PILLAR BIOSCIENCES

WHITEPAPER

oncoReveal® Methylation Panels

Workflow and performance overview of targeted methylation NGS panels

Highlights

- **Recommended converted DNA input: 5-20 ng**
- **Total assay time (from converted DNA): 6-7.5 hours**
- **Recommended depth: ~2,000 clusters/amplicon**
- **Linearity (Pearson's r): >0.99**
- **Targeted methylation panels:**
  (i) oncoReveal® 4 Gene Methylation Panel (*PN# HDA-HR-1009-24*) focusing on *BRCA1*, *BRCA2*, *RAD51C*, and *XRCC3*; for breast and ovarian cancer indications.
  (ii) oncoReveal® *MGMT* & *MLH1* Methylation Panel (*PN# HDA-HR-1010-24*) for glioblastomas, gliomas, colorectal, and endometrial cancer indications.
- **Easy workflow:** A simple NGS library prep workflow that can be completed in a single workday. Total assay time (from converted DNA to sequencer) of 6-7.5 hours.
- **Fast and accurate data analysis:** Get accurate results using PiVAT®, which provides up to 8x faster results compared to standard analysis pipelines.

DNA methylation as a diagnostic biomarker

DNA methylation is a crucial epigenetic modification that regulates gene expression and cell differentiation. This process involves the addition of a methyl ($-CH_3$) group to the cytosine base of DNA, predominantly at CpG dinucleotides. CpG-rich regions (CpG islands), often located in gene promoters and first exons, are key regulatory sites. Methylation of these regions typically inactivates transcription, thereby controlling gene expression. In a normal cell, CpG islands in promoter regions are generally hypomethylated to allow for active transcription, while repetitive regions and the broader genome require hypermethylation to maintain genomic stability. However, the loss of this balance, characterized by the hypo- or hypermethylation of critical genes such as oncogenic drivers and tumor suppressors, can cause genomic instabilities, gene dysregulation, and mitotic missegregation, ultimately leading to cancer. Thus, maintaining precise DNA methylation patterns is critical for normal cellular function and disease prevention[1].

Aberrant DNA methylation patterns are frequently observed across various cancer types and serve as key indicators of therapy response. For example, methylation of the *BRCA1* and *RAD51C*, and less frequently *BRCA2*, promoter has been reported in sporadic breast, ovarian, and pancreatic cancers, leading to gene silencing and the loss of essential tumor suppressor functions[2-4]. Similarly, the hypermethylation of the *MGMT* gene promoter in tumors is a clinically significant marker associated with improved response to alkylating chemotherapy, as the resulting gene silencing prevents repair of chemotherapy-induced DNA damage in tumor cells[5-8].

Since DNA methylation is stable in somatic cells and resilient to changes induced by sample processing and storage, it is a promising candidate for diagnostic applications. Several diagnostic tests based on methylation are currently approved for diagnostic use, with many more in advanced stages of development[9-10]. Locke et al. (2019) identified at least six broad diagnostic areas where a methylation test can be combined with traditional screening and imaging procedures for improved patient outcomes: 1) primary diagnosis prior to traditional testing, 2) triage for patients with unclear diagnosis, 3) therapy selection, 4) monitoring response to therapy and treatment failure, 5) minimal residual disease monitoring, and 6) recurrence detection. Consequently, it is critical that

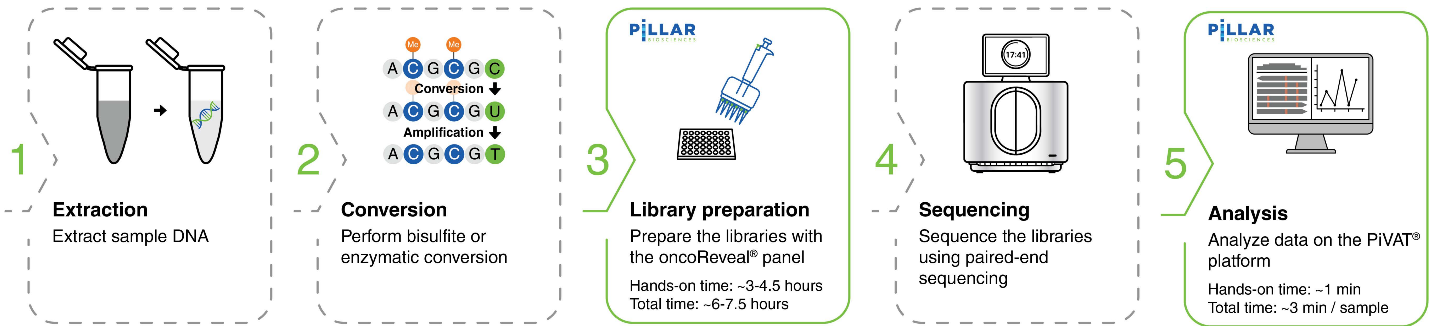

Figure 1. End-to-end workflow for targeted methylation using Pillar oncoReveal® panels with PiVAT® data analysis. The workflow consists of five sequential steps from sample processing to data interpretation. Steps highlighted in **green** (library preparation and data analysis) indicate components facilitated by the oncoReveal® panels and PiVAT® software. **(1) DNA Extraction**: Genomic DNA is isolated from input samples. **(2) Cytosine Conversion**: DNA undergoes either bisulfite or enzymatic conversion, enabling differentiation between methylated and unmethylated cytosines during downstream sequencing. **(3) Library Preparation**: Targeted amplification is performed using an oncoReveal® methylation panel. **(4) Sequencing**: Prepared libraries are sequenced on a high-throughput sequencing platform to generate reads spanning targeted methylation loci. **(5) Data Analysis using PiVAT®**: Sequencing data are processed using PiVAT®, which performs bisulfite-aware alignment and quantitative methylation calling to generate both gene-level and CpG-level methylation metrics.

any methylation test can accurately and reproducibly detect methylation levels in patient samples.

Targeted methylation next-generation sequencing (NGS) panels offer a focused and scalable approach for interrogating clinically relevant promoter regions in genes implicated in cancer biology and therapy response, without the need for whole genome methylation sequencing. In this whitepaper, we describe two Research Use Only (RUO) amplicon-based bisulfite sequencing panels designed to assess promoter methylation in key DNA repair pathways, specifically **homologous recombination repair (HRR) genes** (*BRCA1*, *BRCA2*, *RAD51C*, *XRCC3*)[2-4] and **direct reversal and mismatch repair markers** (*MGMT*, *MLH1*)[5-8]. We outline the associated laboratory and computational workflows (Figure 1) and evaluate their technical performance with clinical specimens and characterized commercially available methylation standards. Two types of conversion methods (bisulfite and enzymatic) were used prior to targeted amplification using either panel. Finally, we examine the concordance of methylation measurements with an established bisulfite sequencing analysis approach and discuss considerations for interpreting methylation levels in the context of heterogeneous tumor samples.

Panel	Gene Target	Functional Pathway	Indication	Reported Clinical/Research Relevance
4 Gene Panel	*BRCA1*	HRR	Breast, Ovarian, Pancreatic	Emerging predictive biomarker for HRR treatment; indicates responsiveness to PARP Inhibitors and Platinum-based therapy.
	RAD51C	HRR	Ovarian	Emerging predictor of PARP Inhibitor efficacy.
	BRCA2	HRR	Breast, Ovarian	Exploratory marker for research panels.
	XRCC3	HRR	Various Solid Tumors	Exploratory marker for research panels.
MGMT & MLH1 Panel	*MGMT*	Direct Repair	Glioblastoma, Glioma	Promoter methylation is used to inform temozolomide (TMZ) based treatment selection.
	MLH1	Mismatch Repair	Colorectal, Endometrial	Promoter methylation can help distinguish between sporadic vs. Lynch syndrome; associated MSI-H/dMMR status may inform checkpoint inhibitor use. (NCCN Colon Cancer)

Table 1. The table provides a summary of panel gene content and associated indications to inform research interpretations related to therapeutic response and patient stratification.

The oncoReveal® workflow

The core of the workflow is a streamlined, two-stage PCR process. Following DNA conversion (bisulfite or enzymatic), the protocol moves from target enrichment to a sequencer-ready library in just a few steps:

1. **Target Enrichment:** A single-tube multiplex PCR reaction simultaneously amplifies all target promoter and enhancer loci.
2. **Indexing:** A second PCR step attaches indexes and sequencing adapters to amplicons.

The entire library preparation requires less than 3-4.5 hours of hands-on time and can be completed in a standard workday in 6-7.5 hours (Figure 1). The oncoReveal® panels are compatible with the two primary methods of cytosine modification:

1. **Bisulfite Conversion:** Remains a widely used and well-established method for methylation analysis, providing high-confidence results for routine samples.
2. **Enzymatic Conversion:** Offers a gentler alternative that preserves DNA integrity by avoiding the harsh chemical conditions of bisulfite treatment. This is particularly advantageous for samples with low input or high degradation, ensuring maximum library complexity and sensitive detection.

Clinical research context and interpretation considerations

The oncoReveal® Methylation Panels provide quantitative insights into the epigenetic landscape of DNA repair genes. Unlike traditional qualitative assays, these panels utilize high-depth amplicon sequencing to provide a granular methylation level (ML), enabling differentiation between low-level background methylation and higher levels that may be associated with gene silencing.

oncoReveal® 4 Gene Methylation Panel: This 4-gene panel focuses on the Homologous Recombination Repair (HRR) related genes (*BRCA1*, *BRCA2*,

<table>
<tr><th>Dataset</th><th>Panel</th><th>DNA</th><th>Instruments Tested</th><th>N</th></tr>
<tr><td rowspan="10">Analytical Stress Test (N=384)</td><td rowspan="7">oncoReveal® 4 Gene</td><td>Anchor Molecular (Unmethylated)</td><td>MiSeq</td><td>5</td></tr>
<tr><td>Enzymatic conversion (Zymo reference)</td><td>NextSeq/MiSeq</td><td>8</td></tr>
<tr><td>Qiagen reference</td><td>NextSeq/MiSeq</td><td>112</td></tr>
<tr><td>Zymo converted (moderate)</td><td>NextSeq/MiSeq</td><td>77</td></tr>
<tr><td>Zymo converted (severe)</td><td>NextSeq/MiSeq</td><td>36</td></tr>
<tr><td>Zymo in-house converted (gDNA)</td><td>NextSeq/MiSeq</td><td>33</td></tr>
<tr><td>Zymo reference (gDNA)</td><td>NextSeq/MiSeq</td><td>93</td></tr>
<tr><td rowspan="3">oncoReveal® MGMT & MLH1</td><td>Zymo converted (moderate)</td><td>NextSeq/MiSeq</td><td>6</td></tr>
<tr><td>Zymo converted (severe)</td><td>NextSeq/MiSeq</td><td>8</td></tr>
<tr><td>Zymo reference (gDNA)</td><td>NextSeq/MiSeq</td><td>6</td></tr>
<tr><td rowspan="6">Quantitative Titration (N=64)</td><td rowspan="5">oncoReveal® 4 Gene</td><td>LGC Methylated</td><td>MiSeq</td><td>2</td></tr>
<tr><td>LGC Unmethylated</td><td>MiSeq</td><td>2</td></tr>
<tr><td>Coriell (Unmethylated)</td><td>MiSeq</td><td>3</td></tr>
<tr><td>NIST Samples A-E (0-100%)</td><td>MiSeq</td><td>10</td></tr>
<tr><td>Qiagen Dilutions</td><td>NextSeq/MiSeq</td><td>24</td></tr>
<tr><td>oncoReveal® MGMT & MLH1</td><td>Qiagen Dilutions</td><td>MiSeq</td><td>23</td></tr>
</table>

Table 2. Summary of datasets used for technical performance evaluation of the oncoReveal® methylation panels. The Analytical Stress Test (N=384) includes samples generated under diverse DNA treatment conditions, including enzymatic and bisulfite conversion, reference standards, and degraded inputs modeling FFPE-like conditions using both fcDNA and gDNA. The Quantitative Titration (N=64) includes samples with defined methylation levels (0-100%) and dilution series to assess quantitative performance. Samples were processed using the indicated oncoReveal® panels and sequenced on MiSeq and NextSeq platforms. The N column indicates the number of samples per condition.

RAD51C, *XRCC3*) and is designed to identify tumors with "BRCAness" – a phenotype where epigenetic silencing mimics the effect of germline or somatic mutations (Table 1).

- *PARP Inhibitor Eligibility:* Promoter hypermethylation of *BRCA1* and *RAD51C* is reported in the literature to be associated with sensitivity to PARP inhibitors and platinum-based chemotherapies in ovarian and breast cancers.
- *Quantitative Measure:* High *RAD51C* methylation (%) has been reported to correlate with HRD phenotypes in some studies. The oncoReveal® workflow enables quantitative assessment of methylation levels across samples.
- *Resistance Monitoring:* Research has shown that the loss of promoter methylation (epigenetic "reversion") is associated with resistance to PARP inhibitors. This panel enables the tracking of these epigenetic shifts in longitudinal samples.

oncoReveal® *MGMT* & *MLH1* Methylation Panel: This panel addresses two of the most critical epigenetic markers in oncology, providing definitive data for both therapy selection and hereditary screening (Table 1).

- *MGMT in Glioma (Therapy Selection): MGMT* promoter methylation is a well-established biomarker associated with response to alkylating agents like temozolomide (TMZ). Our NGS approach profiles multiple CpG sites across the promoter region, enabling higher-resolution assessment of methylation patterns. A higher methylation level across the promoter has been shown in literature to correlate with significantly improved progression-free survival (PFS).
- *MLH1 in CRC/Endometrial (Lynch Syndrome Triage):* For tumors exhibiting Microsatellite Instability (MSI-H), the first clinical question is whether the origin is sporadic or hereditary (Lynch Syndrome). As *MLH1* methylation can contribute to an MSI-H or deficient mismatch repair (dMMR) phenotype, these tumors may be associated with responsiveness to immune checkpoint inhibitors, particularly in colorectal cancer.
    - *Sporadic Identification:* Hypermethylation of *MLH1* (specifically in Region C) is commonly used to support classification of tumors as sporadic rather than Lynch syndrome-associated, potentially sparing the patient and their family from unnecessary germline testing.
    - *Immunotherapy Guidance:* Functional silencing of *MLH1* results in a high mutational burden, making these patients more likely to respond to immune checkpoint inhibitors.

Understanding methylation levels

To assist in the interpretation of the methylation results, we recommend integrating the observed **methylation level (ML)** with an estimate of the **tumor fraction (TF)**. One way of reliably estimating TF is by using variant allele frequency (VAF) of a clonal somatic driver, such as a *TP53* (assuming no copy number changes) mutation. A simplified approximation is:

$$\text{Estimated tumor-specific methylation} = \frac{\text{Observed ML}}{\text{Estimated TF}}$$

Examples:

Sample A: MGMT observed ML = 15%; estimated TF = 80%

Interpretation: ~18% of tumor cells are methylated. This may reflect subclonal methylation or low biological significance.

Sample B: *MGMT* observed ML = 15%; estimated TF = 16%

Interpretation: ~94% of tumor cells are methylated. This suggests a high proportion of methylated tumor cells and may be associated with gene silencing.

Technical performance assessment

The oncoReveal® Methylation Panels enable sensitive detection of promoter methylation while maintaining the rapid turnaround times required for high-throughput research workflows. The following data demonstrates the analytical precision and computational efficiency of the end-to-end workflow.

► *Technical Performance Evaluation Across Diverse Conditions*

To ensure the oncoReveal® system delivers consistent results across the diverse conditions encountered in clinical laboratories, a comprehensive evaluation was conducted using a total of 80 samples (Table 2). This experimental design rigorously tested both

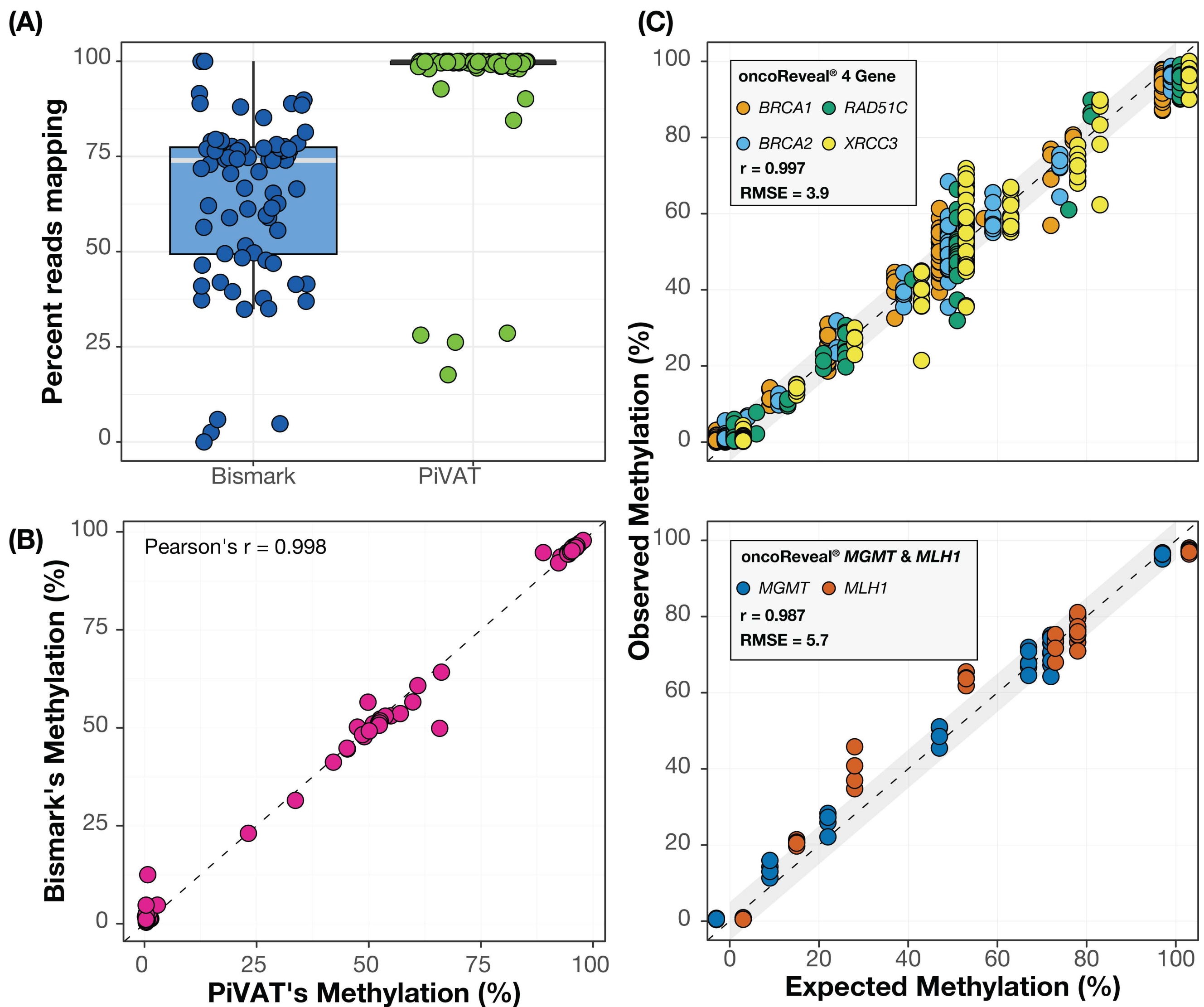

Figure 2. Technical performance of the oncoReveal® methylation panels and PiVAT® analysis workflow. (A) Percent reads mapping across DNA treatment conditions (aggregated), comparing PiVAT and a Bismark-based pipeline. Observed methylation percentages are jittered by gene to improve visualization of overlapping data points. **(B)** Concordance of methylation levels between PiVAT and Bismark across samples (Pearson's r = 0.998). **(C)** Observed versus expected methylation levels for controlled titration samples, demonstrating quantitative performance for the 4-gene panel (*BRCA1*, *BRCA2*, *RAD51C*, *XRCC3*) and the *MGMT* & *MLH1* panel. Correlation (r) and Root Mean Square Error (RMSE) values are shown for each panel.

oncoReveal® Methylation panels against varied conversion chemistries, sample types, and sequencing configurations. The evaluation cohort was comprised of two primary datasets:

- **Analytical Stress Test** (N=384): This dataset utilized a collection of samples generated through multiple DNA treatment procedures to evaluate performance under varying conditions, including both bisulfite and enzymatic conversion. The cohort integrated a range of reference standards, including Qiagen EpiTect PCR Control DNA Set, Zymo Human DNA Standard, Horizon DNA sets, Coriell reference standards, Anchor Molecular, and NIST cell-free DNA methylation challenge samples. To simulate challenges encountered in clinical specimens, Horizon fcDNA was used to model varying levels of degradation corresponding to

moderate and severe FFPE conditions, while Horizon gDNA served as the undamaged control.

- **Quantitative Titration (N=64):** A controlled set of experiments was performed using samples with known methylation levels of 0%, 12%, 25%, 50%, 75%, and 100% to establish the assay's linear range and quantitative accuracy.

All samples were processed and sequenced using Illumina NextSeq 550 and MiSeq v2 chemistry, demonstrating the flexibility of the chemistry for different laboratory scales.

► *Bioinformatics Performance: PiVAT® vs. Standard Pipelines*

The accuracy of methylation profiling is fundamentally dependent on the pipeline's ability to efficiently process bisulfite- or enzymatically-converted sequencing data. The oncoReveal® analysis workflow, powered by the Pillar Variant Analysis Toolkit (PiVAT®), was benchmarked against the industry-standard Bismark[9] pipeline to assess mapping efficiency, accuracy, and throughput (Figure 2). The key differences are

A. Enhanced Read Capture and Mapping Efficiency: A critical metric for data utilization and coverage is the effectiveness of read mapping. In 83% of the samples analyzed, PiVAT® successfully mapped a higher proportion of sequence reads compared to Bismark. On average, the PiVAT® mapping rate was **26% higher** than the industry standard, indicating improved utilization of input sequencing reads. This efficiency ensures higher depth of coverage across critical CpG targets, even in low-input or damaged samples.

B. Excellent Quantitative Accuracy and Concordance: A subset of the controlled titration samples was run on both PiVAT® and Bismark, for the purpose of computational performance benchmarking. Both PiVAT® and Bismark demonstrated high correlation between predicted and expected methylation levels. PiVAT® provided greater precision with a Pearson's r of 0.998 (vs. 0.993 for Bismark) and a substantially lower error rate, achieving a Root Mean Squared Error (RMSE) of 4.3, compared to 6.2 for Bismark. Furthermore, analysis of the DNA treatment dataset showed a near-perfect concordance (Pearson's r = 0.998) between the two pipelines, confirming that PiVAT® demonstrates high concordance with the established bisulfite analysis pipeline.

C. Faster Computational Throughput: When executed on identical hardware (Intel Xeon 6-core server with 64 GB RAM), PiVAT® analyzed the entire 48-sample treatment dataset in approximately **26 minutes**. In contrast, the standard Bismark pipeline required approximately **216 minutes** (~3.5 hours). This represents an **8.3x reduction in runtime**, enabling laboratories to move from sequencing completion to data interpretation in a fraction of the time required by traditional methods.

Conclusions

This technical performance assessment of the oncoReveal® 4 Gene Methylation Panel and oncoReveal® MGMT & MLH1 Methylation Panel demonstrates consistent performance across evaluated conditions for epigenetic profiling. By utilizing the input read set more efficiently and delivering accurate, high-depth methylation results in significantly less time than traditional NGS pipelines, the oncoReveal® workflow provides a scalable and precise solution for research applications involving targeted methylation analysis.

Panel	Part Number
oncoReveal® 4 Gene Methylation Panel	HDA-HR-1009-24
oncoReveal® MGMT & MLH1 Methylation Panel	HDA-HR-1010-24

References

1. Kulis M, Esteller M. DNA methylation and cancer. Adv Genet. 2010; 70:27-56.
2. Kondrashova O, et al. Methylation of all BRCA1 copies predicts response to the PARP inhibitor rucaparib in ovarian carcinoma. Nat Commun. 2018; 9:3970.
3. Xu L, et al. High-level tumour methylation of *BRCA1* and *RAD51C* is required for homologous recombination deficiency in solid cancers. NAR Cancer. 2024;6(3): zcae033.
4. Nesic K, et al. Acquired RAD51C promoter methylation loss causes PARP inhibitor resistance in high grade serous ovarian carcinoma. Cancer Res. 2021;81: canres.0774.2021.
5. Esteller M, et al. Inactivation of the DNA-repair gene *MGMT* and the clinical response of gliomas to alkylating agents. N Engl J Med. 2000; 343(19):1350-4.
6. Hegi ME, et al. MGMT Gene Silencing and Benefit from Temozolomide in Glioblastoma. N Engl J Med. 2005; 352:997-1003.
7. Bettstetter M, et al. Distinction of hereditary nonpolyposis colorectal cancer and sporadic microsatellite instability-high colorectal cancer through analysis of MLH1 methylation. Clin Cancer Res. 2007; 13(11):3221-3228.
8. André T, et al. Pembrolizumab in Microsatellite-Instability-High Advanced Colorectal Cancer. N Engl J Med. 2020; 383:2207-2218.
9. Locke WJ, et al. DNA Methylation Cancer Biomarkers: Translation to the Clinic. Front Genet. 2019; 10:1150.
10. Taryma-Leśniak O, et al. Current status of development of methylation biomarkers for in vitro diagnostic IVD applications. Clin Epigenetics. 2020; 12(1):100.
11. Krueger F, et al. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics. 2011; 27(11):1571-2.

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