TECHNICAL NOTE

User-definable parameters in the Illumina DNA Prep with Enrichment workflow

- Increasing PCR denaturation time improves coverage
- Changing enrichment hybridization and wash temperature impacts coverage and variant detection
- Using single SPRI cleanup conserves library fragments in low-quality FFPE samples

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Introduction

Illumina DNA Prep with Enrichment is the fastest and most flexible targeted next-generation sequencing (NGS) solution for DNA in the Illumina library preparation portfolio. It supports a broad DNA input range (10–1000 ng) and multiple sample types, including blood, saliva, genomic DNA, and formalin-fixed, paraffin-embedded (FFPE) tissue.

Accommodating various study requirements, the Illumina DNA Prep with Enrichment solution delivers consistent insert sizes, uniform coverage, and optimized performance. The bead-based technology minimizes fragmentation bias and other opportunities for error, resulting in highly reproducible data across all Illumina sequencing systems.

The Illumina DNA Prep with Enrichment reagents and protocol are highly optimized to provide the most robust on-target enrichment solution. However, in some use cases and with some panels, advanced users may want to adjust select parameters to improve coverage of challenging sequences, such as targeted sequencing of GC- and AT-rich content or to address poor-quality samples, such as FFPE tissue.

This technical note provides information on the impact on NGS data outcomes resulting from changes to user-defined parameters within the workflow. Specifically, we look at the effects of changing temperature during the hybridization and wash steps of the enrichment, as well as altering denaturation time during the post-capture PCR. We also examine different conditions for the solid-phase reversible immobilization (SPRI) process when working with degraded FFPE samples. Any adjustments to the optimized protocols should be carefully considered as they can impact performance and available support options.

Changing hybridization and wash temperature affects coverage and variant detection

During enrichment, biotinylated probes are used to capture specific sequences from the indexed NGS library for analysis. The recommended temperatures for the hybridization and wash steps depend on the sequence content of the panel. We tested changes to the hybridization and wash temperatures and assessed the resulting performance changes in the TruSight™ Hereditary Cancer Panel and Illumina Exome Panel (Table 1).

Table 1: Enrichment hybridization and wash temperatures tested

	Hybridization/ wash temp (°C)
Illumina Exome Panel[a]	58
Illumina Exome Panel, Hyb62	62
TruSight Hereditary Cancer Panel[b]	62
TruSight Hereditary Cancer Panel, Hyb58	58

a. Recommended Illumina DNA Prep with Enrichment conditions for Illumina Exome Panel using germline DNA
b. Recommended Illumina DNA Prep with Enrichment conditions for TruSight Hereditary Cancer Panel using germline DNA

Testing for hybridization and wash temperatures, and PCR denaturation times was performed using 50 ng input of commercially available genomic DNA (gDNA; Coriell Institute, Catalog no. NA12878). Targeted sequencing panels tested included the TruSight Hereditary Cancer – Enrichment Oligos Only (Catalog no. 20029551) and the Illumina Exome Panel - Enrichment Oligos Only (Catalog no. 20020183).

The recommended hybridization and wash temperature for the TruSight Hereditary Cancer Panel is 62°C. Changing this temperature to 58°C resulted in lower AT dropout (Figure 1). The recommended 62°C wash temperature showed lower GC dropout, higher padded unique read enrichment, and higher insertion deletion (Indel) recall and precision (Figure 1). The lower 58°C hybridization and wash temperature resulted in improved variant allele frequency (VAF) distribution for variants at, or below, 50% (Figure 2), with minimal change to VAF distribution above 50%.

For the Illumina Exome Panel, the recommended hybridization and wash temperature is 58°C. At this temperature the Illumina Exome Panel shows better uniformity of coverage, target coverage at 20×, AT dropout, and SNV/indel recall. Changing the hybridization and wash temperature to 62°C showed improvements in padded unique read enrichment and GC dropout (Figure 3).

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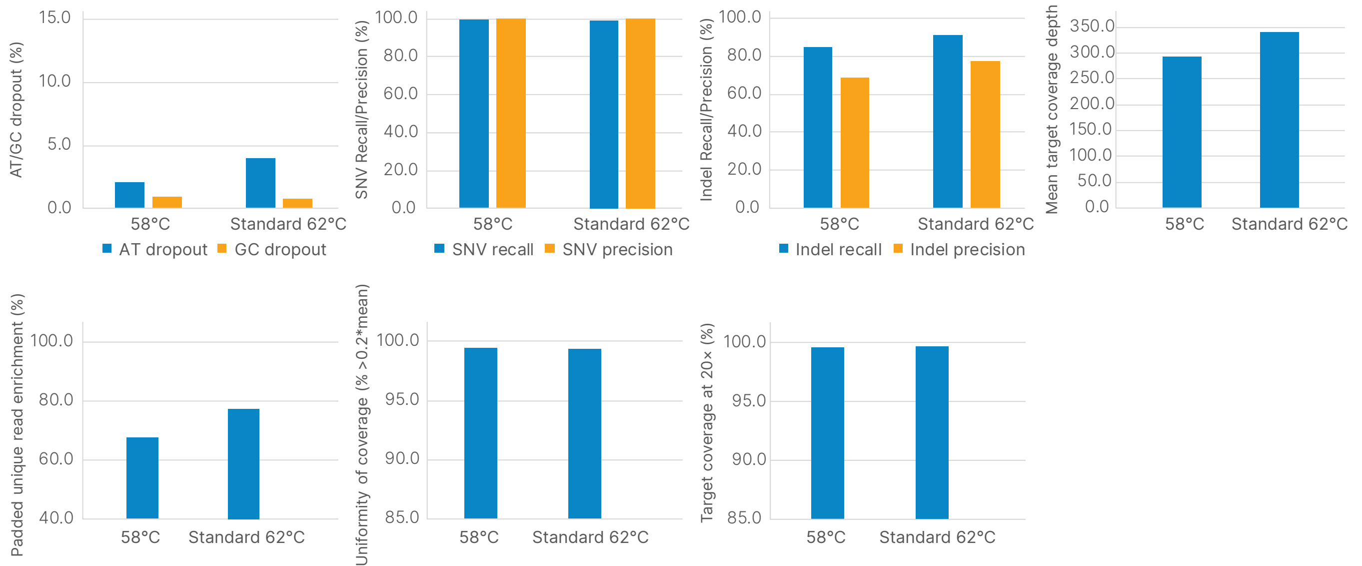

Figure 1: TruSight Hereditary Cancer Panel enrichment results for tested hybridization and wash temperatures—Hybridization and wash temperatures of 62°C and 58°C were tested with the TruSight Hereditary Cancer Panel library enrichment protocol. Changes in sequence coverage were observed with the temperature adjustment, including shifts in AT and GC dropout, indel recall and precision, and padded unique reads. Data are presented to inform decisions for experimental needs.

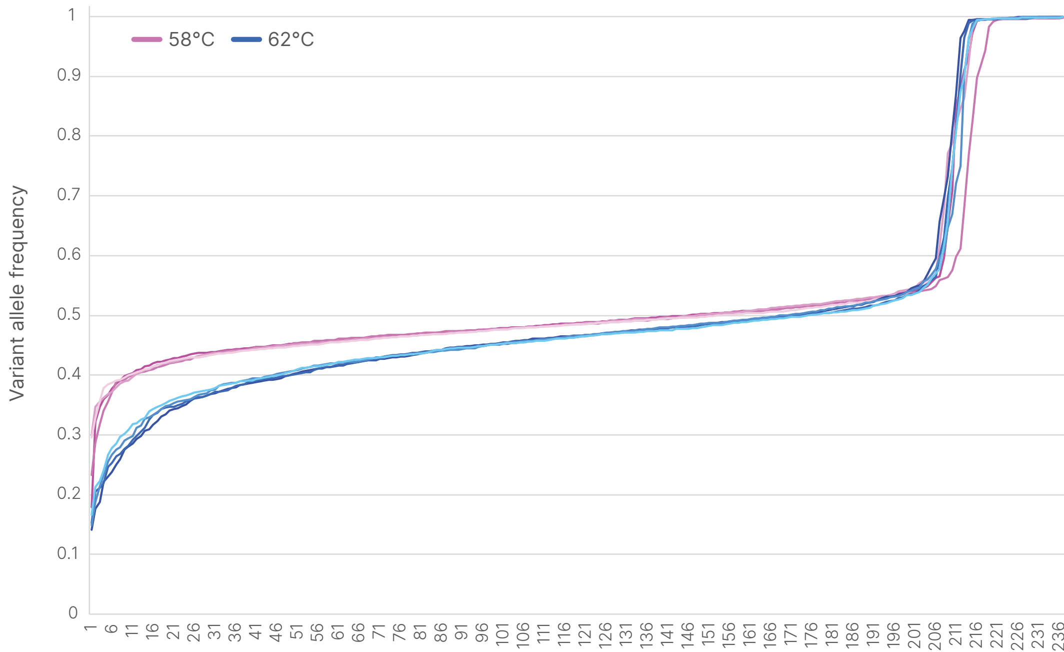

Figure 2: TruSight Hereditary Cancer Panel VAF detection results for adjusted hybridization and wash temperatures—Data shown for all variants reported in the TruSight Hereditary Cancer Panel enriched library from NA12878 gDNA. X-axis values indicate observed variants placed in numerical order from lowest VAF to highest VAF.

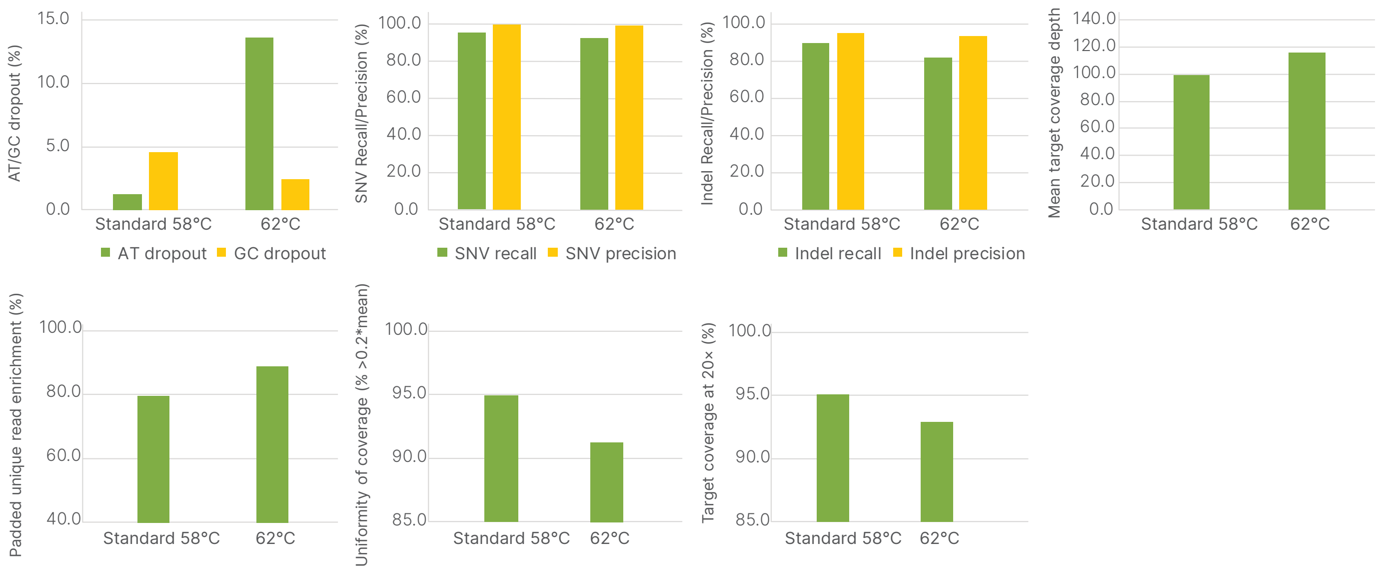

Figure 3: Illumina Exome Panel enrichment results for tested hybridization and wash temperatures—Hybridization and wash temperatures of 62°C and 58°C were tested with the Illumina Exome Panel library enrichment protocol. Changes in sequence coverage were observed with the temperature adjustment, including shifts in AT and GC dropout, indel recall and precision, and padded unique reads. Uniformity of coverage and target coverage at 20× are higher at the standard 58°C temperature. Data are presented to inform decisions for experimental needs.

Extending probe hybridization time to 16 hours was tested using both panels and did not significantly improve enrichment in these experiments (data not shown).

Increasing post-capture PCR denaturation time improves coverage

Following the capture and wash steps, libraries undergo a limited number of PCR amplification cycles. The Illumina DNA Prep with Enrichment PCR parameters have been extensively tested and optimized to produce the most uniform and complete NGS library sequence coverage. However, it is possible to adjust denaturation times in the post-capture PCR protocol without affecting other aspects of the PCR chemistry. Extending denaturation times can improve access to target DNA by adapter priming sequences. We tested long denaturation (LD) times during the post-capture PCR by extending the initial denaturation time from 30 to 45 seconds and the denaturation times for cycles 2–13 from 10 to 30 seconds (Table 2).

Table 2: Denaturation conditions tested for post-capture PCR

	Initial denaturation time (sec)	Denaturation time, cycles 2–13 (sec)
Illumina Exome Panel[a]	30	10
Illumina Exome Panel, LD	45	30
TruSight Hereditary Cancer Panel[b]	30	10
TruSight Hereditary Cancer Panel, LD	45	30

a. Recommended Illumina DNA Prep with Enrichment post-capture PCR denaturation conditions for Illumina Exome Panel using germline DNA
b. Recommended Illumina DNA Prep with Enrichment post-capture PCR denaturation conditions for TruSight Hereditary Cancer Panel using germline DNA

Due to higher coverage depth and a smaller number of target sequences in the panel, LD cycles did not result in significant coverage differences with the small TruSight Hereditary Cancer Panel. Subtle differences were observed in AT/GC dropout in the TruSight Hereditary

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Cancer Panel when using the LD protocol variation (Figure 4). In contrast, using LD conditions defined in Table 2 with the larger Illumina Exome Panel we observed improved uniformity of coverage, target coverage at 20×, and single-nucleotide variant (SNV)/Indel recall, with minimal increase in the duration of the PCR (Figure 5). For both the large and small panels, the results show some benefit of the LD conditions with virtually no negative impact, with more benefits being observed for the larger Illumina Exome Panel where sequencing depth is lower.

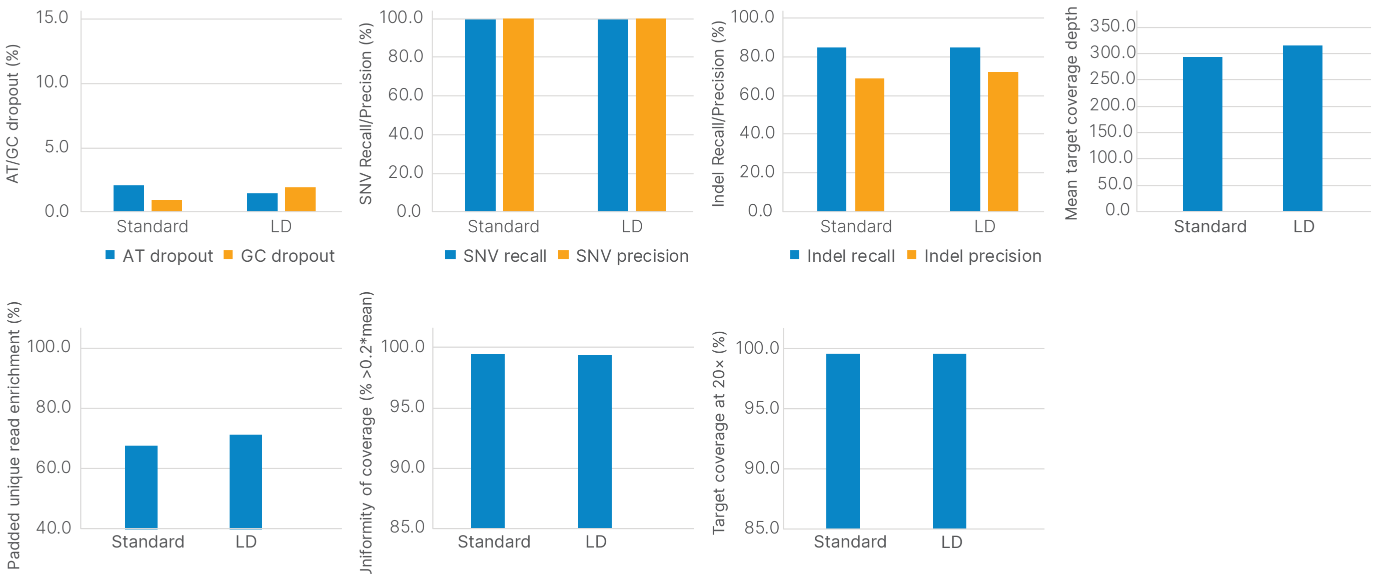

Figure 4: TruSight Hereditary Cancer Panel results for LD and normal PCR denaturation steps—Changes in NGS library coverage and variant detection observed following the standard Illumina DNA Prep with Enrichment protocol or LD steps.

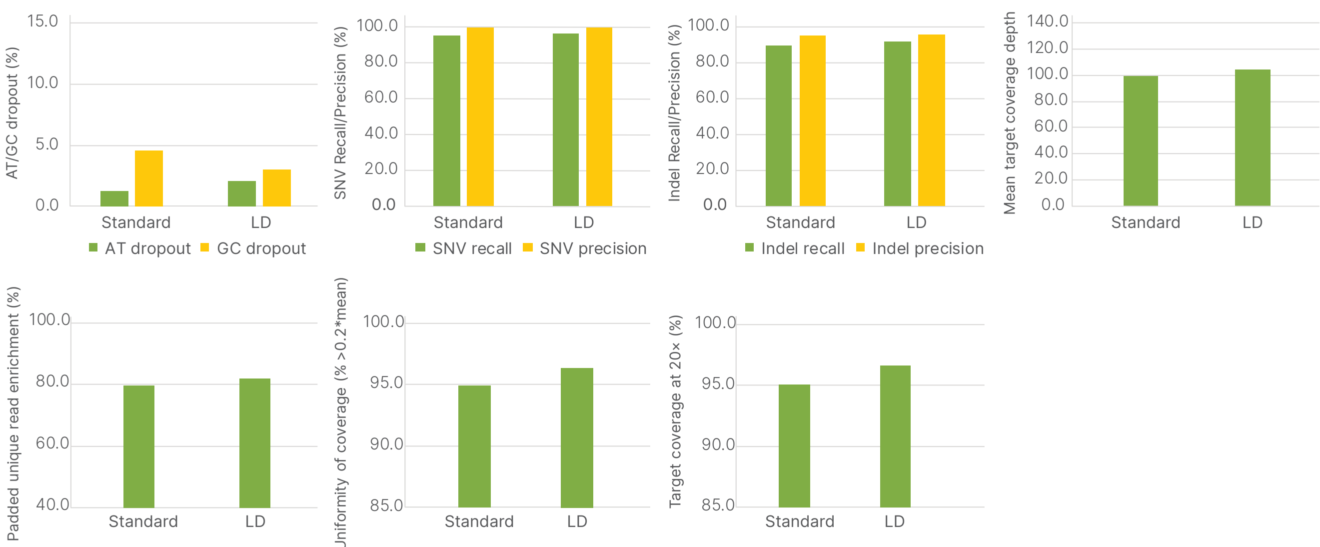

Figure 5: Illumina Exome Panel results for LD and normal PCR denaturation steps—Changes in NGS library coverage and variant detection observed following the standard Illumina DNA Prep with Enrichment protocol or LD steps. Uniformity of coverage, target coverage at 20×, and single-nucleotide variant (SNV)/Indel recall were improved with the longer denaturation time.

Single SPRI cleanup preserves library content in low-quality samples

For low-quality samples such as FFPE tissue, library fragments are generally shorter following tagmentation. In these samples, the standard double SPRI cleanup results in lower pre-enrichment library yields and removes a large amount of fragment diversity from the library (Figure 6).

For SPRI analysis, comparisons were performed using Horizon Tru-Q DNA Reference Standards for high-quality DNA (Horizon Catalog no. HD730), moderate FFPE DNA (Horizon Catalog no. HD799), and severe FFPE DNA (Horizon Catalog no. HD803) (Table 3). Sequencing libraries were prepared and enrichment performed using the TruSight One Sequencing Panel. Enrichment was performed at 58°C with a 90-min hybridization.

For high-quality DNA and moderately damaged FFPE (ΔCq 1.5) samples, double SPRI cleanup produced usable quantities of sequencing-length fragments. For the more damaged HD803 FFPE sample (ΔCq 4.5), the library yield was reduced. For the severely damaged HD803 sample, the double SPRI process reduced library yield to the point that important data were likely lost. Following a single SPRI cleanup, library yield was significantly higher for all three gDNA sample types (Figure 6). The HD803 data also showed significant improvements in observed vs. expected variant calls with severely degraded FFPE samples when using the single SPRI cleanup (Figure 7).

The Illumina DNA Prep with Enrichment guide recommends a single SPRI cleanup step for damaged DNA samples.[1] This data confirms that, when working with highly degraded samples such as those found in FFPE tissues, use of a single SPRI cleanup step during library prep will improve library yields and sequencing coverage.

Summary

Under normal circumstances, following the validated Illumina DNA Prep with Enrichment protocol will deliver the best performance with Illumina NGS sequencing panels. In some use cases, advanced users may want to adjust the parameters covered in this document.

The data shown demonstrate that the increased PCR denaturation time yielded sequencing coverage benefits with the Illumina Exome Panel. Increasing PCR denaturation times also showed benefits for performance, especially with GC-rich regions. Changing the denaturation time had little impact on the overall workflow time and did not negatively affect other aspects of sequencing.

Changing the wash and hybridization temperatures during target enrichment improved variant allele frequency calling in the TruSight Hereditary Cancer Panel. The hybridization and wash temperatures also affected other aspects of performance, such as padded unique read enrichment and AT dropout.

Table 3: Samples tested for single- and double-sided SPRI conditions

Sample name	Sample type	Input (ng)	SPRI condition tested
HD730	gDNA	10	Double SPRI
HD730	gDNA	10	Single SPRI (1.8×)[a]
HD799	Moderate FFPE (ΔCq 1.5)	50	Double SPRI
HD799	Moderate FFPE (ΔCq 1.5)	50	Single SPRI (1.8×)
HD803	Severe FFPE (ΔCq 4.5)	50	Double SPRI
HD803	Severe FFPE (ΔCq 4.5)	50	Single SPRI (1.8×)

a. 1.8× is the ratio of SPRI volume to DNA volume

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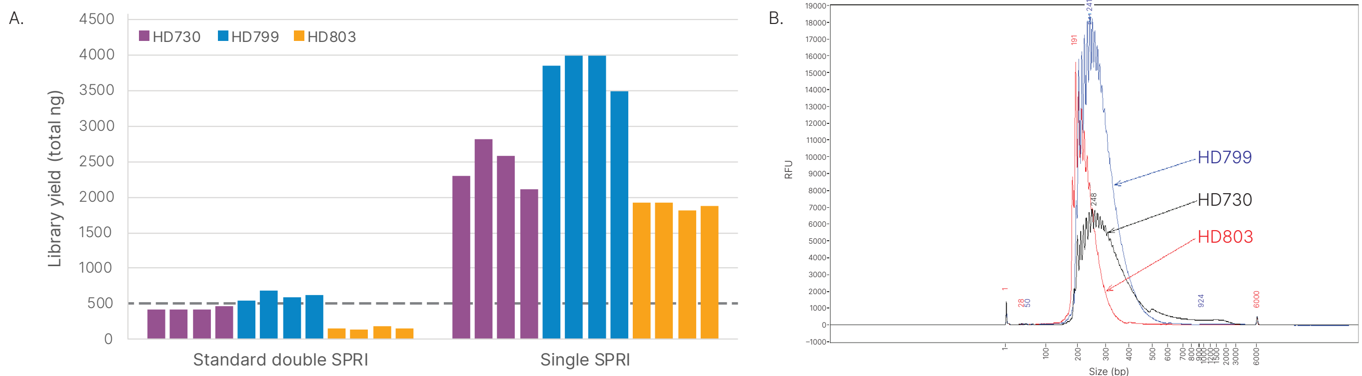

Figure 6: Library quantification following SPRI cleanup of tagmented libraries—(A) NGS library yields from high-quality and FFPE-damaged DNA libraries following double SPRI or single SPRI cleanup. Dotted line represents 500 ng target. gDNA input was 10 ng for HD730 and 50 ng for HD799 and HD803. (B) Fragment Analyzer data showing distribution of library fragments following single SPRI cleanup.

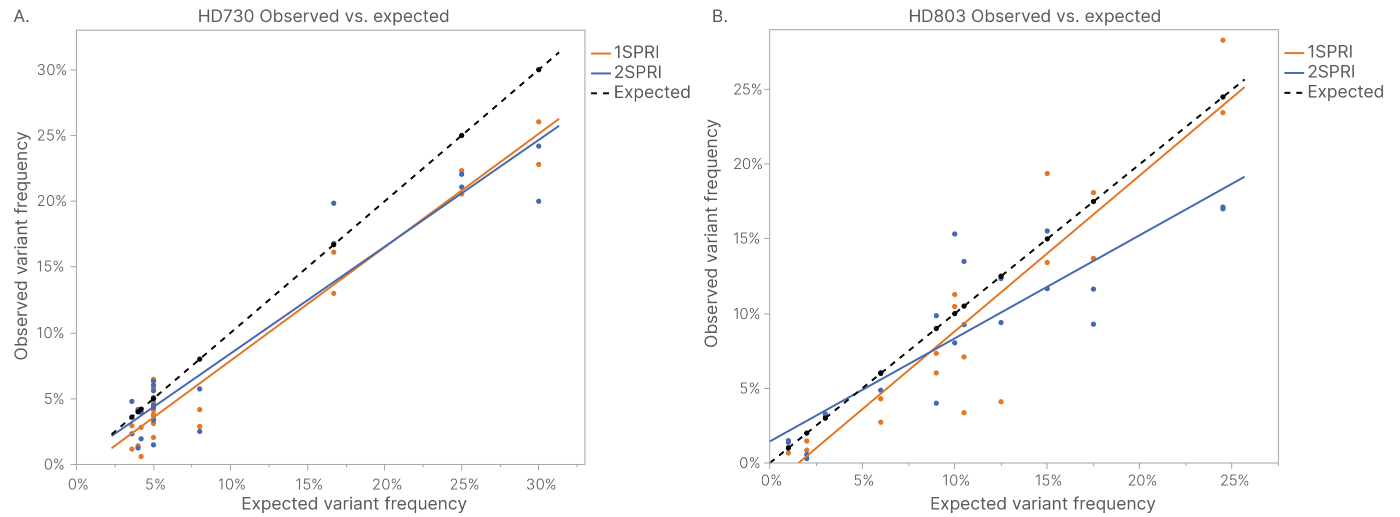

Figure 7: Minor allele frequency calling after single (1.8×) and double SPRI cleanup—(A) Expected and observed variant calls for high-quality HD730 gDNA sample. (B) Expected and observed variant calls for severe FFPE-damaged HD803 gDNA sample.

For the SPRI cleanup step, we focused on the benefits to low-quality DNA samples such as DNA damaged by FFPE storage conditions. For high-quality DNA, double SPRI is recommended. A single SPRI step improved library yields and variant frequency calling for severely damaged FFPE samples, which is an important measure in applications such as cancer studies. Therefore, for FFPE samples, a single SPRI cleanup step is recommended as described in the Illumina DNA Prep with Enrichment Reference Guide.[1]

Glossary

AT dropout—a measure of how undercovered ≤ 50% GC regions are relative to the mean

GC dropout—a measure of how undercovered ≥ 50% GC regions are relative to the mean

Recall—truth true positives/(truth true positives + truth false negative)

Precision—query true positives/(query true positives + query false positives)

References

1. Illumina DNA Prep with Enrichment Reference Guide. Illumina website. June 2020. Accessed March 3, 2021. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/illumina_prep/illumina-dna-prep-with-enrichment-reference-1000000048041-06.pdf

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