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Validation and Performance of Quantitative *BRCA1* and *RAD51C* Promoter Hypermethylation Testing in Breast and Ovarian Cancers

J. Lynn Fink,[*] Binny Jaradi,[*] Nathan Stone,[*] Brittany Sanker,[*] Fan Zhang,[†] Alexander Dobrovic,[†] Sophie Kirschner,[‡] James Hadfield,[‡] Olga Kondrashova,[§] and Paul M. Waring[*‡]

From XING Genomic Services,[*] Sinnamon Park, Queensland, Australia; Beacon Biomarkers Laboratory,[†] University of Melbourne Department of Surgery at Austin Health, Heidelberg, Victoria, Australia; Translational Medicine,[‡] AstraZeneca, Cambridge, United Kingdom; and Department of Genetics and Computational Biology,[§] QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia

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Address correspondence to J. Lynn Fink, Ph.D., LifeStrands Genomics, Bldg. A (G.01), 18-24 Ricketts Rd, Mount Waverley, VIC 3149, Australia.
E-mail: lynn.fink@lifestrandsgx.com.au.

Poly (adenosine diphosphate-ribose) polymerase (PARP) inhibitors represent a significant advancement in the treatment of epithelial ovarian cancer, triple-negative breast cancer, pancreatic cancer, and castrate-resistant prostate cancer, and they are poised to improve treatment in an increasing number of other cancer types. PARP inhibitor efficacy as monotherapy has been primarily observed in tumors with deleterious genetic variants in genes involved in the homologous recombination repair pathway. Tumors without these variants have also been shown to respond; notably, those with hypermethylation at all alleles of the *BRCA1* or *RAD51C* promoter can respond to PARP inhibitors. These epigenetic biomarkers therefore represent a patient population that may also benefit from this targeted therapy. However, no robust test has been conducted to identify these biomarkers in routine clinical specimens that is amenable to implementation for decentralized testing. This study describes the analytical and clinical validation of a *BRCA1* and *RAD51C* promoter methylation test that can be run with a single-day library preparation workflow for sequencing on any next-generation sequencing platform. The results show that this test can accurately quantitate the level of promoter methylation at the *BRCA1* and *RAD51C* genes using formalin-fixed, paraffin-embedded samples, even when the extracted DNA is extremely degraded or the input amount is limited. This test increases the precision of diagnostic tests aimed at identifying patients who are likely and unlikely to respond to PARP inhibitor therapy. *(J Mol Diagn 2025, 27: 139—153; https://doi.org/10.1016/j.jmoldx.2024.11.004)*

Favorable responses to poly (adenosine diphosphate-ribose) polymerase inhibitors (PARPis) as monotherapy have been observed in patients with cancer who carry deleterious germline or somatic genetic variants in genes involved in the homologous recombination repair (HRR) pathway, most commonly *BRCA1* and *BRCA2*.[1] However, some cancer patients without germline or somatic HRR variants also respond to PARPis, suggesting that defects in HRR can be caused by mechanisms other than genetic variants in *BRCA1*, *BRCA2*, or other HRR genes. Silencing of the *BRCA1* or *RAD51C* genes by complete promoter hypermethylation in breast and ovarian carcinomas has been shown to lead to HRR deficiency and PARPi sensitivity.

BRCA1 promoter methylation was first discovered in breast cancer in 1997,[2] proposed as a mechanism of disease in breast and ovarian cancer in subsequent years,[3–6] and established as a hallmark of HRR deficiency (then referred to as "BRCAness") in 2004.[7] The first evidence of epigenetic modulation of platinum and PARPi response was shown in a cohort of 115 sporadic primary ovarian carcinomas and in *in vitro* and xenograft testing with the PARPi rucaparib.[8,9] The development of resistance to PARPi via loss of *BRCA1* promoter

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hypermethylation further substantiated the role of epigenetic silencing as a determinant of HRR deficiency (HRD).[10]

Based on a comparative molecular analysis of the foundational Study 19 (Assessment of Efficacy of AZD2281 in Platinum Sensitive Relapsed Serous Ovarian Cancer; NCT00753545, *https://clinicaltrials.gov/study/NCT00753545*, last accessed November 17, 2024), a randomized phase 2 trial assessing the PARPi olaparib as maintenance therapy after response to platinum in high-grade serous ovarian carcinoma, *BRCA1* methylation was not associated with response duration.[11] Similarly, in a smaller study, deleterious *BRCA1* variants were associated with increased sensitivity to platinum chemotherapy while *BRCA1* methylation was not.[12] Notably, these studies were performed using methylation-sensitive PCR, which is a qualitative or, at best, semi-quantitative test. It was not until a 2018 study[13] using methylation-sensitive droplet digital PCR (MS-ddPCR), a fully quantitative methylation test, that it was shown that all copies of *BRCA1* must be methylated to confer PARPi sensitivity. Similar results were found for *RAD51C*,[14] although *RAD51C*-hypermethylated tumors are rarer.[15]

The incidence of *BRCA1* and *RAD51C* promoter methylation varies depending on the cohort, tumor type, disease stage, and prior treatments. For example, approximately 3% of unselected primary breast cancers are *BRCA1* methylated, while approximately 26% of early-stage triple-negative breast cancers (TNBC) are *BRCA1* methylated. Nonetheless, it is becoming clear that patients with complete epigenetic silencing of either *BRCA1* or *RAD51C* represent a patient population that would likely benefit from PARPi,[15–18] in which "complete" silencing is operationally defined as at least 70% methylation (adjusted for tumor content). The EMBRACE clinical trial (*https://anzctr.org.au/Trial/Registration/TrialReview.aspx?ACTRN=ACTRN12617000855325*, last accessed December 18, 2024) was the first to prospectively recruit patients based on *BRCA1* or *RAD51C* promoter methylation.[19] Initial results from the EMBRACE, DORA (Olaparib with or without Durvalumab), and PAOLA-1 (PAOLA-1/ENGOT-ov25) clinical trials suggest that these tumors are likely HRR deficient and could have a similar clinical benefit as tumors that are classified as HRR deficient according to *BRCA1* variant status.[18–20] Importantly, *BRCA1* and *RAD51C* promoter methylation is mutually exclusive of both germline and somatic pathogenic variants,[21–23] indicating that these patients represent an unmet medical need and potential new indication for PARPis.

A critical facet of promoter hypermethylation and PARPi response is the dynamic nature of epigenetic silencing. Unlike genetic variants, promoter methylation may be readily lost when a cell is challenged with a therapy such as PARPi or platinum-based compounds. If methylation is lost at a single *BRCA1* or *RAD51C* allele, the cell will revert to an HRR-proficient phenotype, which would likely result in resistance to treatment.[10,17] Capturing the moment of this event during the course of treatment, and adjusting the treatment strategy accordingly, could minimize the use of ineffective therapy, although ideally this would be performed on serial blood sample–derived circulating tumor DNA rather than tumor tissue.[24] Moreover, the previously claimed lack of association between *BRCA1* promoter methylation and PARPi response is likely due to these studies not being able to differentiate completely methylated cases from partially methylated cases based on tests that were available at the time; it could also be because testing may have been performed on specimens obtained before treatments that can cause loss of promoter methylation.

Although ddPCR has been shown to be a suitable method for quantitating *BRCA1* methylation and retrospectively predicting PARPi response,[13] ddPCR is not a standard testing platform in general use in pathology laboratories. Pyrosequencing was recently shown to also be an effective quantitative test for *BRCA1* methylation,[25] but this is also not a standard clinical testing platform. In addition, these tests require relatively large amounts of input DNA (100 to 300 ng), which can be prohibitive for small tissue samples that also require molecular genetic testing for somatic variants. For quantitative promoter methylation testing to become routine, a test that can be performed in any modern molecular pathology laboratory on the full range of sample types and quality encountered in clinical practice is required. The most practical approach is next-generation sequencing (NGS), as this is an established technology for clinical somatic cancer testing. However, the test must be robust to bias,[26,27] poor DNA quality associated with formalin-related degradation, and low input amounts from small samples such as core biopsies or specimens with low or diffuse tumor content.

This article reports a quantitative NGS-based *BRCA1* and *RAD51C* promoter methylation that is highly sensitive and specific and has high correlation with an established MS-ddPCR test. This test was analytically and clinically validated by using reference samples, research samples, and clinical trial samples. It can be run with a single-day library preparation workflow for any NGS platform, enabling widespread decentralized testing capabilities for determining epigenetic factors, modulating HRR proficiency, and, ultimately, therapy response or resistance, in breast and ovarian cancers.

Materials and Methods

Reference Samples

The CpGenome Human Methylated & Non-Methylated DNA Set was used to create samples with different levels of methylation. Briefly, 100% methylated DNA (catalog number S8001 mol/L; Sigma-Aldrich, MilliporeSigma, Burlington, MA) was diluted in a background of non-methylated DNA (catalog number S8001U; Sigma-Aldrich, MilliporeSigma) or DNA extracted from the buffy coat sample of a patient in which no methylation of the targeted

regions was expected. Titrated mixtures included 0%, 0.1%, 1%, 2.5%, 5%, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, and 100%.

The Horizon Discovery Quantitative Multiplex Reference Standards for Formalin-Compromised DNA (mild, moderate, and severe) were tested with the QuantiMIZE assay (as described in *DNA Integrity Assessment*) to characterize the extent of formalin-related DNA degradation on total and amplifiable DNA concentration (catalog numbers HD798, HD799, and HD803; MetaGene Pty Ltd., Brisbane City, Queensland, Australia).

Clinical Samples

Formalin-fixed, paraffin-embedded (FFPE) specimens, as well as some paired blood samples, from patients with cancer were tested. Supplemental Table S1 provides a full list of unique samples, Table 1 lists the attributes of the samples used for validation against a ddPCR test, and Table 2 presents the samples used in test performance assessment. A total of 182 unique samples were tested, and 297 total samples were tested.

DNA Extraction

FFPE-stained slides were assessed by a pathologist to estimate tumor content and to identify regions for macrodissection to enrich for tumor content. Tumor sections were then macrodissected, when indicated, from one or more corresponding unstained slides. The pooled macrodissected FFPE tissue was then digested and purified by using the QIAamp DNA FFPE Tissue Kit (catalog number 56404; Qiagen, Clayton, Victoria, Australia). DNA was eluted in 30 μL of elution buffer and quantified by using the Qubit dsDNA HS Assay kit (catalog number Q33231; Thermo Fisher Scientific, Brisbane, Queensland, Australia).

DNA Integrity Assessment

The QIAseq DNA QuantiMIZE kit (catalog number 333414; Qiagen) was used to quantify and qualify amplifiable DNA. This kit uses two real-time quantitative PCR assays that interrogate 40 genomic loci to determine the amounts of amplifiable DNA fragments in a sample. Briefly, samples or control genomic DNA were mixed with a real-time quantitative PCR master mix and QuantiMIZE primer pairs. Real-time PCR was performed according to the manufacturer's instructions, and threshold cycle values were analyzed to determine concentration and absolute quantities of amplifiable DNA. Samples were tested in triplicate.

Table 1 Study Population Characteristics for Test Validation

	Total, $N = 60$	
No. of samples, ddPCR vs NGS	*n*	%
Diagnosis		
Ovarian cancer	43	72
Breast cancer	9	15
Not provided	7	13
Promoter methylation status		
BRCA1 methylated	19	32
RAD51C methylated	6	10
Not methylated	35	58

ddPCR, droplet digital PCR; NGS, next-generation sequencing.

Table 2 Study Population Characteristics for Test Validation and Performance

Samples	*n*	%
Unique samples	182	
Total samples (including replicates)	297	
Reference samples	24	13
Clinical samples	158	87
Diagnosis, clinical samples		
Ovarian cancer	65	41
Breast cancer	66	41
Lung cancer	12	8
Prostate cancer	2	1
Not provided	7	4
Blood	6	4
Promoter methylation status, clinical samples		
BRCA1-methylated only (raw % $\geq$ 2.5)	38	24
RAD51C-methylated only (raw % $\geq$ 2.5)	9	6
BRCA1- and *RAD51C*-methylated (raw % $\geq$2.5)	6	4
Not methylated	105	66

Library Preparation and Sequencing

The oncoReveal HRD kit (catalog number HDA-HR-1003-96; Pillar Biosciences, Natick, MA) and the oncoReveal BRCA1 & RAD51C Methylation (BRM) kit (catalog number HDA-HR-1006-96; Pillar Biosciences) were used to prepare sequencing libraries for short read sequencing of amplicons using paired-end 150 bp reads. The HRD kit is a multi-gene test that targets 27 genes, 19 of which are involved in HRR (*ATR, ATM, BARD1, BRCA1, BRCA2, BRIP1, CHEK2, RAD50, RAD51, RAD51C, RAD51D, FANCA, FANCC, FANCD2, FANCE, FANCF,* and *MRE11A*; hot spot regions of *PALB2* and *NBN*) and eight other genes of diagnostic utility in subclassifying breast and ovarian cancer, including *ARID1A, BRAF, CNTTB1, KRAS, PIK3CA, PPP2RIA, PTEN,* and *TP53*. The BRM kit targets the promoter regions of *BRCA1* and *RAD51C* and interrogates 35 CpG sites (10 in *BRCA1* promoter, 25 in *RAD51C* promoter).

Before library preparation with the BRM kit, up to 50 ng of genomic DNA was bisulfite-converted (catalog nos. D5030 and D5031; Zymo Research, Irvine, CA). The NEBNext Enzymatic Methyl-seq Conversion Module (catalog number E7125; New England BioLabs, Ipswich, MA) was also used for some samples with a genomic DNA input amount of 20 ng. For BRM library preparation, 10 pg to 13.92 ng of bisulfite-converted DNA (bismodDNA) was

used; 5 to 20 ng of genomic DNA was used for HRD library preparation.

Libraries were sequenced on the Illumina NextSeq 550 and MiSeq sequencers (Illumina, Melbourne, Victoria, Australia). Because some of these samples were sequenced in batches with routine clinical samples, a uniform level of coverage across all samples was not achievable. However, obtaining a minimum of 3500× mean coverage was suggested by the kit manufacturer.

Short Read Alignment and Variant Detection: HRD Test

Short read alignment, coverage analysis, and variant calling were performed by using the Pillar Biosciences proprietary PiVAT bioinformatics platform (v2020.2.2) with default settings. Briefly, PiVAT performs short read alignment of non-overlapping 150 bp paired-end reads to a reference genome (hg19); local realignment and/or *de novo* read assembly to ensure capture of long insertions/deletions; quality- and noise-weighted variant calling; variant selection using heuristic thresholds; and variant annotation [PiVAT User Manual (RUO) v.2023.1.0, Pillar Biosciences]. Variants called by PiVAT were filtered to remove those with a variant allele frequency (VAF) <3%. The validated limit of detection (LOD) of the HRD test is 4% based on a minimum tumor content of 20%, although variants with VAFs between 3% and 4% may be reported if coverage depth and tumor content are sufficiently high. Variants with <200× coverage were removed before variant annotation and interpretation. Samples were considered to pass post-sequencing quality control if they had a mean coverage of at least 2000× with at least 98% of targeted regions receiving at least 200× coverage.

Short Read Alignment and Variant Detection: BRM Test

The genomic regions targeted by the BRM test are short amplicons (117 to 149 bp) with overlapping 150 bp read pairs. Read alignment, coverage analysis, and variant calling were performed by using a custom in-house pipeline (Supplemental Figure S1). Briefly, adapters and low-quality ends were trimmed from the sequencing read pairs in FASTQ files using Trim Galore, v0.6.8dev (*https://github.com/FelixKrueger/TrimGalore*, last accessed April 23, 2024). Trimmed reads were then aligned to the reference human genome (hg19) and an *in silico* bisulfite-converted version of the same genome using Bismark.[28] Samtools[29] was used to create a pileup of aligned reads that includes the reads that align to both reference genomes.[30] A Browser Extensible Data file, which specifies the genomic coordinates of the CpG sites of interest, was used to extract the pileup data for each site, and the number of each nucleotide at that site was counted. Coverage was calculated by adding up the total number of bases. Percentage of methylated bases at each CpG site was calculated by dividing the unconverted cytosines by the sum of cytosines and thymines (adjusted for strand in the case of *BRCA1* sites).

Promoter methylation was calculated for both *BRCA1* and *RAD51C* independently and reported as a mean methylation percentage (methylation %) across all evaluated CpG sites. For clinical interpretation, the mean methylation % was adjusted by the tumor cell content as estimated by an anatomical pathologist or, if the HRD test was run on the same sample and a pathogenic somatic *TP53* alteration was detected, the mean methylation % was instead adjusted by *TP53* variant allele frequency using an equation adapted from other sources[13,14]: $\text{AdjustedMethylation} = [(\text{RawMethylation} \times [\text{TumorContent} \times \text{GeneCopyNumber} + (100 - \text{TumorContent}) \times 2])/(\text{TumorContent} \times \text{GeneCopyNumber})]$, where TumorContent is either the *TP53* variant allele percentage or the pathologist's estimation of tumor content.

Methylation-Sensitive Droplet Digital PCR

Promoter methylation of *BRCA1* and *RAD51C*, determined by MS-ddPCR, was performed as previously described.[13] Briefly, primers were designed to amplify regions of the *BRCA1* and *RAD51C* promoter regions. MGB probes hybridizing to the fully methylated (VIC labeled) and the fully unmethylated sequences (FAM labeled) were used. ddPCR was performed on the Bio-Rad QX-200 system (Bio-Rad, Hercules, CA).

Clinical Reporting

Based on HRD variant panel results and adjusted mean methylation %, samples were assigned, in combination with quality control metrics, patient histopathology reports, and other identified somatic alterations, to a category of likely PARPi response. These responses comprised likely sensitive, sensitivity uncertain, and likely not sensitive, based on findings reported in patients treated with PARPi.[13,14,31]

Statistical Methods for Comparing Tests

Methylation levels were compared between the two tests by using Deming regression and Bland-Altman approaches.[32,33] Deming regression is an errors-in-variables model that fits a line describing the relationship between two variables and is suitable when there is measurement error in both variables. Deming regression is often used for method comparison studies, especially when a gold standard method is not available. The Bland-Altman plot is used to compare the agreement between two quantitative methods of measurement. Correlation and linear regression are frequently proposed methods for comparison. However, these approaches compare the relationship between one variable and another, not the differences, and are not recommended for assessing the comparability between tests.

Data Analysis

Data were analyzed using the R language and environment for statistical computing (R version 4.2.2; R Foundation for Statistical Computing, *https://www.r-project.org*). Packages used included: blandr v0.5.3 (*https://github.com/deepankardatta/blandr*), data.table v1.14.8 (*https://github.com/Rdatatable/data.table*), dplyr v1.1.1 (*https://github.com/tidyverse/dplyr*), ggformula v0.10.4 (*https://github.com/ProjectMOSAIC/ggformula*), ggplot2 v3.4.2[34] (*https://ggplot2.tidyverse.org*), Hmisc v 5.0-1 (*https://hbiostat.org/R/Hmisc*), mcr v1.3.2 (*https://CRAN.R-project.org/package=mcr*), patchwork v1.1.2 (*https://github.com/thomasp85/patchwork*), plyr v1.8.8,[35] scales v1.2.1 (*https://github.com/r-lib/scales*), and tidyfst v1.7.6,[36] tidyr v1.3.0 (*https://github.com/tidyverse/tidyr*).

Results

DNA Quality, Input Amount, and Concentration

The kit manufacturer recommends a minimum DNA input of 5 ng if the DNA is not severely degraded. If the FFPE DNA is severely degraded, they recommend increasing the DNA input to a maximum of 75 ng. To characterize the quality of the DNA, the QuantiMIZE assay was used to estimate the amplifiable DNA concentration (before bisulfite conversion) of both the clinical samples and the Horizon reference standards, which have a qualitative assignment of mild, moderate, or severe formalin-induced degradation. Figure 1A shows the distribution of amplifiable DNA concentrations of 82 clinical samples that were tested in the context of the concentrations estimated from the three reference samples. The amplifiable DNA concentration of these standards provides a quantitative range of DNA degradation that can be used to categorize the quality of the clinical sample DNA. The majority of samples tested with the HRD and/or BRM tests were below the amplifiable DNA concentration estimated for the reference sample of severely degraded DNA, suggesting that these samples were of very poor quality. However, all samples characterized with the QuantiMIZE assay were successfully tested with the BRM test, which shows that this test is robust to extremely poor-quality DNA. Because this is consistent with a previous performance evaluation of SLIMamp-based kits,[37] the ability of the BRM test to successfully interrogate these samples was not surprising.

A range of DNA input amounts was also used to assess the performance of the test. In many cases, especially during routine clinical testing, material was limited and the manufacturer's recommendation of a lower limit of 5 ng was not always possible to achieve. The range of bismodDNA input amounts used, including reference samples, was 10 pg to 13.92 ng (mean, 2.58 ng) (Figure 1B). The lowest amount of DNA successfully tested was 40 pg. The only sample that was not successfully tested was a 0.1% dilution of 10 ng/μL 100% methylated DNA, which had an estimated input DNA of 10 pg.

Bisulfite conversion yield was measured for 62 samples, including clinical and reference samples (Figure 1C). The average yield for clinical samples was 24%, with a range of 5.7% to 70%. Interestingly, the reference samples consistently had low yields compared with clinical FFPE tissue samples with an average yield of 8.3% and a range of 2.4% to 14%. Bisulfite modification of DNA is considered to be harsh, resulting in significant degradation of the DNA.[38] However, even with low conversion yields and any additional bisulfite-induced degradation, the BRM test was still successful.

Concentration of libraries generated from bismodDNA with the BRM test was also measured (Figure 1D). A concentration of 0.15 ng/μL was found to be the lower limit for successful test results. The two samples with library concentrations below this limit were generated from 1% to 0.1% dilutions of 10 ng/μL 100% methylated DNA. A relatively accurate test result (90% methylation at *BRCA1*, 100% methylation at *RAD51C*) was produced with 1% diluted DNA, although coverage (960×) was lower than the manufacturer's recommended level of 3500×. Use of 0.1% diluted DNA failed to produce a library or any sequencing reads.

Correlation with Reference Standards and Titrated Controls

Observed methylation % was compared with the expected methylation % based on the titrated 0%:100% mixtures, and the Pearson correlation coefficient was calculated. Observed methylation % data were also fitted with a linear regression model to determine how well they correlate with the expected percent methylation as defined by the mixtures that were tested. The correlations between runs for the methylation mixtures that were common to most runs (Supplemental Table S2) ranged between 0.9988 and 0.9998. All runs and samples had a very high correlation, indicating that the test results are extremely highly reproducible with reference DNA.

The linear regression model fit to the data had an R^2 value of 0.9733, suggesting that the data fit well with the expected percentages and were generally linear (Supplemental Figure S2). However, the observed methylation % for mixtures between 2.5% and 75% were slightly overestimated and observed methylated at 100% was slightly underestimated. It should be noted that, although the CpGenome 100% methylated DNA purports to be 100% methylated, this may not actually be the case. The CpGenome standards vendor notes that the nonmethylated DNA standard is not exactly 0% methylated but is <5% methylated, which could explain the slight overestimation of methylation in the 2.5% mixture. A similar phenomenon could be applicable to the 100%

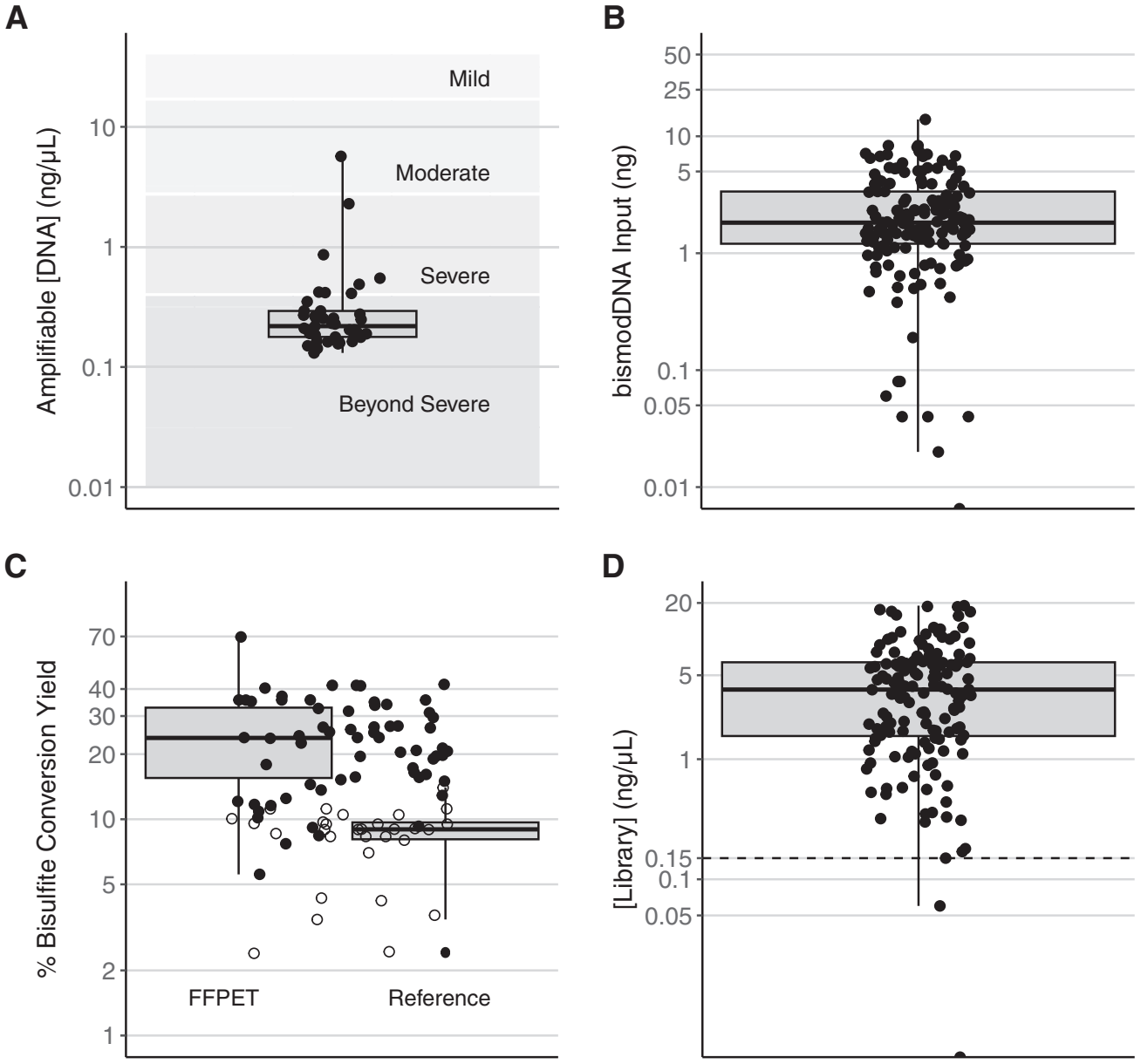

Figure 1 Performance characteristics of input DNA and sequencing libraries. Characteristics describing the DNA quality and quantities are shown here for any samples that were evaluated. Each plot has a different number of samples because all metrics were not captured for every sample. **A:** DNA quality, assessed by using the QuantiMIZE assay for formalin-fixed, paraffin-embedded tissue (FFPET) samples. Levels of formalin-induced degradation are based on the Quantitative Multiplex Formalin Compromised DNA Reference Standards (mild, moderate, and severe) and are shown in **gray rectangles**. Samples shown here were all used for test validation and produced interpretable results. **B:** Amount of bisulfite-converted DNA (bismodDNA) used for sequencing library preparation. Amounts were determined by DNA availability. All but one sample shown here produced interpretable results, and most are samples that were tested postvalidation. The one sample that did not yield interpretable results was 100% methylated CpGenome DNA diluted in water to 0.1% of the initial concentration of 10.4 ng/μL (XGS119). **C:** Yield of DNA from bisulfite conversion for FFPET (**black circles**) and reference DNA (**white circles**) samples. All samples shown here produced interpretable results and comprise mainly clinical trial samples (tested either prospectively or retrospectively, depending on the trial). Most samples shown here were tested postvalidation. Reference DNA samples are CpGenome 100% methylated DNA and various titrated mixtures based on that material. **D:** Library concentration, assessed by using the Qubit assay. Libraries below a concentration of 0.15 ng/μL did not generate interpretable results. The two samples shown here with a library concentration below that level are 100% methylated CpGenome DNA diluted in water to 1% and 0.1% of the initial concentration of 10.4 ng/μL (XGS118 and XGS119). Most samples shown here were tested postvalidation. The **horizontal dashed black line** represents the library concentration that was found to the lower limit for successful test results (0.15 ng/μL).

methylated DNA. Overall, however, the test seems to quantitate expected methylation % values accurately and is generally linear across the range of 0% to 100%. Interestingly, when the titrated controls were created by using genomic DNA from the buffy coat sample of a patient with prostate cancer, the bias disappeared (Supplemental Figure S2). However, only one experiment was performed with the full range of mixtures. The observed bias

with the 0%:100% CpGenome mixtures may be related to the reference materials used, not to the BRM test itself.

Validation Against a Gold Standard ddPCR Test

Sixty clinical samples were tested by using a ddPCR test (which is accepted as the gold standard method for quantitating promoter methylation) and the BRM test. The samples are shown in Supplemental Table S1 with raw methylation levels in bold and classified in the "True Positive/True Negative Methylation Status" Column. The bismodDNA input amounts used ranged from 60 pg to 4.24 ng with a mean of 1.43 ng. One sample (XGS034) had very low sequencing coverage relative to all other samples, although it was still concordant and did not result from the lowest amount of input DNA. Rather, the bismodDNA amount was above the mean input amount, which may suggest that the DNA was exceptionally degraded. Because many samples were provided as bismodDNA, there was no opportunity to perform the QuantiMIZE assay on the original genomic DNA and assess DNA quality. The sample with the lowest input amount of bismodDNA (XGS035, 60 pg) still generated sufficient sequencing coverage.

Methylation levels were compared between the two tests by using Deming regression and Bland-Altman approaches.[32,33] Figure 2A shows the Deming regression between the BRM test and the ddPCR test. The regression indicates that there is strong agreement between both methods and suggests that there is very little systematic difference between them. There is less confidence in the agreement at high levels of methylation, but there are also fewer points of measurement in that range, presumably because it is rare to find clinical samples that are 100% tumor content and 90% to 100% methylated at either gene promoter. The Pearson correlation coefficient is 0.972.

Figure 2B presents a Bland-Altman plot. Here, the percent methylation level is shown on the *x* axis, and the differences between the ddPCR methylation level and the NGS methylation level are plotted on the *y* axis. This method can highlight anomalies in method agreement or trends in overestimation or underestimation of measurements. No anomalies in agreement were observed related to level of methylation. There does seem to be a general, but slight, underestimation of methylation by the NGS method compared with ddPCR as the mean level of agreement is just below 0. The points that are related to the most disagreement between methods are samples with the lowest amounts of input DNA (60 and 80 pg bismodDNA). Overall, there is strong agreement between tests, and this shows that the NGS-quantitated methylation levels can be considered accurate and precise. Table 3 summarizes the binary diagnostic test results, considering presence or absence of clinically relevant levels of methylation as a positive or negative call. Sensitivity and specificity are both 100%.

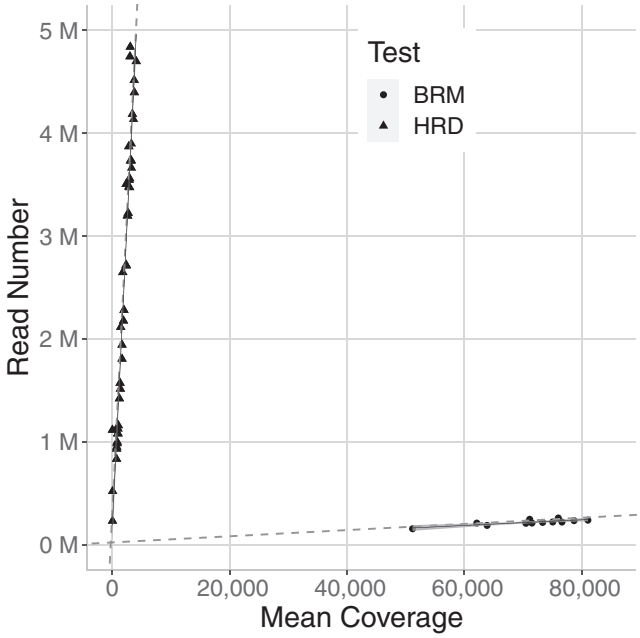

Figure 2 Relationship between mean coverage and number of read pairs. The line fit by the coverage balancing equation is shown as a **dashed gray line**. The 95% confidence interval is shown in dark gray. The oncoReveal BRCA1 & RAD51C Methylation (BRM) samples are shown as filled circles; homologous recombination repair deficiency (HRD) samples are shown as filled triangles. M, million.

Read Depth Coverage Analysis

To determine if sequencing depth has a significant effect on reproducibility of the quantitated methylation, an *in silico* down-sampling experiment was performed with three samples. One was a titrated reference mixture (XGS002; 50% CpGenome 100% methylated DNA:50% CpGenome 0% methylated DNA), and two were clinical samples that were previously shown to be methylated at either the *BRCA1* or *RAD51C* promoter (XGS004, 74% *TP53* VAF-adjusted *BRCA1* methylation; XGS005, 71% *RAD51C* tumor content–adjusted methylation). These samples had high coverage (36,055×, 29,717×, and 22,939×, respectively). Sequencing reads were down-sampled at intervals of 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 2.5%, 1%, 0.5%, 0.4%, 0.3%, 0.2%, and 0.1% of the original number of reads. The down-sampled data were then re-analyzed with the analytical pipeline. The correlation coefficients between the methylation % of the original data and the observed methylation % in the down-sampled data were calculated. Supplemental Figure S3 shows the relationship between correlation and level of down-sampling. The correlation between observed and expected methylation % was stable across all levels of coverage down to 0.1% of the original data for the clinical samples (29 and 21 reads for XGS004 and XGS005, respectively), although the 50% mixture only had stable correlation down to

Table 3 Binary Diagnostic Test Results Comparing ddPCR and NGS Methods Based on Qualitative Results

Variable	Test result, *n*
No. of samples	60
True positives	26
True positives, *BRCA1* methylated	20
True positives, *RAD51C* methylated	6
False positives	0
False negatives	0
True negatives (no promoter methylation at either gene)	34
Sensitivity	1
Specificity	1
False-positive rate	0
False-negative rate	0
Positive predictive value	1
Negative predictive value	1
False omission rate	0
False discovery rate	0
Prevalence	0.43
Proportion correctly classified	1
Proportion incorrectly classified	0
Overall accuracy	2
Positive likelihood ratio	Infinity
Negative likelihood ratio	0
Diagnostic odds ratio	Infinity

ddPCR, droplet digital PCR; NGS, next-generation sequencing.

2.5% of the original data (891 reads). Considering the differences in behavior observed between FFPE tissue and reference DNA when evaluating bisulfite conversion yields, the differences here are perhaps unsurprising. Overall, the test seems to be robust across a wide range of sequencing depths, but correlation was observed to start decreasing around 1000× mean coverage. Although the manufacturer initially recommended a depth of 3500×, a lower, yet still conservative, threshold of 2000× mean coverage was set as the minimum required sequencing depth for this test.

To determine how many sequencing reads would be needed to ensure a minimum mean coverage of 2000×, a retrospective analysis of results from samples sequenced from HRD and BRM libraries was performed. Figure 3 shows the relationship between mean coverage and total number of read pairs for both tests. Strikingly, the relationship is strongly linear where the slope of the line is effectively the number of amplicons in the test (HRD, 1070 amplicons; BRM, 4 amplicons). Careful examination of the relationship between coverage and read number shows that the fitted line does not pass through the origin. Error modeling of these data was performed, and an equation was created that accurately predicts the number of read pairs needed for any desired level of coverage for either test where N = number of reads required, S = number of amplicons in test panel, and C = mean coverage desired.

$$y = N$$

$$m = S + 0.0927S - 7.2165$$

$$x = C$$

$$b = 27280 * e^{0.0026s}$$

$$N = (S + 0.0927S - 7.2165)C + 27280e^{0.0026S} \quad (1)$$

This relationship was extremely robust across 171 unique samples that were tested with 17 sequencing runs, 4 operators, 2 sequencers, 2 sites, 2 different sequencing chemistries (4-channel, 2-channel), 4 tests (all Pillar Biosciences NGS amplicon tests, including BRM and HRD), and 3 sample types (reference DNA, FFPE tissue DNA, and buffy coat DNA) (complete data not shown). Specifically, the number of read pairs required for 2000× mean coverage of BRM libraries is 21,874, the number required for 2500× mean coverage of tumor sample HRD libraries is 3,345,538, and for 750× coverage of buffy coat libraries (representing a paired normal sample) it is 1,312,087. Furthermore, all libraries can be balanced for sequencing on the same flow cell to ensure that all samples receive the level of required coverage by determining the fraction of the total number of reads required for each test and adding that fraction of the library to the pool of libraries. Sequencing BRM and HRD libraries on the same flow cell was especially useful because it obviated the need for PhiX spike-in DNA to create enough sequence complexity for accurate base calling from bismodDNA and simultaneously enabled efficient batch processing of patient samples.

Determination of Bisulfite Conversion Completeness

The level of bisulfite conversion of DNA was assessed to confirm that the methylation % values calculated for the *BRCA1* and *RAD51C* promoters are not underestimated due to incomplete conversion. Methylation was calculated at all bases covered by the amplicons in the test with a depth of at least 200 reads, excluding the CpG sites of interest. Supplemental Figure S4 shows the percentage of cytosines that were converted to uracils for each sample for both *BRCA1* and *RAD51C*, respectively. Sites that received coverage of <200 reads were excluded from analysis. Conversion of non-CpG cytosines seemed to be effectively complete, based on this analysis, for all samples. The lowest mean conversion rates observed were 96% and 93% for *BRCA1* and *RAD51C*. Some CpG sites were consistently converted less completely than others regardless of the sample or overall conversion rate, suggesting that these sites may not be reliable indicators of conversion. These observations were consistent across all 192 samples evaluated. Overall, bisulfite conversion was effectively complete and did not seem to be a confounding factor in the accurate determination of promoter methylation levels.

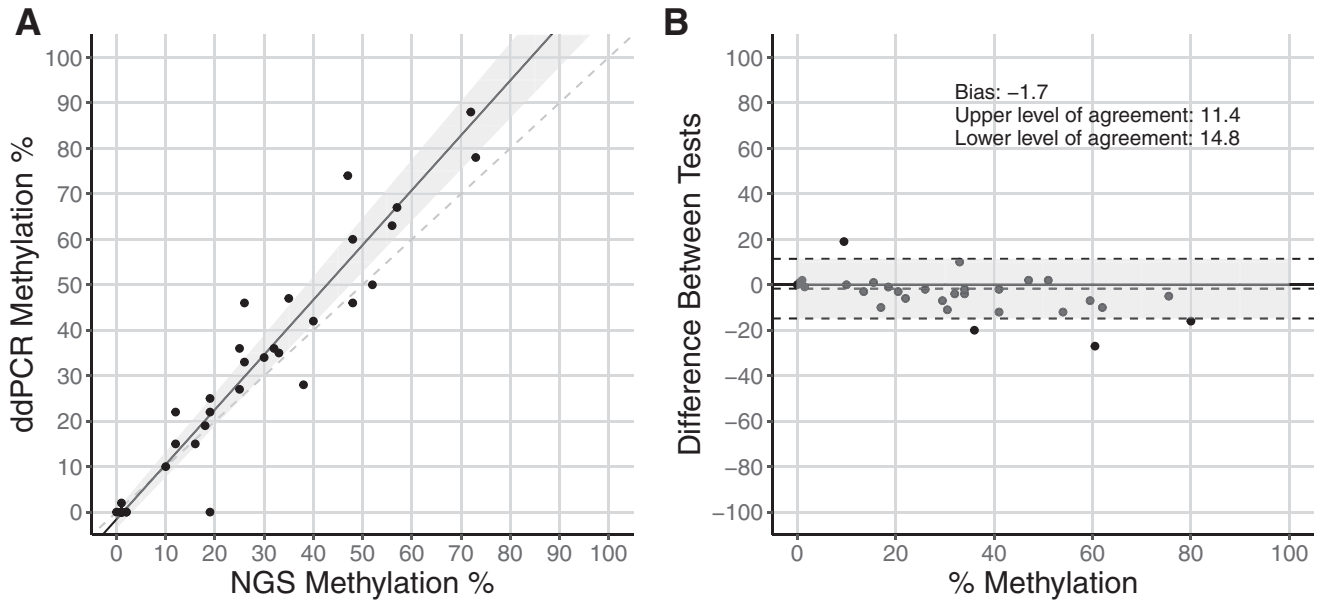

Figure 3 Comparison of the oncoReveal BRCA1 & RAD51C Methylation test with a droplet digital PCR (ddPCR) test of 60 clinical samples. **A:** Deming regression: this plot shows the concordance of mean percent promoter methylation calculated by the ddPCR and next-generation sequencing (NGS) tests. The **black line** shows the regression line; the **gray area** shows the confidence intervals around the regression line; and the **dotted line** represents what would be perfect concordance between the methods. The confidence interval widens toward higher methylation levels; this may be due, at least in part, to the underrepresentation of samples with very high methylation. In actual clinical samples, it is unlikely that any will demonstrate 100% methylation because tumor specimens rarely, if ever, are composed solely of tumor cells. **B:** Bland-Altman plot: this plot shows the level of agreement of mean percent promoter methylation calculated between NGS and ddPCR tests across the possible range of mean methylation (0% to 100%). The NGS methylation level is shown on the *x* axis, and the difference between the ddPCR methylation level and the NGS methylation level are plotted on the *y* axis. The **top**, **central**, and **bottom dotted lines** show the upper, mean, and lower levels of agreement between the two methods, respectively; the **gray area** highlights the samples between the upper and lower levels of agreement. No anomalies in agreement related to level of methylation are observed as the points representing the differences are not clustered around a particular methylation level but are spread horizontally across the plot. There does appear to be a general, but slight, underestimation of methylation by the NGS method compared with ddPCR as the mean level of agreement is just below 0. It is important to note, however, that the NGS method measures more CpG sites in both promoters than the ddPCR method. The points that are related to the most disagreement between methods are samples with the lowest amounts of input DNA.

Determination of Measurement Uncertainty

To determine methylation % range to distinguish true-positive methylated CpG sites from false-positive methylated CpG sites, which may be a result of noise caused by sequencing errors or formalin-related cytosine deamination, the level of methylation was calculated at all cytosines covered by the amplicons in the BRM test, excluding the CpG sites of interest (528 bases) (Supplemental Figure S4). Supplemental Figure S5 shows the background level of noise. This noise has a mean of 1.6% and a standard deviation of 9.6% methylation, across 135 samples and 125 sites). The level of background noise is below the analytical LOD (as discussed in *Determination of LOD*), and thus false-positive findings resulting from noise are not expected.

Determination of LOD

Several replicates (technical and biological) of titrated mixtures of reference DNA were evaluated to determine the analytical LOD of the test (Supplemental Table S2 and Supplemental Figure S6). All mixtures correlated with their expected methylation % except the 0% methylated DNA. Presumably due to noise in the sequencing, PCR amplification process, or reference DNA itself, the expected 0% methylation was always slightly above 0% when observed. The LOD and level of sensitivity were set at the lowest methylation % that was accurately and precisely quantitated. In addition, clinical samples that were previously characterized by ddPCR were evaluated to ensure that the LOD was appropriately set. During test validation, no samples between 2.5% and 10% were evaluated. However, many samples with no methylation (as quantitated by ddPCR) were evaluated and, similar to the results seen with the mixture samples, the methylation % was noisy but always <2%. When compared with the ddPCR test results, the test achieved 100% specificity (Table 3) for determining promoter methylation based on the analytical LOD of 2.5%.

Curiously, two patients (XGS013 and XGS014/XGS127) were methylated at both *BRCA1* and *RAD51C* between 9% and 16%. Interestingly, both patients were diagnosed with high-grade serous ovarian carcinoma but neither possessed a *TP53* mutation, and XGS014/XGS127 had a somatic *KRAS* mutation, which is more consistent with a diagnosis of low-grade serous ovarian carcinoma. Low-level mosaic

constitutive *BRCA1* promoter methylation occurs in normal tissues,[39] which may explain this unusual finding. Accordingly, a clinical LOD for somatic tumor cell–specific methylation detection was set at 10% to avoid false-positive findings from contaminating methylated normal cells in samples with low tumor content.

Determination of Analytical Repeatability and Reproducibility

To determine precision between results of the test when performed on the same sample by one or more operators, on the same instrument, and under the same conditions (within- and between-run precision/batch repeatability), on different days and in different batches, three titrated mixtures of the CpGenome methylation standards (2.5%, 50%, and 100%) were evaluated. Five experiments were compared that included at least three replicates of all mixtures, although only one sample (100% methylated DNA) was included in all experiments (Supplemental Figure S7). Methylation at each CpG site was 100% repeatable at all three methylation levels included here, including 2.5% (the analytical LOD). For all three DNA samples, the mean methylation % across all CpG sites was within $\leq$10% of the expected methylation % of the titrated mixture. The 50% methylation mixture had the highest variation. Detection of methylated CpG sites was 100% repeatable across tests performed by the same operator with the same conditions and was within 10% of the expected value, which is considered acceptable. Detection of methylated CpG sites was 100% reproducible across tests performed by different operators and was within 10% of the expected value, which is considered acceptable.[32,33]

CpG Methylation Heterogeneity

The *RAD51C* promoter has been reported to be heterogeneously methylated.[14,40] The data shown here support this as the *RAD51C* promoter methylated CpG sites within each amplicon were heterogeneously methylated (Supplemental Figure S8) within the methylated haplotype block[41] (Supplemental Figure S9).

Clinical Interpretation

Quantitation of methylation at the *BRCA1* and *RAD51C* promoter regions is performed to predict if either gene is effectively transcriptionally silenced. If either gene is completely silenced through methylation of all alleles, the tumor will likely be HRR deficient and respond to PARPi therapy. However, interpretation of the methylation % is based on a number of factors, including tumor cell content, genetic alterations (somatic or germline) in genes involved in HRR, and other clinical factors. The findings of the BRM and HRD tests were interpreted as follows.

First, the mean methylation % at a gene promoter is adjusted based on either estimated tumor cell content or, preferably, by the *TP53* VAF. Loss of heterozygosity of *TP53* occurs in most high-grade serous ovarian carcinoma and TNBC cases and can be used as a proxy for tumor content.[42] The tumor cell methylation % changes relative to the raw level because most, if not all, tumor specimens are not completely composed of tumor cells and may contain between 5% and 90% non-tumor cells. Using adjusted methylation levels, a relative level of "completeness" of methylation can be applied. Because the tests that were performed to infer HRD status did not interrogate gene copy number, actual zygosity cannot be determined.

Using 29 samples that were found to be methylated at either gene promoter, a histogram of the adjusted methylation levels was created to explore the distribution of percent methylation (Figure 4). The histogram shows two peaks, one around 40% to 50% methylation and another around 70% to 80% methylation, with a valley between 60% and 70% (similar to the "complete" methylation threshold of 70% used by other investigators[13,14,17,43,44]). These groups presumably represent "partial" promoter methylation and "complete" promoter methylation, corresponding to heterozygous (monoallelic) and homozygous (biallelic) methylation, respectively. These qualitative levels can be then used in combination with variants in HRR genes to predict HR status and PARPi sensitivity: tumors with

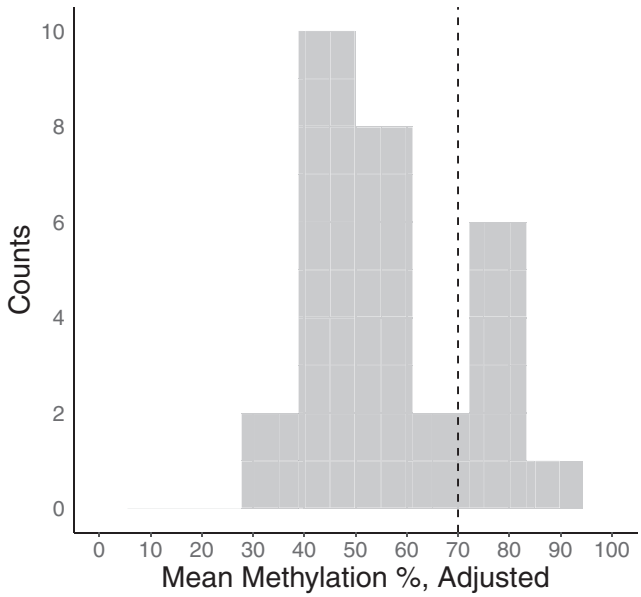

Figure 4 Distribution of mean methylation percentage (methylation %) per sample after adjustment by *TP53* variant allele frequency or estimated tumor cell content. A histogram of the adjusted mean methylation % values is shown across all samples that had promoter methylation of either the *BRCA1* or *RAD51C* promoter to show the distribution of samples with either "complete" or "partial" methylation. Methylation levels were adjusted for 29 samples (12 breast cancer, 17 ovarian cancer) that had promoter methylation. Twenty-eight samples had somatic *TP53* variants; adjustment based on variant allele frequency was therefore used preferentially to adjust by tumor cell content while the estimate of tumor content by pathologist assessment was used in the absence of a *TP53* variant. The **vertical dashed black line** occurs at 70% mean methylation.

"complete" methylation at either *BRCA1* or *RAD51C* are considered to be likely HR deficient and therefore likely sensitive to PARPi; tumors with "partial" methylation and a pathogenic variant in the same HRR gene may also be HR deficient and PARPi-sensitive; and, tumors with "partial" or no methylation and no pathogenic HRR gene variants are likely HR proficient and not sensitive to PARPi.

In the 158 clinical samples tested with the BRM test, adjusting for either *TP53* VAF and/or tumor content and using 70% as the "complete" methylation threshold, 11 were found to be "completely" methylated (7%); 39 were found to be "partially" methylated; (24%) and 113 were found to be not methylated (69%) at either gene promoter. Although it is difficult to perform a direct comparison of these proportions versus those found in other studies because of differences in patient population, cancer types, treatment history, gene promoters interrogated, and thresholds for complete methylation, reported proportions of complete, partial, and no methylation range between 2.5% and 29%, 1.9% and 21%, and 67% and 94%, respectively.[13,14,17,43–45] Because promoter methylation is dynamic and can change in response to therapy, the broad ranges of reported complete and partial methylation statuses are not surprising. The patient population interrogated in the current study is generally consistent with what has been previously reported.

One breast cancer sample (XGS172) displayed high levels of both *BRCA1* (chromosome 17q21.31) and *RAD51C* (chromosome 17q23) promoter methylation (36%, 62% adjusted by tumor cell content; 55%, 100% adjusted by *TP53* VAF) that could not be readily explained by contaminating methylated normal cells. Simultaneous methylation of both promoters seems to be a rare event but was reported in four patients (<1%) enrolled in the PAOLA-1 study,[18] suggesting that complete epigenetic silencing of different HRR genes is not mutually exclusive and may occasionally span both genes.[19,20]

Discussion

The importance of *BRCA1* promoter methylation as an epigenetic mechanism in breast cancer was elucidated more than two decades ago.[2] Since then, a number of *in vitro* studies and clinical trials have refined the understanding of HRR gene promoter methylation in both tumorigenesis and therapy response, specifically in relation to the PARPi class of drugs. More recently, it has become clear that the level of promoter methylation, rather than merely its presence, can inform patient response to PARPi.[13,14] In TNBC and ovarian cancer, it has been shown that both *BRCA1*- and *RAD51C*-methylated tumors with a high level of methylation also have high HRD scores and/or the presence of mutational signature 3, which is associated with HRD.[31,45–48]

The results from the NGS-based *BRCA1* and *RAD51C* promoter methylation test presented here show that it can accurately quantitate the level of promoter methylation at the *BRCA1* and *RAD51C* genes using FFPE samples, even when the extracted DNA is degraded or input amount is limited. Although the test has an analytical LOD of 2.5%, based on serial dilution of reference DNA, a clinical LOD of 10% was determined from clinical samples and applied to avoid false-positive findings resulting from low-level mosaic constitutive methylation of contaminating normal cells in samples with low tumor cell content. Validation of this test showed that the test has strong linearity and precision and is robust to noise contributed by PCR amplification errors, sequencing errors, and variable, although slight, incompleteness of bisulfite conversion. Comparison with a gold standard quantitative test found that it is concordant with expected results and has high sensitivity, specificity, and accuracy.

The need to adjust the raw methylation frequency for tumor cell content is an important step in being able to reduce the apparent heterogeneity into dichotomous clinically actionable results. Our attempts to do this using the *TP53* VAF, as a more accurate surrogate measure of tumor purity than a pathologist's subjective estimate, showed some improvement but did not result in clear-cut 0%, 50% (monoallelic), and 100% (biallelic) groups. Further improvements using loss of heterozygosity allele frequencies and *BRCA1* and *RAD51C* gene copy number measurements, generated from whole-exome sequencing, are underway as correlative translational studies in the EMBRACE clinical trial (Sjoquist et al, unpublished data).

The ddPCR comparator test measured methylation at six CpG sites across both genes while the NGS test measured 35 sites. It has been observed here and by others that methylation across the *BRCA1* promoter is homogeneous (ie, all CpG sites methylated), whereas the *RAD51C* promoter seems to be heterogeneously methylated[14,40] (Supplemental Figure S8). Although averaging the methylation levels of three *RAD51C* CpG sites, measured by ddPCR, and 25 sites, measured by NGS, produced similar values, measuring several sites remains important to avoid sampling bias and a potentially incorrect quantitation of methylation at the promoter. Regardless of the heterogeneity, patients with hypermethylation of the *RAD51C* promoter are still able to respond to PARPi therapy. Perhaps after many more *RAD51C*-methylated patients have been tested, a pattern of critical CpG sites will emerge.[18]

Although the testing conducted in the current study was performed only on tumor samples, constitutional methylation of *BRCA1* and *RAD51C* has been reported.[2,39,49–63] Constitutional methylation in normal tissues is invariably mosaic and below the clinical LOD of 10%.[49] Constitutional *BRCA1* methylation seems to be mutually exclusive with germline *BRCA1*/*BRCA2* pathogenic variants[23] and may be a risk factor for both TNBC and high-grade serous ovarian carcinoma and may also be true of *RAD51C* as well. This assay could also be used for constitutive promoter

methylation testing of blood samples to identify additional individuals at higher inherited risk of these cancers. Moreover, the constitutional *BRCA1* c.-107A > T epimutation site[64] is also covered by this assay, allowing this genetic variant to be identified with the same test, although it seems to be rare.[49,65–67] No patients have been identified to date with a pathogenic germline or somatic *BRCA1* or *RAD51C* mutation in one allele and germline or somatic promoter silencing by methylation of the other, again pointing to mutually exclusive mechanisms.

BRCA2 promoter methylation was reported, during the conduct of this work, in a single study[68] at a prevalence of 4.6% in patients with ovarian cancer and is now included as a target in the next generation of the methylation test used here. To date, it seems that no other HRR genes have been shown to be silenced by promoter methylation in breast or ovarian cancer.[31,32]

The potential clinical utility of this test has been shown in two clinical trials. Samples XGS141-XGS181 were from patients enrolled in the DORA clinical trial, which evaluated olaparib maintenance therapy in patients with platinum-sensitive TNBC.[20] The initial retrospective results showed that 22% of patients tested had some level of either *BRCA1* or *RAD51C* promoter methylation, whereas 20% of patients had a deleterious somatic *BRCA1* variant (several of whom also had a germline *BRCA1* variant), and that these patients were mutually exclusive. Overall, the median progression-free survival on maintenance therapy was longer in the presence of any HRR gene variant, and three of the four patients with the most durable responses were observed in patients with either *BRCA1*- or *RAD51C*-methylated tumors.

One of the samples used in this validation series (XGS120) was from a patient with TNBC whose tumor was exquisitely and durably platinum sensitive.[31] This patient's tumor had previously been tested with a large gene panel, and no genetic cause for this response was identified. However, BRM testing revealed the tumor to be completely methylated at the *BRCA1* promoter, which likely explained the patient's response to therapy.[31]

Including *BRCA1* or *RAD51C* promoter methylation in HRR gene panel testing would identify an additional approximately 3% to 10% of patients (depending on cancer type) who could potentially benefit from PARPi therapy. Notably, the BRM test was designed to be used for prospectively prescreening patients and quantitating the level of *BRCA1* and *RAD51C* methylation in the EMBRACE trial, a single-arm, signal-seeking phase 2 trial evaluating olaparib as a monotherapy in germline *BRCA1/2* wild-type patients with HRR-deficient TNBC or high-grade serous ovarian carcinoma.[20] The trial is now closed, and the results will be published shortly.

A decision tree was created to provide a structure to guide interpretation of the results of the BRM test in the context of other HRR gene variants or signatures (Supplemental Figure S10). This was used for retrospective analysis and prospective screening and recruitment of patients in PARPi clinical trials.[19,20]

Due to the rapid workflow, robustness regarding DNA input quality, and quantity and ease of integration into a modern pathology laboratory, this test is well positioned to enable both retrospective testing of precious samples and routine clinical testing of patients with breast and ovarian cancer. This test increases the precision of diagnostic tests aimed at identifying patients who are likely to respond to PARPi therapy as well as those who are unlikely to benefit. In particular, when this test is paired with an HRR gene panel test, a more comprehensive understanding of the molecular determinants of HRR deficiency in a tumor can be achieved.

Conclusions

In summary, the BMR test was analytically and clinically validated by using MS-ddPCR as a comparison test. This showed that the BRM test can accurately and robustly quantitate methylation levels, even in challenging clinical samples in which DNA input amount is limited or DNA is highly degraded due to formalin fixation. Guidance for interpreting the test results was also established and a robust and efficient workflow was designed to enable fast turnaround times in a clinical setting to enable a more accurate precision diagnostic test that identifies predictive biomarkers for PARPi response.

Acknowledgments

All patients signed research consent forms before sample testing. All testing was performed in ISO15189-accredited laboratories. For EMBRACE (X17-0159) trial screening samples, the ethics application was approved by the RPAH Human Research Ethics Committee (HREC/17/RPAH/232); funding was from Cancer Australia and the NBCF (PS-15-048). EMBRACE was led by the National Health and Medical Research Council Clinical Trials Centre, University of Sydney, in collaboration with the Australia New Zealand Gynaecological Oncology Group, Breast Cancer Trials—Australia and New Zealand, and the Genomic Cancer Clinical Trials Initiative. The authors thank the DORA clinical trial team and National Cancer Centre of Singapore; the Beacon Biomarkers Laboratory (University of Melbourne); the Australian Translational Genomics Centre; and AstraZeneca for providing samples for testing and expert discussion and Pillar Biosciences for providing oncoReveal kits.

This publication is dedicated to the memory of Alex Dobrovic, who passed in the final months of this study. His discovery of *BRCA1* methylation,[2] description of its prevalence in breast and ovarian cancer,[2,6] demonstration of the importance of biallelic silencing,[13,69] and the development of the comparator quantitative MS-ddPCR test[39] inspired and enabled this study.

Author Contributions

J.L.F. and P.M.W. conceived and designed the study; J.L.F., B.J., and P.M.W. developed the methodology; J.L.F. wrote the original draft of the article; J.L.F., B.J., A.D., S.K., J.H., O.K., and P.M.W. reviewed and revised the article; B.J., N.S., and B.S. performed sequencing-related molecular experiments; F.Z. and A.D. performed MS-ddPCR experiments; J.L.F. curated data, performed bioinformatics and statistical analyses, and generated data visualizations; J.L.F., A.D., S.K., J.H., and P.M.W. interpreted data; and A.D., S.K., J.H., and O.K. provided technical and/or material support. All authors read and approved the final paper.

Disclosure Statement

P.M.W. is on the Scientific Advisory Board and is a shareholder in Pillar Biosciences. P.M.W. and J.L.F. have received consulting fees and conference travel support from Pillar Biosciences. O.K. has received consulting fees from XING Genomic Services and a travel honorarium from AstraZeneca.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2024.11.004*.

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