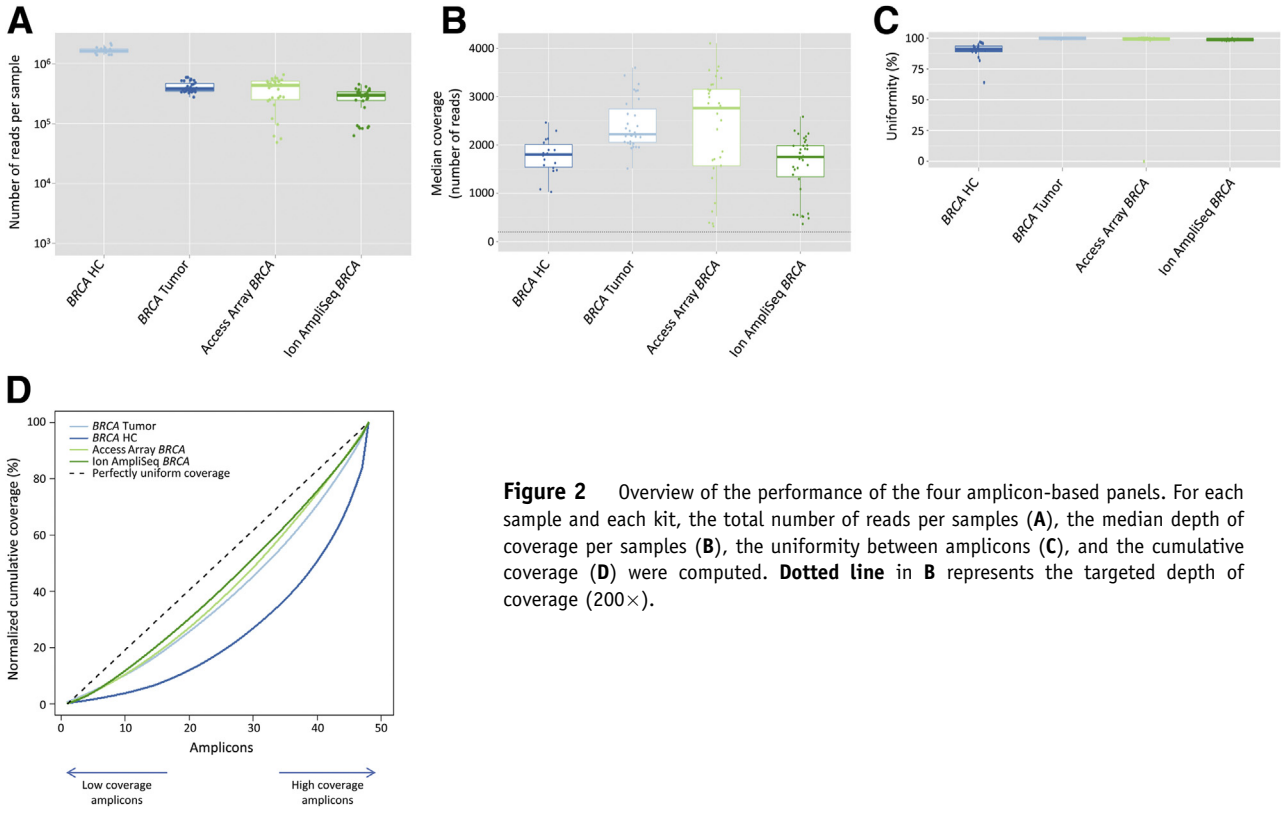

Figure 2 Overview of the performance of the four amplicon-based panels. For each sample and each kit, the total number of reads per samples (**A**), the median depth of coverage per samples (**B**), the uniformity between amplicons (**C**), and the cumulative coverage (**D**) were computed. **Dotted line** in **B** represents the targeted depth of coverage (200×).

In contrast, *BRCA* HC featured the lowest coverage uniformity (Figure 4). The Access Array *BRCA* panel exhibited good coverage uniformity, except for run 2, where two amplicons did not amplify (Figure 3). This issue cannot be attributed to panel design, because it is not present in run 1 and may be attributable to a technical problem while loading the chip. Finally, for the Ion AmpliSeq *BRCA* panel, exons 20 and 23 of *BRCA2* showed a systematic decrease in coverage (Figure 3). This issue was observed consistently in all samples from multiple runs, indicating a weakness in panel design. To avoid FN variants in these regions, additional experiments (such

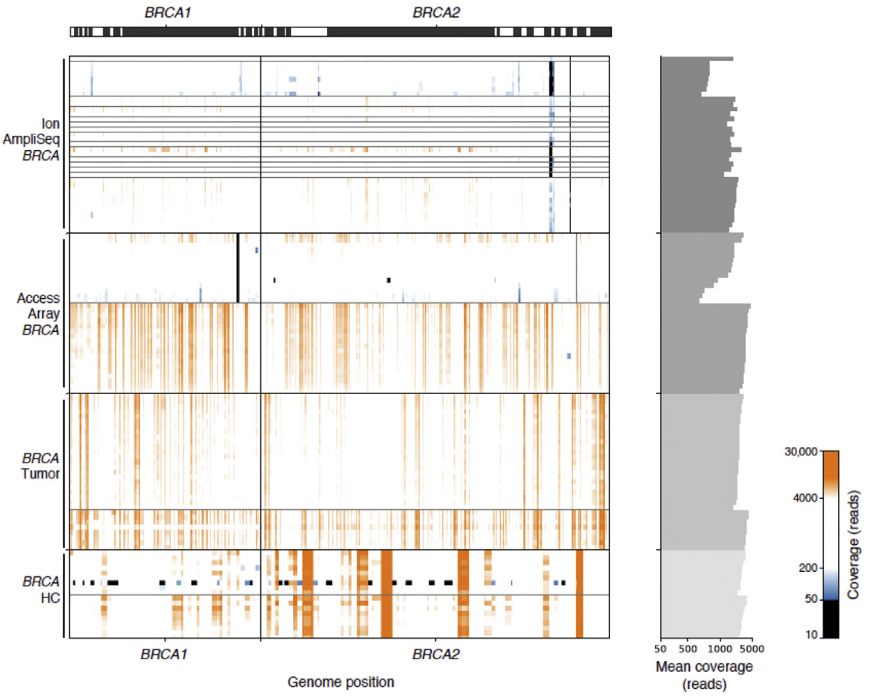

Figure 3 Absolute coverage depth profiles observed in *BRCA1* and *BRCA2* regions targeted by *BRCA* HC, *BRCA* Tumor, Access Array *BRCA*, and Ion AmpliSeq *BRCA* panels. Heat map showing the coverage depth per bp observed in different samples (*y* axis) at different genomic positions (*x* axis). Low coverage regions (coverage, <50×) are shown in black (see color bar). The horizontal bar chart shows the average coverage depth in different samples.

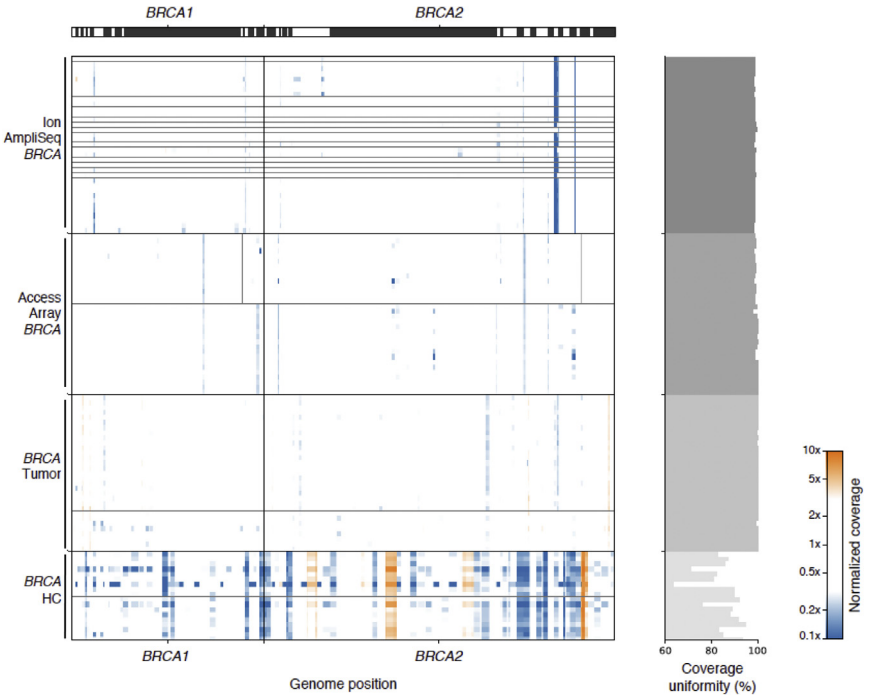

Figure 4 Mean-normalized coverage depth profiles observed in *BRCA1* and *BRCA2* regions targeted by *BRCA* HC, *BRCA* Tumor, Access Array *BRCA*, and Ion AmpliSeq *BRCA* panels. The coverage depth data shown in Figure 3 were normalized on a sample-by-sample basis by dividing by the mean coverage. As a result, the coverage depth is expressed in units of mean coverage. Genomic regions with a coverage depth of 10 times smaller or larger than the average mean are shown in blue (0.1×) or orange (10×), respectively (see color bar). The horizontal bar chart shows the coverage uniformity score measured in different samples.

as Sanger sequencing) are required to complement NGS results. Overall, these results indicate that *BRCA* Tumor and Access Array *BRCA* panels provide the highest performance in terms of coverage uniformity.

BRCA1 and *BRCA2* Mutation Detection

To establish the analytical accuracy of the four amplicon-based approaches for the detection of SNVs and indels, their reliability levels were assessed and compared with the results obtained by Sanger sequencing (Table 3).

With the Sophia DDM platform, all the SNVs and indels previously detected were correctly identified by NGS regardless of the panel used. Some variability in the VAF was, however, observed among the different kits (Supplemental Table S1). The largest and the most recurrent differences between the expected and the observed VAFs were observed with Access Array *BRCA* and Ion AmpliSeq *BRCA* panels. This point must be considered

Table 3 Correlation between Results Obtained Using the Amplicon-Based Panel Approaches and Sanger Sequencing for SNV and Indel Detection

	Sophia DDM platform				SeqNext software			
Variable	*BRCA* Tumor	*BRCA* HC	Access Array *BRCA*	Ion AmpliSeq *BRCA*	*BRCA* Tumor	*BRCA* HC	Access Array *BRCA*	Ion AmpliSeq *BRCA*
Total number of bases analyzed*	557,088	338,232	557,088	557,088	557,088	338,232	557,088	557,088
TP†	271	149	271	271	271	149	271	261
TN‡	556,817	338,083	556,817	556,813	556,817	338,083	556,817	556,801
FP	0	0	0	4	0	0	0	26
FN	0	0	0	0	0	0	0	0
Performance§								
Sensitivity, %	100	100	100	100	100	100	100	100
Specificity, %	100	100	100	99.999	100	100	100	99.995
Accuracy, %	100	100	100	99.999	100	100	100	99.995

*Number of bases interrogated by both next-generation sequencing techniques and Sanger sequencing.
†For TP, all polymorphic positions (positions with a difference compared with the genome of reference) across all samples were considered.
‡For TN, all called positions similar to the genome of reference across all samples were considered.
§Performance metrics were defined as follows: Sensitivity = TP/(TP + FN); Specificity = TN/(TN + FP); Accuracy = (TP + TN)/(TP + FP + TN + FN).
FN, false negative; FP, false positive; Indel, insertion/deletion; SNV, single-nucleotide variation; TN, true negative; TP, true positive.

with caution because it could lead to FN variants or a misinterpretation of the zygosity status. For example, the VAF of a heterozygous SNV detected in sample S20 using the Access Array *BRCA* data was 98%. In this case, the variant would have been erroneously considered as homozygous. Moreover, as previously reported by groups that used the Ion AmpliSeq *BRCA* Panel,[24–26] four false-positive (FP) variants (three indels and one SNV) were observed for *BRCA2* with relatively high VAF (Table 3 and Supplemental Table S2). Thus, the detection of FP variants, particularly *BRCA2* c.2175dup (VAF of 50%, S18), which is classified as deleterious in the specialized *BRCA* databases, may have dramatic consequences for a patient's medical care if no validation is performed by another technique.

The same data sets were then analyzed with the SeqNext software. All of the expected mutations were likewise correctly detected. However, compared with the Sophia DDM platform, the VAFs obtained with SeqNext presented larger deviations from the expected VAFs (Supplemental Table S3). Most important, a larger number of FP variants was obtained with the Ion AmpliSeq *BRCA* panel ($n = 26$) (Table 3 and Supplemental Table S4). Some of these FP variants (eg, *BRCA1* c.5289del, *BRCA2* c.1689del, and *BRCA2* c.9739del) were present in several samples, with a VAF of approximately 50% (Supplemental Table S4), and can thus be attributed to systematic artifacts. However, because some of them are annotated as deleterious in the databases, careful validation must be performed to avoid delivering an erroneous diagnosis.

Finally, the fact that different FP variants were reported with Sophia DDM and SeqNext demonstrates that the quality of NGS-based clinical diagnostics does not simply depend on the experimental methods used for enrichment and sequencing, but also on the bioinformatics solutions.

BRCA CNV Detection

The performance of the four molecular approaches was next evaluated for the detection of CNVs by comparing the NGS results with those obtained by MLPA assays. For the Access Array *BRCA* method, this analysis could not be performed, because the library preparation protocol included a C_0t PCR amplification. This particular PCR method induces the homogeneous amplification of each amplicon with a leveling of rare sequences,[27] rendering this technique unsuitable for the detection of CNVs. Regarding the Ion AmpliSeq BRCA panel, the kit supplier did not provide the composition of the PCR plexes, rendering the CNV analysis not feasible by the SeqNext software (Table 4).

For all panels, the CNV results obtained by NGS demonstrated good/excellent concordance with the conventional techniques (Table 4). Notably, although the amplicon-based method is not the most appropriate approach to highlight CNVs, perfect accuracy (100%) was obtained when the *BRCA* HC or the Ion AmpliSeq *BRCA* Panel was used in combination with the Sophia DDM platform. With the *BRCA* Tumor Kit, only one FP CNV was reported when the analysis was performed with the Sophia DDM platform (Supplemental Table S5). On the other hand, CNV calling with SeqNext, while achieving 100% sensitivity, produced seven and five false positives with the *BRCA* Tumor and *BRCA* HC kits, respectively (Table 4 and Supplemental Table S5).

These results suggest that, regardless of the enrichment kit used for library preparation, Sophia DDM outperforms SeqNext for CNV analysis.

Table 4 Correlation between Results Obtained Using the Amplicon-Based Panel Approaches and MLPA for CNV Detection

	Sophia DDM platform				SeqNext software			
Variable	*BRCA* Tumor	*BRCA* HC	Access Array *BRCA*	Ion AmpliSeq *BRCA*	*BRCA* Tumor	*BRCA* HC	Access Array *BRCA*	Ion AmpliSeq *BRCA*
Absolute number of CNVs analyzed*	1652	944†	NA	1652	1652	1003	NA	NA
TP	29	23	NA	29	29	24	NA	NA
TN	1622	921	NA	1623	1616	974	NA	NA
FP	1	0	NA	0	7	5	NA	NA
FN	0	0	NA	0	0	0	NA	NA
Performance‡								
Sensitivity, %	100	100	NA	100	100	100	NA	NA
Specificity, %	99.938	100	NA	100	99.569	99.489	NA	NA
Accuracy, %	99.939	100	NA	100	99.576	99.501	NA	NA
Precision, %	96.667	100	NA	100	80.556	82.759	NA	NA

*Number of CNVs analyzed by both next-generation sequencing techniques and MLPA method. Each exon analyzed was considered as an independent event.

†One sample (S26) could not be analyzed and was excluded from the analysis.

‡Performance metrics were defined as follows: Sensitivity = TP/(TP + FN); Specificity = TN/(TN + FP); Accuracy = (TP + TN)/(TP + FP + TN + FN); Precision = TP/(TP + FP).

CNV, copy number variation; FN, false negative; FP, false positive; MLPA, multiplex ligand probe-dependent amplification; NA, not applicable; TN, true negative; TP, true positive.

Table 5 Performance of the Combination of the *BRCA* Tumor Panel and the Sophia DDM Analysis Tested on an Independent Set of Samples ($n = 152$)

Variable	*BRCA* Tumor and Sophia DDM platform	
	SNPs and indels	CNVs*
Number of alterations analyzed[†]	3,024,192	8437
TP[‡]	1382	47
TN[§]	3,022,810	8385
FP	0	5
FN	0	0
Performance[¶]		
Sensitivity, %	100 (99.73–100)	100 (92.45–100)
Specificity, %	100 (100–100)	99.93 (99.86–99.98)
Accuracy, %	100 (100–100)	99.93 (99.86–99.98)

*Nine samples could not be analyzed and were excluded from the analysis.

[†]Number of alterations analyzed by both next-generation sequencing techniques and Sanger sequencing or multiplex ligand probe-dependent amplification.

[‡]For TP, all polymorphic positions (positions with a difference compared with the genome of reference) across all samples were considered.

[§]For TN, all called positions similar to the genome of reference across all samples were considered.

[¶]Performance metrics were defined as follows: Sensitivity = TP/(TP + FN); Specificity = TN/(TN + FP); Accuracy = (TP + TN)/(TP + FP + TN + FN).

CNV, copy number variation; FN, false negative; FP, false positive; indel, insertion/deletion; SNP, single-nucleotide variation; TN, true negative; TP, true positive.

Validation of the Workflow on an Independent Set of Samples

Overall, our analysis performed on set 2 suggests that, in terms of variant detection accuracy in *BRCA1* and *BRCA2*, the *BRCA* Tumor and the *BRCA* HC panels are the most appropriate solutions. Because the *BRCA* HC panel features a lower coverage uniformity, the *BRCA* Tumor Panel was preferred for the validation step. Regarding the bioinformatics solution, the best results were obtained with the Sophia DDM platform. Thus, to further assess the performance of clinical diagnostics based on NGS, the *BRCA* Tumor panel, combined with the Sophia DDM platform, was blindly tested on a larger cohort of 152 samples (Supplemental Table S6).

All of the SNVs and indels previously detected by Sanger sequencing were correctly identified, and no FP variants were reported. These results thus confirm the remarkable sensitivity, specificity, and accuracy of this workflow (Table 5). Regarding the detection of CNVs, all were also correctly identified (seven and one affecting *BRCA1* and *BRCA2*, respectively) (Supplemental Table S6). However, as previously observed in the training cohort, some FP CNVs were also reported ($n = 5$). Finally, nine samples (5.9%) were rejected, because of their elevated level of coverage noise (Supplemental Table S6).

Altogether, the workflow was tested on an independent cohort of 152 samples that harbored alterations commonly analyzed in clinical laboratory diagnostics (ie, 8 samples with CNVs, 10 samples with indels, 16 samples with SNVs, and 118 samples without alterations). These results demonstrated good/excellent accuracy for both the detection of SNPs and indels (100%; CI, 100%–100%) and the detection of CNVs (99.93%; CI, 99.86%–99.98%). Thus, although the process was less accurate for CNV detection than for SNV and indel identification (Table 5), the use of the *BRCA* Tumor Kit combined with the Sophia DDM platform provides a powerful tool for reliable detection of genetic alterations in *BRCA1* or *BRCA2*.

Discussion

Increasing evidence indicates that information about the *BRCA1* and *BRCA2* germline alteration status, especially if available in a timely manner, will enable patients and health-care providers to make informed decisions about cancer prevention, screening, and treatment. Although Sanger sequencing and MLPA remain the conventional techniques for the detection of SNVs, indels, and CNVs, the implementation of NGS approaches in clinical laboratories has modified the landscape of molecular diagnostic practices. However, the use of NGS methods for clinical genetic testing requires advanced knowledge of the entire process to ensure the correct and reliable detection of multiple alterations. The panel design, experimental process, sequencer, and bioinformatics analysis must be considered and accurately controlled. To our knowledge, numerous studies have reported high consistency between commercial multiplex PCR-based targeted NGS methods and Sanger sequencing for the detection of SNVs or indels[15,23,25,26,28–32]; however, a direct and systematic comparison of these NGS approaches has so far never been performed. Moreover, extensive analyses of the feasibility to detect CNVs using PCR-based panels have been poorly investigated.[23,28]

Herein, we aimed to benchmark four commercial multiplex PCR–based methods and two bioinformatics approaches for the detection of SNVs, indels, and CNVs and determine the best combination for the diagnostic screening of *BRCA1* and *BRCA2*. The specifications of the four selected PCR-based panels were first analyzed. Extensive knowledge of the regions targeted by the panel used is necessary to control the alteration detection limits. Therefore, particular attention was paid to the theoretical coverage and the level of overlap between amplicons, and it was noted that the Access Array *BRCA* Panel was the lowest-confidence assay. However, because this molecular approach used a specific technology that allowed complete customization of the regions targeted, this concern can easily be overcome by users. For the Ion AmpliSeq *BRCA* Panel, the coverage remains more problematic. Indeed, because low overlap between amplicons may yield FN

results,[15,16] a new panel design should be developed to address the fact that half of the amplicons have tiling of <6 nt.

The analytical performance of each kit was next assessed in terms of depth and coverage uniformity on a first set. From our perspective, the most problematic concern arose with the Ion AmpliSeq *BRCA* Panel, which presented a systematic weakness in depth coverage for exons 20 and 23 of *BRCA2*. This issue has also been previously reported by Buzolin et al,[26] who described that 90% of analyzed samples exhibited poor coverage for these specific regions (<20×). Because the specialized databases report deleterious alterations in these regions, the use of the AmpliSeq *BRCA* Panel for routine diagnosis may miss those requiring additional techniques to accurately fill the gaps.

To challenge the different panels in terms of alteration detection, the samples included in set 1 were carefully selected to harbor a broad range of alterations (SNVs, deletions of 1 to 10 nt, insertions or duplications of 1 to 28 nt, and CNVs). Because the bioinformatics software and algorithms are of utmost importance for the detection of such alterations, the results obtained with each assay were blindly analyzed with two independent commercial solutions (the Sophia DDM platform and the SeqNext software). Of interest, all true-positive variants, including CNVs, were successfully detected regardless of the kit or the bioinformatics solution used. FP SNVs/indels were also reported by using the Ion AmpliSeq *BRCA* Panel, independently of the bioinformatics analysis. Although no FN variants were noted in this study, other groups have reported some when using the same technology.[24–26] FP and FN concerns have been mainly attributed to the PGM sequencing chemistry.[24,33,34] Currently, the consensus NGS technique for the detection of CNVs is the use of capture-based methods.[12] Because CNVs account for approximately 7% of all inherited *BRCA* alterations,[35] a workflow that allows their accurate detection at the same time as SNVs and indels is essential. In addition, although PCR-based panels are relatively easy to implement in clinical laboratory analysis, the reliable detection of CNVs by an amplicon-based approach has remained challenging. To date, only one study has reported a good concordance between MLPA and their PCR-based panel.[28] In the present study, the feasibility of detecting CNVs with high confidence using the Sophia DDM platform, regardless of the enrichment kit used for library preparation, was emphasized. Finally, the *BRCA* Tumor Panel and the Sophia DDM platform were found to be the most relevant combination for concomitant and optimal CNV, SNV, and indel detection.

Complete control and knowledge of the limits of amplicon-based panel processes are required to ensure the reliable detection of different types of alterations. Our results demonstrated that, even if the various *BRCA* assays exhibited suitable data, the most relevant results were obtained by using the *BRCA* Tumor Panel combined with the Sophia DDM platform.

Moreover, the CE-mark–certified *BRCA* Tumor was specifically designed for short amplicon generation and, therefore, somatic sample analysis.[36,37] Such design is of interest for clinical laboratories that would like to implement a common experimental workflow for both somatic and germline sample testing. Finally, our results highlight the necessity of controlling the whole process, including the molecular approach and the bioinformatics software, to achieve the most relevant process for *BRCA1* and *BRCA2* alteration detection in a clinical setting and to limit the number of discrepant alterations.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2018.06.003*.

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