Pillar Biosciences oncoReveal™ methylQuant Panels: Accurate and easy-to-use methylation NGS assays leveraging SLIMamp technology

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ABSTRACT

Introduction. Cancer-associated DNA methylation changes are promising biomarkers for screening, diagnosis, prognosis, treatment prediction and monitoring. Despite progress in studies correlating DNA methylation with cancer, the adoption of methylation tests for solid tumors in clinical trials and patient testing remains challenging due to assay complexity, informatics burden, high DNA input requirement, improper CpG site selection, and incorrect methylation level readouts. We developed SLIMamp® based multiplex PCR-based bisulfite NGS panels which are simple, economical, and highly specific. These research-use-only (RUO) kitted assays can assess methylation levels in BRCA1, RAD51C, BRCA2, XRCC3, MGMT and MLH1, focusing on several key CpG sites spanning gene promoters and first exons. The PCR primers are designed to bind equally to bisulfited-converted methylated and unmethylated DNA sequences, but not to un-converted gDNA. Following NGS, the methylation level [100 * C/(C + T)] at each CpG site can be determined with great precision. Here, we report our analytical verification study to evaluate the assay performance with a set of reference materials and well-characterized FFPE samples from CAP.

Methods. Moderately and severely formalin compromised DNAs (Horizon Discovery), and both methylated and unmethylated human standards (Zymo Research Corp.) were converted using either the Zymo EZ DNA Methylation-Lightning Kit or the Qiagen Epitect Fast Bisulfite Kit. Primers were designed with small amplicons ranging from 85-154bp, and with gene compatibility in one primer pool. Inputs into conversion were 250ng for reference standards and 25ng for CAP samples. Post bisulfite treatment, a protocol consists of two rounds of PCR and subsequent DNA purifications. DNA methylation levels from 0-100%, Horizon standards and CAP samples were tested in duplicate. Libraries were sequenced on Illumina MiSeq and NextSeq machines, and sequencing data was analyzed through Bismark and PiVAT® (Pillar Biosciences' Variant Analysis Toolkit).

Results. Concordant uniformity and methylation calls were observed across conversion kits and library prep inputs (2.5-20ng), with 66-83% of amplicons having mean coverage in the 0.3-1.8x range. Observed methylation levels matched expected levels, with a Pearson's r>0.99 and root mean squared error of 3.5 across all samples. Lastly, PiVAT reported methylation levels were highly concordant with Bismark's, but had improved mapping rates (~91.6% PiVAT vs ~64.9% in Bismark).

Conclusion. Pillar Biosciences' oncoReveal methylQuant panels can accurately detect methylation levels from 0% to 100% and effectively target moderately and severely formalin compromised DNA at library prep inputs of 5-20ng. Furthermore, PiVAT produces concordant results to Bismark with improved mapping rate.

EXPERIMENT DESIGN

- Two methylation panels were designed, targeting specific CpG sites in:
  - BRCA1, RAD51C, BRCA2, and XRCC3
  - MGMT and MLH1
- Primers were designed to amplify methylated and unmethylated bisulfite treated DNA in an unbiased manner.
- DNA was converted using the Qiagen Epitect Fast Bisulfite Kit or the Zymo EZ DNA Methylation Lightning Kit.
- Library preparation input ranged from 5-20ng of bisulfite converted DNA.
- The multiplex PCR library preparation procedure consists of two rounds of PCR and subsequent DNA purifications.
- Prepared DNA libraries were sequenced on Illumina's MiSeq platforms across several runs.
- All secondary analysis were performed in PiVAT.

- **Positive standard reference samples:**
  - Zymo Bisulfite Converted Universal Methylated Human Standard (n=10)
- **Other samples:**
  - Bisulfite converted Horizon moderate (n=12)
  - Bisulfite converted Horizon severe (n=10)
  - Bisulfite converted CAP FFPE sample with matching normal (n=2)
  - Bisulfite converted HCT DKO human methylated and unmethylated DNA at methylation levels:
    - 0% (n=4), 12.5% (n=6), 25% (n=4), 50% (n=8), 100% (n=8)

RESULTS

Pillar Biosciences oncoReveal methylQuant Panels Overview

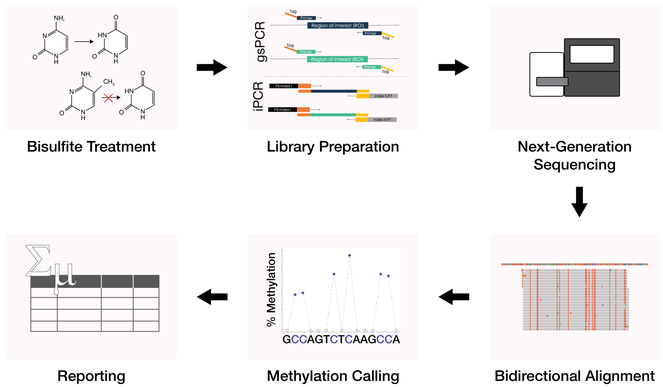

Figure 1. Overview of Pillar Biosciences oncoReveal methylQuant panels. Broadly, the molecular biochemistry steps of methylQuant panel can be broken into: (1) bisulfite treatment of input DNA, (2) library preparation using 2 rounds of PCR and DNA purification, (3) next-generation sequencing of the library. The generated sequencing data is processed through PiVAT which performs a (4) bi-directional alignment step to capture molecules originating from either strand of the genome, (5) refinement of the alignment, merging of paired-end reads and performing methylation calling, and (6) summarizing the results in an output report.

Methylation Call Comparison With Expectation

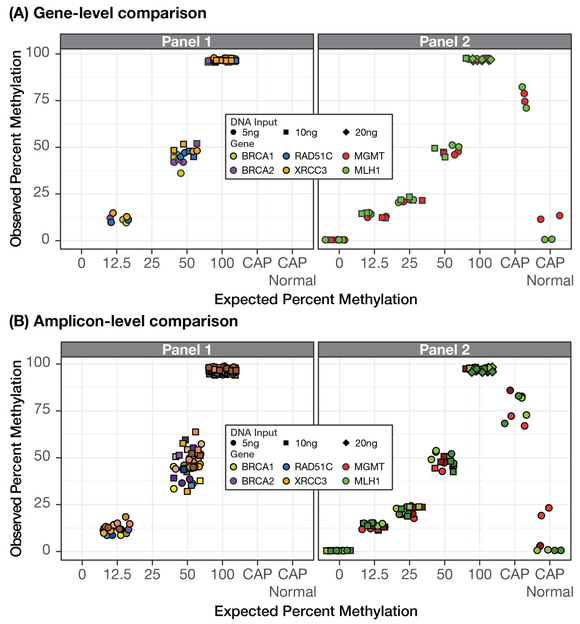

Figure 2. Observed vs expected percent methylation calls compared at gene and amplicon levels. Observed methylation calls from PiVAT were compared to the expected methylation levels across all samples from the two panels. Methylation calls were calculated at each site and then averaged at (A) gene-level, and (B) amplicon-level. Different DNA input are shown in different shapes, with the colors representing the targeted genes. For amplicons targeted within each gene, colors from the same family are used for visual identification. High correlation were observed at both the gene-level (Pearson's r=0.998) and amplicon-level (r=0.996)

RESULTS

Uniform Coverage Across Amplicons

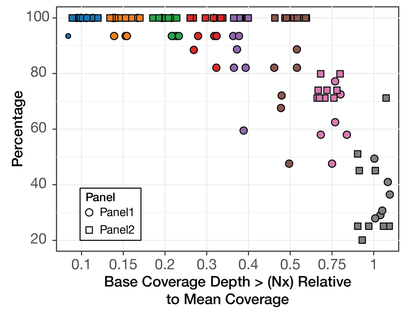

Figure 3. Uniform amplicon coverage was observed across the two panels. The percentage of amplicons in Pillar Biosciences oncoReveal methylQuant panels with coverage greater than varied fractions of mean coverage (across the x-axis) is displayed in figure 3. Points are jittered horizontally to show their distribution, and the fill color represents the x-axis percentage of mean coverage they represent. For both panel, most of the sequencing data (>80%) had relative coverage >0.4x of the mean, demonstrating a fairly uniform amplicon coverage across the panels. For panel 2, all the amplicons had a relative coverage >0.5x of the mean.

Comparison of PiVAT's Methylation Calls with Bismark

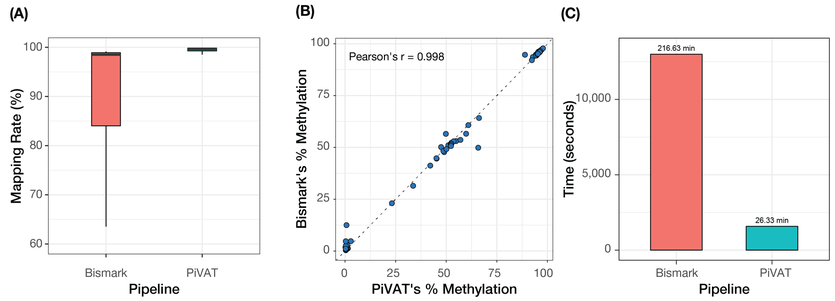

Figure 4. PiVAT's methylation calling performance compared with Bismark. PiVAT's methylation pipeline is compared to Bismark in three ways: (A) mapping rate, (B) called percent methylation, and (C) runtime compared when run on the same machine. (A) PiVAT consistently maps more reads (not shown) at a higher mapping percentage as shown in the boxplot. The average mapping rate for PiVAT is 99.52 ± 0.05%, compared to Bismark's 89.89 ± 1.5%. (B) Both PiVAT and Bismark's methylation calls were observed to be concordant, with a Pearson's r of 0.998. However, (C) PiVAT was significantly faster in processing the sequencing data, taking ~8x less time when compared to Bismark.

CONCLUSIONS

- Pillar oncoReveal™ methylQuant panels use a simple protocol involving 2 rounds of PCR and DNA purification of bisulfite converted DNA.
- The methylQuant panels accurately call expected methylation levels from 0-100% at DNA inputs ranging from 5-20ng in gDNA, and both moderately and severely compromised DNA.
- The methylQuant panels have >90% amplicon uniformity above 0.2x mean coverage and >80% amplicon uniformity above 0.3x mean coverage.
- PiVAT's methylation calling is concordant with Bismark's with ~8x faster processing and a higher mapping rate.
- Larger studies would be needed to establish the clinical utility of these panel