Minimizing Off-Target Capture in Targeted Methylation Sequencing Through Panel Design and Enhancer Reagents

INTRODUCTION

Methylation Sequencing and Capture

Methylation sequencing uses enzymatic or chemical methods to convert unmethylated cytosines to uracil. In both cases, the process leaves methylated cytosines intact (Figure 1). During amplification, each uracil is paired with adenine on the complementary strand, leading to the inclusion of thymines in the original positions of unmethylated cytosines. The final product is asymmetric, yielding two different double-stranded DNA (dsDNA) molecules after conversion (top row, Figure 1); the same process for methylated DNA generates another two dsDNA molecules (bottom row, Figure 1).

Target enrichment can be performed before or after the conversion process. While presenting fewer challenges for probe design, post-capture conversion often requires large quantities of starting DNA material because PCR amplification does not preserve methylation patterns. Therefore, pre-capture conversion, enabling the amplification of the converted material, is often the method of choice for low-input, sensitive applications such as liquid biopsies assaying minute amounts of cell-free DNA. Nevertheless, pre-capture conversion presents several challenges, including more complex panels as probes must be designed to target each of the four possible converted sequences for optimal capture (Figure 1).

Effect of Conversion on Sequence Complexity

Historically, hybrid capture of methylation sequencing libraries leads to increased off-target capture and reduced capture uniformity. These issues arise as the conversion of unmethylated cytosines to thymines causes the target genome to become reduced in sequence complexity and GC content (Figure 2).

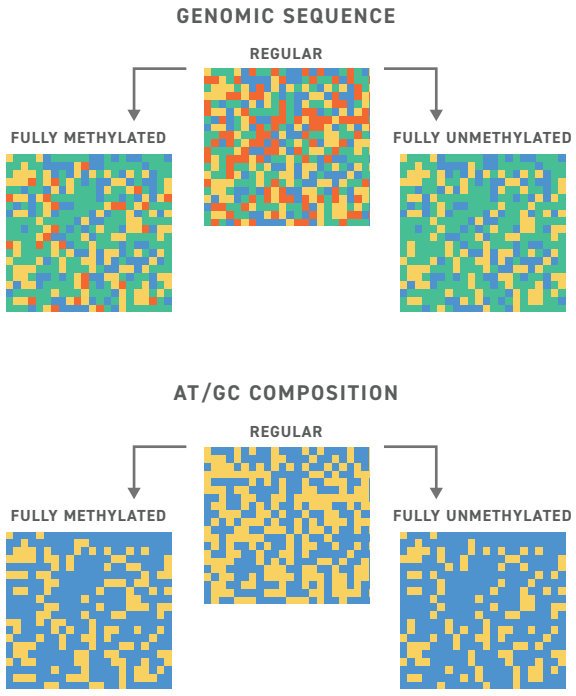

Figure 2. The effect of conversion on sequence complexity and GC content. Compared to the regular nucleotide composition of the genome, conversion of methylated cytosines causes a notable reduction in sequence complexity. An example is shown above for a random genomic sequence where four colors represent each of the four nucleotides (top) or two colors corresponding to G/C or A/T (bottom). This reduction in sequence complexity and %GC content after conversion is exacerbated in GC rich functional sequences and methylation targets, leading to challenges including increased repeat content, off-target capture, and widespread changes to the melting temperature and kinetics of the system.

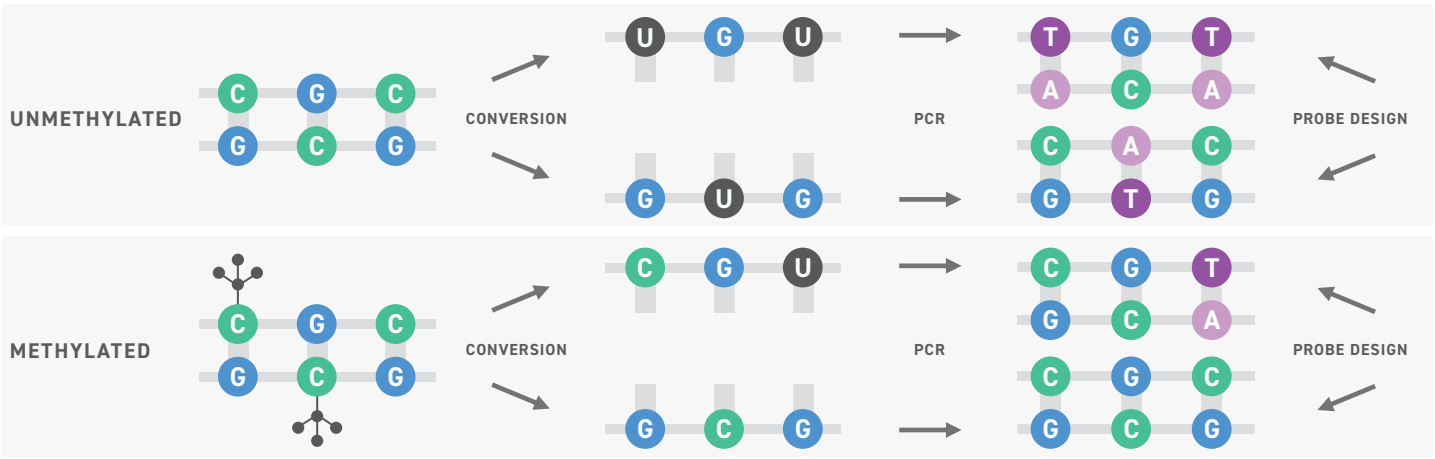

Figure 1. The process of methylation conversion. Differences in sequence during the conversion of unmethylated and methylated DNA..

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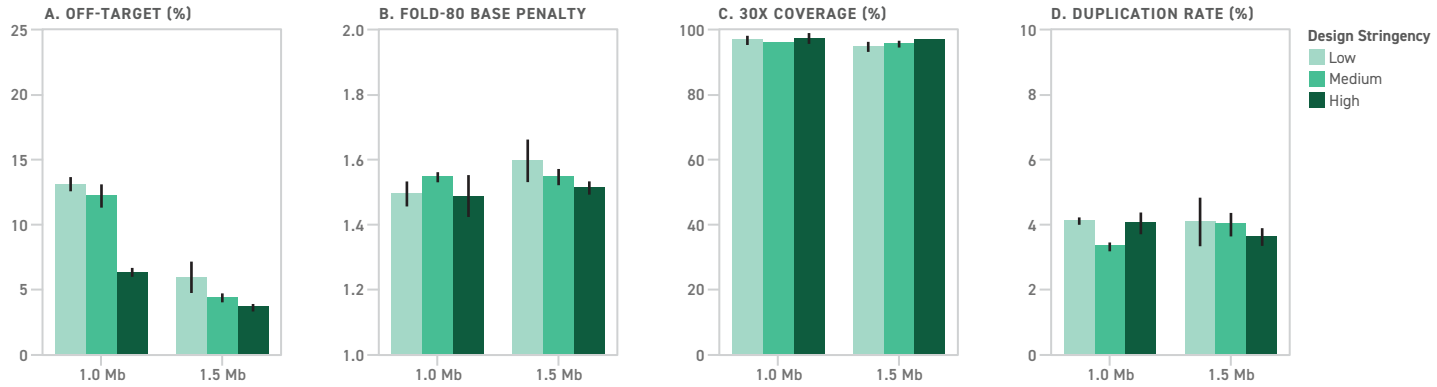

Figure 3. The effect of design stringency on sequencing performance. Increased design stringency reduces off-target capture. Low, medium, and high stringency designs were compared using 1 Mb, and 1.5 Mb custom methylation panels and the Twist Targeted Methylation Sequencing Protocol. The following recommended capture conditions were used: 2 µl of Methylation Enhancer, a Wash Buffer 1 temperature of 65°C, and a 2-hour hybridization time. Throughout this note, results were obtained by 1) sequencing with a NextSeq® 500/550 High Output v2 kit to generate 2x76 paired-end reads, and 2) down-sampling to 200x aligned coverage relative to the panel target size, mapping using the Bismark Aligner, and the use of Picard HS Metrics.

Twist Bioscience has worked to overcome the challenges of targeted methylation sequencing with two complementary advances that enable an unprecedented level of off-target control and capture performance with methylation panels:

1. Optimized panel design with different levels of filtering stringency, controlling the level of rigor with which probes are removed to fine-tune performance vs. retained to enable coverage of critical targets in difficult regions.
2. Twist Methylation Enhancer: a novel reagent that maximizes the efficiency of post-conversion capture systems.

To develop the above methods, data was collected from several tens of thousands of methylation targets and used to determine how the complexity, repetitiveness, and context of the converted genome contributes to panel performance. Several conventional metrics used for filtering saturated. For this reason, informative sequence features were derived and used to develop new optimizations which underpin the performance advances presented in this technical note.

While static arrays are still widely used in methylation detection, the type of high-quality target enrichment panels enabled by the advances described in this note, provide an attractive alternative for exploring dynamic and cell-specific methylation targets at single-base-pair resolution.

RESULTS

Reducing Off-target Capture with Panel Design

Changes in genomic complexity after conversion mean that the filtering of probe sequences for methylation panels is considerably more challenging than for their standard, unconverted counterparts. The presence of low-complexity, low GC, repetitive sequences in methylation detection libraries forces users to balance the inclusion of challenging targets with the potential for increased off-target capture, decreased coverage, and reduced confidence in variant calls across the targeted region.

Often, this means that a panel would require iterative development using empirical capture data to further improve design at cost to the user. As a solution, we developed methods to enable three different levels of filtering stringency during custom methylation panel design to achieve highly optimized target enrichment out of the box. Users are able to tune targets vs. filtering stringency according to their requirements during the ordering process.

Two panels of 1 Mb and 1.5 Mb were used to test default panel designs with low, medium, and high filtering stringency. The results show increased control of off-target rates with filtering stringency achieving rates of off-target as low as 4% with the removal of <1% of probes for the medium tier, and the removal of 3–5% of probes for the high stringency tier. Importantly, other metrics remained broadly unaffected by changes in design stringency (Figure 3).

Reducing Off-target Capture with the Methylation Enhancer

Target enrichment systems typically include blockers that minimize cross-hybridization during capture. The inclusion of blockers in hybridization reactions limits non-specific DNA interactions that can ultimately result in the pull-down of non-targeted library sequences. While effective at reducing off-target capture in standard target enrichment applications, commercially available blockers are not optimized for hybrid capture of methylation sequencing DNA libraries. Twist Bioscience has created a proprietary blocker set called Methylation Enhancer that is designed specifically for targeted methylation sequencing. This product effectively suppresses the capture of repetitive off-target library sequences generated by enzymatic or bisulfite conversion. In addition, this product does not cause detriment to any other crucial hybrid selection metrics.

The Twist Methylation Enhancer reduces off-target capture in a variable fashion depending on the custom panel target region and the methylation state of the input genomic DNA, without adversely impacting hybrid selection metrics. The Twist Methylation Enhancer was applied to Twist's Targeted Methylation Sequencing Workflow using the medium stringency 1.0 Mb and 1.5 Mb methylation panels in single-plex reactions. Results showed a reduction in off-target rates as high as two-fold, with off-target values as low as 4%. Other key capture metrics, including

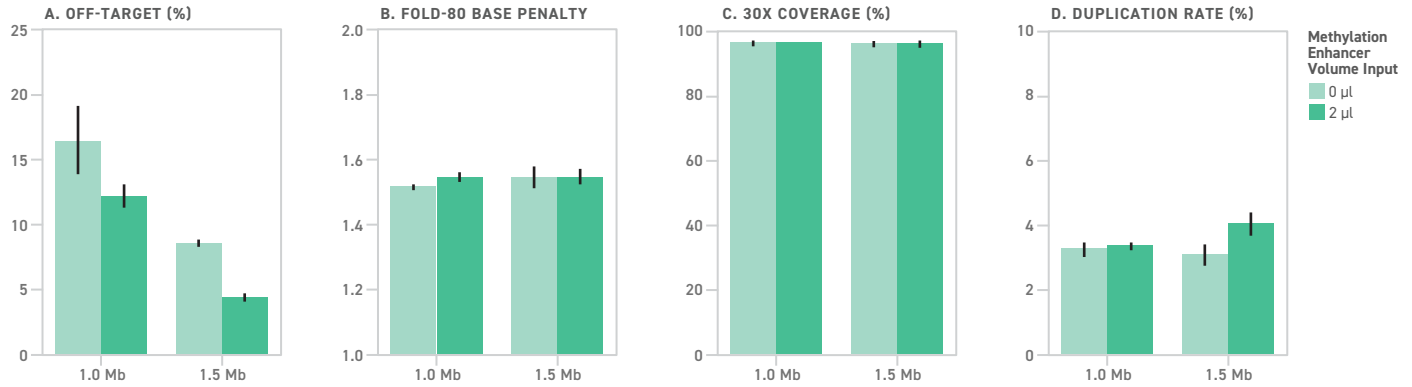

Figure 4. The effect of Twist Methylation Enhancer on sequencing performance. Twist Methylation Enhancer effectively reduces off-target capture by blocking nonspecific off-target capture. Hybrid captures were performed using 1.0 Mb and 1.5 Mb medium stringency designed custom methylation panels and 200 ng of library (NA12878, Coriell). Twist recommended capture conditions were used, including 2 µl of Twist Methylation Enhancer, a Wash Buffer 1 temperature of 65°C, and a 2-hour hybridization time for all reactions (see Figure 3. for additional methods).

uniformity (fold-80 base penalty), 30x coverage, and duplication rates, remained broadly unaltered (Figure 4).

While conventional blockers are not optimally tuned for methylation, they do still exhibit blocking activity for converted sequences. Below we compare the blocking capability of the Twist Methylation Enhancer vs. standard blockers on methylated DNA. Both blockers were titrated separately in a single-plex hybridization reaction captured with an unoptimized 1.5 Mb custom methylation panel to compare their performance. Each blocker was titrated such that equivalent amounts were added to the reaction for comparison. In this instance, the Twist Methylation Enhancer reduced off-target capture by approximately 40%, whereas conventional standard blockers reduced off-target capture by approximately 15% (Figure 5). Twist Bioscience recommends users start with 2 µl of Twist Methylation Enhancer in each hybridization reaction and increase this amount to up to 5 µl depending on the needs of each specific custom methylation panel.

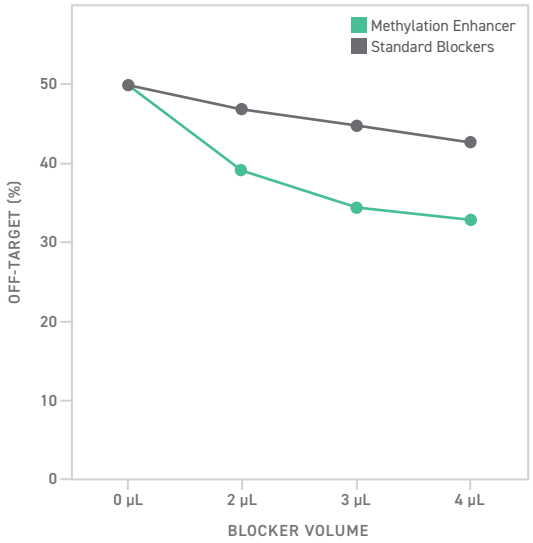

Figure 5. Titration of the Twist Methylation Enhancer and Standard Blockers. The Methylation Enhancer works to reduce off-target more effectively than standard blockers at similar mass inputs. These data were generated using the two blocker systems independently. Equivalent amounts of each blocker were used in each reaction. Hybrid captures were performed using a 1.5 Mb unoptimized custom methylation panel and 200 ng of library (NA12878, Coriell). See Figure 3. for additional methods.

CONCLUSION

Targeted methylation sequencing for low input samples has classically posed considerable challenges, owing to the reduction in genomic sequencing complexity caused by converting unmethylated cytosines. Twist's Methylation Detection System effectively overcomes these barriers through panel design capabilities that enable different levels of optimized filtering stringency, and the introduction of the Methylation Enhancer, a tailor-made blocker that synergistically interacts to improve system performance through the reduction of off-target. Both optimizations substantially improve the observed sequencing performance of panels for methylation detection and are part of the advances underlying Twist's Methylation Detection System enabling sensitive applications such as liquid biopsies.