TECH NOTE

Optimizing the Performance of the Twist Targeted Methylation Sequencing Workflow

INTRODUCTION

Each Twist Custom Panel is unique. For methylation detection, the panel design depends on several factors specific to the customer's research, including size, GC content, and the target region's methylation level. Flexible methylation detection systems are needed to accommodate such panels. With the Twist Targeted Methylation Detection Workflow, experimenters can tune hybrid capture conditions to optimize final sequencing metrics for each custom panel. This technical note highlights the impact multiplexing, hybridization timing, and wash stringency have on the final sequencing outcome.

RESULTS

The Twist Targeted Methylation Sequencing Workflow is an end-to-end methylation detection solution that experimenters can perform in a single day using the Twist Fast Hybridization Target Enrichment System. The Twist Targeted Methylation Sequencing Workflow proceeds as follows:

Steps featured in the Twist green denote those that experimenters can tune to optimize hybridization stringency and hands-on time. For example, multiplexing reduces reagent use, hands-on time, and pipette use during sample preparation. In the hybridization step, experimenters can adjust the capture timing to accommodate a given schedule. Finally, experimenters can tailor the wash step stringency to each specific custom panel by altering the wash temperature.

OPTIMIZING HYBRIDIZATION REACTION: MULTIPLEXING

Throughput and cost-efficiency are essential to many researchers, regardless of the area of study. Multiplexing increases both by minimizing the amount of time and reagents spent preparing samples. The Twist Targeted Methylation Sequencing Workflow supports multiplexing (up to eight-plex) and uses unique dual index (UDI) barcodes incorporated during library generation to deconvolute samples.

Eight-plex and single-plex hybrid captures were compared using an unoptimized custom 1.0Mb methylation panel to demonstrate multiplexing in the Twist Targeted Methylation Sequencing Workflow. Compared to their single-plex counterparts, eight-plex captures showed a decrease in off-target (Figure 1A) and an increase in the number of unique sequences found in the final capture (Figure 1D). Uniformity (Figure 1B; fold-80 base penalty) and the proportion of target region covered at least 30x (Figure 1C) were similar between the two capture types. In a single-plex system, a total mass of 200 ng library input is added, while in a multiplex system, a total mass of 1500 ng is added. Therefore, we suspect the decrease in off-target percentage and increase in unique sequences resulted from the increased total library mass used for hybrid capture. These data support the multiplexing capability of the Twist Targeted Methylation Sequencing Workflow.

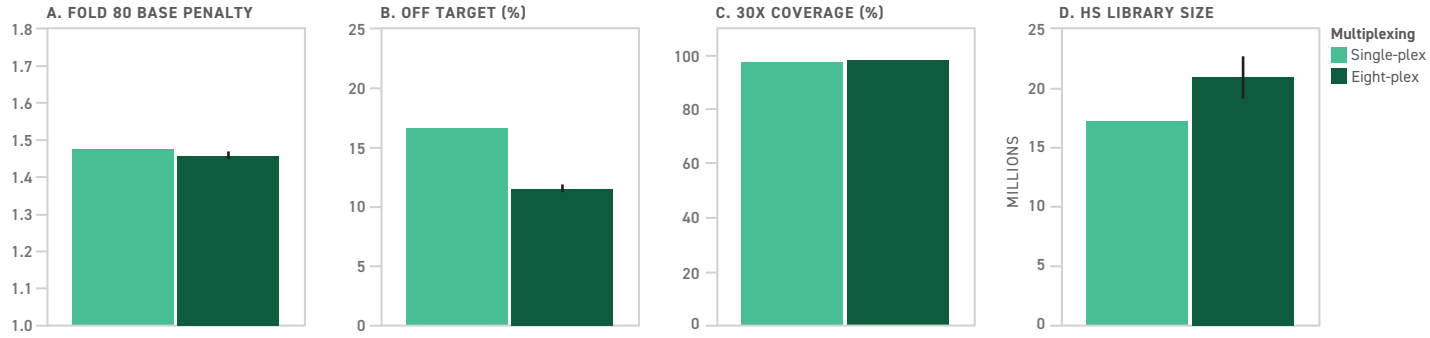

Figure 1. The effect of multiplexing on Picard metrics. For multiplex data points, eight libraries were pooled at equal mass (1500 ng total mass) before use in the Twist Fast Hybridization Target Enrichment System (NA12878, Coriell) to generate an eight-plex capture pool. Hybrid capture was performed using an unoptimized 1.0Mb custom methylation panel. Recommended capture conditions, including 2 µl of Methylation Enhancer, a Fast Wash Buffer 1 temperature of 65°C, and a 2-hour hybridization time was used for all reactions. 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.

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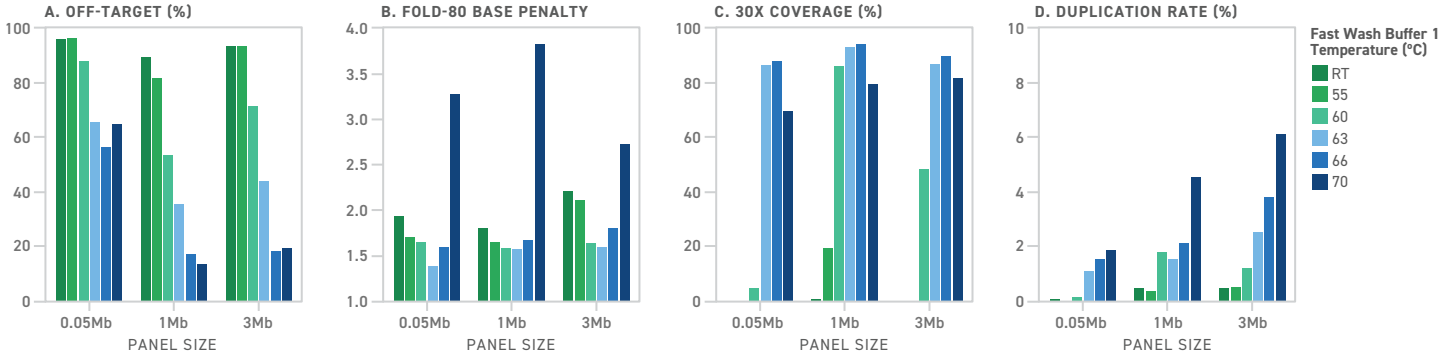

Figure 2. The effect of Fast Wash Buffer 1 temperatures on final Picard metrics. Hybrid capture was performed using different sized custom methylation panels and 200ng of library (NA12878, Coriell) and a 4-hour hybridization time. Custom methylation panels were designed without stringency filters to determine how off-target is impacted. See Figure 1. for additional methods.

OPTIMIZING HYBRIDIZATION TIME

In the broader application of using hybrid capture to detect for specific target and exome regions, hybridization time impacts the number of on-target molecules captured when using the Twist Fast Hybridization Target Enrichment Protocol. During the library preparation and conversion process for methylation detection, sequence complexity is greatly reduced, resulting in much lower GC content than would typically be observed in a non-converted library.

Lower GC content from conversion, in addition to the reduced complexity from library creation, enables the application of shorter, more efficient hybridization times. For the Twist Targeted Methylation Sequencing Workflow, optimal hybridization times will range from 30 minutes to 4 hours, enabling greater flexibility around a user's schedule and available hands-on-time. Twist Bioscience recommends starting with two hours for hybridization and performing subsequent optimizations. If a custom methylation panel targets an area >50Mb, increasing the hybridization time can further improve capture specificity and the number of unique molecules captured, potentially improving downstream Picard metrics.

OPTIMIZING WASH TEMPERATURE

The conversion of unmethylated cytosines to thymines during library preparation reduces library complexity and decreases the DNA molecule's melting temperature. As a result, high off-target rates and poor uniformity during hybrid capture may be encountered.

To accommodate the lower sequence complexity of post-conversion libraries, the Twist Targeted Methylation Sequencing Workflow uses a hybridization temperature of 60°C. As a result, the system pulls down more molecules, including non-specific target regions, which contribute to the off-target rate during sequencing.

The system balances this by using more stringent washes (i.e., a higher temperature during washing) to reduce non-specific capture. The target region, panel size, and gDNA sequence dictate the optimal washing temperature. Twist Bioscience recommends using a temperature of 65°C for Fast Wash Buffer 1, as this produces consistently low off-target metrics. This temperature can be adjusted within the range of 63°C and 66°C as needed. In general, as the temperature used for Fast Wash Buffer 1 increases (i.e., as the wash becomes more stringent), non-specific capture decreases. However, increasing the temperature too high can adversely impact uniformity, coverage, and the duplication rate, as shown in Figure 2.

CONCLUSION

The data presented here highlight the unique capabilities of Twist Target Enrichment Panels for methylation detection. Twist Custom Methylation Panels offer both highly optimized designs out of the box and the flexibility to tune panel performance depending on the experiment's needs, including hybridization time and wash temperature. The Twist Targeted Methylation Sequencing Workflow begins with gDNA and ends with sequencing-ready libraries for methylation detection using any Illumina-based platform. With optimized protocols and a highly efficient hybridization technology, researchers can go from gDNA to aligned sequencing reads in less time and with better performance than ever before.