illumina®

APPLICATION NOTE

A single NGS assay to detect methylation and somatic variants in archived tumor samples

Recommendations for using Illumina 5-Base DNA Prep for analyzing FFPE DNA

Maximize discovery power from FFPE samples using gentle chemistry that preserves DNA integrity

Gain high-accuracy genome and methylome insights from 40 ng input FFPE DNA of varying quality

Generate robust results from lower input FFPE DNA samples with simple assay modifications

Introduction

Cancer is a disease of both the genome and the epigenome.[1] Identifying the genomic mutations and aberrant DNA methylation that drive tumor growth is crucial for modeling cancer initiation, progression, and therapeutic responses. Formalin-fixed, paraffin-embedded (FFPE) tissue samples are a valuable source of archived genomic material for cancer research. However, comprehensive molecular profiling of these samples is challenging due to the degraded quality of FFPE DNA.

Illumina 5-Base DNA Prep uses unique chemistry and optimized analysis to detect five bases—adenine (A), thymine (T), guanine (G), cytosine (C), and 5-methylcytosine (5mC)—combining whole-genome sequencing (WGS) and methylation sequencing into one streamlined assay.[2] The gentle single-step methylated base conversion preserves DNA integrity to maximize discovery power from FFPE samples. In this application note, we demonstrate use of Illumina 5-Base DNA Prep with FFPE tumor samples and share protocol recommendations to obtain high-accuracy methylome and somatic variant calling.

Methods

Genome and methylome analysis

Genomic DNA was extracted from standard FFPE tissue blocks for 10 samples of various tumor types, including lung cancer, melanoma, colorectal cancer, colon cancer, breast cancer, and kidney cancer (Table 1). Quality (ΔCq) of FFPE DNA was determined using the TruSight™ FFPE QC Kit (Illumina, Catalog no. 20139070). For each FFPE sample, 40 ng input DNA was prepared using Illumina 5-Base DNA Prep (Illumina, Catalog no. 20140364). For three FFPE samples, additional inputs of 25 ng, 10 ng, and 1 ng were prepared with minor alterations to the standard protocol (Table 2). All 5-base FFPE libraries were sequenced on the NovaSeq™X Plus System with 2 × 151 bp read lengths and sequencing of 500M clusters (1B paired-end reads). Secondary analysis was performed with the DRAGEN™ Somatic pipeline (v4.4.6).

Table 1: FFPE tumor samples analyzed

FFPE sample	Tissue type	Quality (ΔCq)	Input DNA
1	Lung cancer	1.00	40 ng, 25 ng, 10 ng, 1 ng
2	Lung cancer	1.00	40 ng
3	Lung cancer	1.00	40 ng
4	Melanoma	1.21	40 ng
5	Colorectal cancer	1.50	40 ng
6	Colon cancer	2.01	40 ng, 25 ng, 10 ng, 1 ng
7	Colon cancer	3.40	40 ng
8	Breast cancer	4.11	40 ng
9	Melanoma	4.11	40 ng, 25 ng, 10 ng, 1 ng
10	Kidney cancer	5.00	40 ng

Tumor–normal somatic variant analysis

The HCC1395 breast cancer cell line (ATCC, Catalog no. CRL-324) and the matched normal HCC1395BL cell line (ATCC, Catalog no. CRL-2325) were cultured and processed either as fresh frozen cell pellets or converted to FFPE material before DNA extraction. Libraries were prepared using Illumina 5-Base DNA Prep, with two replicates using the standard protocol and two replicates prepared without the Illumina methylation conversion enzyme as WGS-only controls. Libraries were sequenced on the NovaSeq X Plus System and data analyzed with DRAGEN Somatic v4.5. Tumor–normal variant calling was compared between 5-base data and WGS data at equivalent read depth. Tumor–normal variant calling for 5-base data was also examined at deeper coverage.

Results

High-performance genome and methylome sequencing for FFPE DNA samples of varying quality

Illumina 5-Base DNA libraries from FFPE tumor DNA of varying quality at the recommended 40 ng input show robust sequencing performance with a high percentage of mapped reads (Figure 1A) and at least 30× coverage for most samples (Figure 1B). Lower quality samples (ΔCq ≥ 3) may require more sequencing to achieve 30× coverage. Median insert size was ~110–150 bp with lower quality FFPE samples showing shorter insert sizes (Figure 1C).

Illumina 5-Base DNA libraries from FFPE tumor samples show high-accuracy methylation detection (Figure 2). The expected global CpG* methylation percentage for normal human tissue is 70%–80%. Tumor samples are characterized by genome-wide hypomethylation compared to normal tissue, therefore the ~50%–70% methylation percentage observed is expected (Figure 2A). Low levels of CHG and CHH* methylation (< 2%) are observed and expected (Figure 2A). Lambda and pUC19 classified reads from the genomic DNA methylation control (GDMC) added to the FFPE samples demonstrate high true positive rate and low false positive rate across samples (Figure 2B).

* CpG, cytosine-guanine dinucleotide; other contexts for cytosine methylation include CHG and CHH, where H is A, C, or T.

Table 2: Protocol changes and conditions for Illumina 5-Base DNA Prep for using low-input FFPE samples

Protocol step		Shearing[b]	Ligation		Index PCR	Post-PCR clean up
Protocol change		Volume GDMC[b]	Volume UMI3[c]	Volume RSB[c]	No. of PCR cycles[c]	SPRI ratio[d]
FFPE DNA input amount	40 ng[a]	2.5 µl	5 µl	–	8	0.8×
	25 ng	2.5 µl 1:10 diluted GDMC[b]	2 µl	3 µl	9	0.8×
	10 ng		1 µl	4 µl	10	0.8×
	1 ng		1 µl	17 µl[c]	12	0.8×

a. 40 ng is the recommended input amount for FFPE DNA with Illumina 5-Base DNA Prep.
b. Shearing conditions and reaction volume optimized for FFPE. Add either 2.5 µl stock genomic DNA methylation control (GDMC) for 40 ng, or 2.5 µl GDMC diluted 1:10 with resuspension buffer (RSB) for inputs < 40 ng. For ultralow FFPE DNA inputs (1 ng), further dilution may be warranted to reduce reads from control DNA. Omit "Prepare Size-Select Sheared DNA" step, which is intended to remove smaller fragments that should be retained when processing FFPE. Bring the sample to optimal volume for end repair using RSB.
c. Only add 5 µl diluted adapter UMI3. Adapter input and PCR cycles adjusted to prevent adapter dimer and attain sufficient yield without overamplification. UMI, unique molecular identifier; RSB, resuspension buffer.
d. Ratio adjusted to remove any residual adapter dimer. SPRI, solid-phase reversible immobilization.

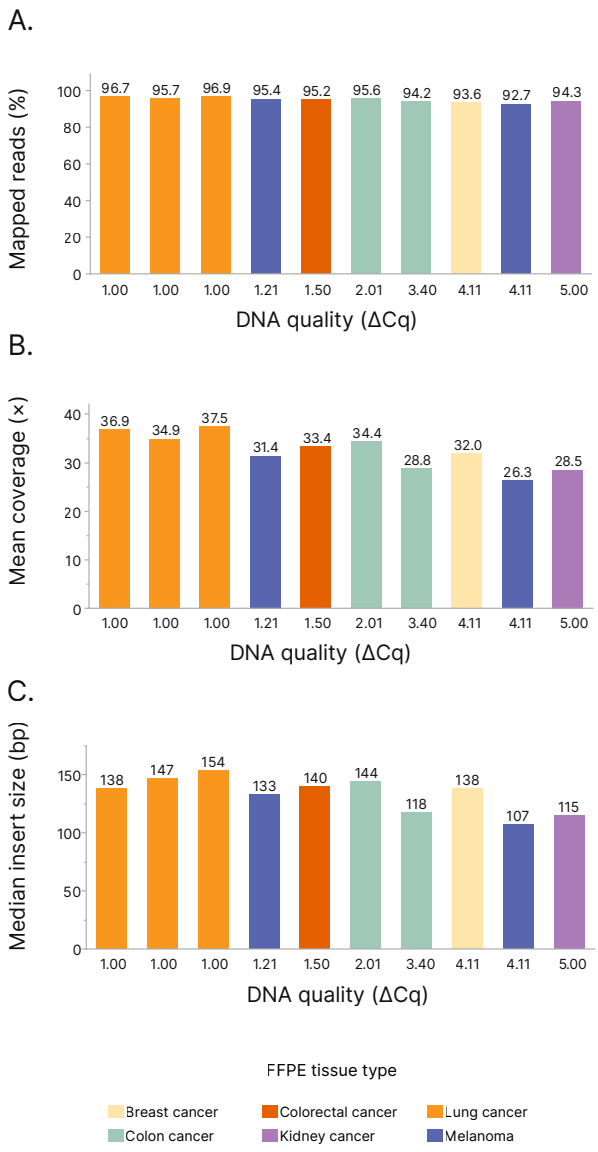

Figure 1: Robust sequencing performance across FFPE DNA of varying quality with Illumina 5-Base DNA Prep

DRAGEN Somatic library metrics for 10 FFPE tumor samples with 40 ng input (Table 1) using Illumina 5-Base DNA Prep show (A) high mapping rates across tissue types and quality (ΔCq). Mapped read calculations do not include the lambda and pUC19 classified reads originating from the genomic DNA methylation control (GDMC). (B) Mean coverage ranged from 26×–38× across samples tested. Lower quality FFPE samples (ΔCq ≥ 3) may require more sequencing depth to achieve 30× coverage. (C) Median insert size was ~110–150 bp with lower quality FFPE samples showing shorter lengths. Libraries were sequenced at 2 × 151 bp and downsampled to 500M clusters.

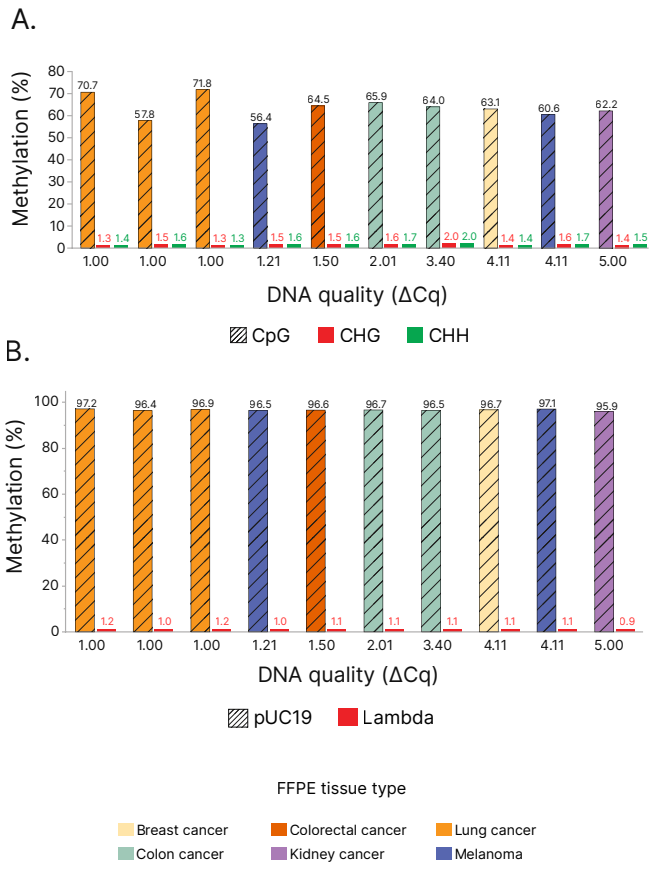

Figure 2: Robust methylation detection across FFPE DNA of varying quality with Illumina 5-Base DNA Prep

Methylation metrics for 10 FFPE tumor samples with 40 ng input (Table 1) using Illumina 5-Base DNA Prep show (A) percent methylation at cytosines in CpG, CHG, and CHH context at expected ranges. (B) Lambda and pUC19 classified reads from the genomic DNA methylation control (GDMC) show high true positive and low false positive rates across samples.

Robust genome and methylome sequencing for low-input FFPE DNA

For users with limited FFPE material to analyze, DNA input requirements can be constraining. With simple assay modifications, including UMI3 adapter dilution, GDMC dilution, and PCR cycle adjustment, users can assay samples at lower inputs (Table 2). Applying these adjustments, Illumina 5-Base DNA Prep produces library yield sufficient for sequencing (≥ 4 nM) across inputs and varying sample quality (Figure 3).

For three FFPE tumor samples tested at inputs lower than 40 ng (Table 1), sequencing metrics and methylation detection were robust at 25 ng and 10 ng DNA inputs (Figure 4, Figure 5). At 1 ng input, lower percent mapped rates are observed due to increased fraction of lambda and pUC19 genome relative to FFPE DNA in the final library. This may be mitigated further by omitting the GDMC methylation control. Lower coverage and higher duplicates are also expected at 1 ng input (Figure 4).

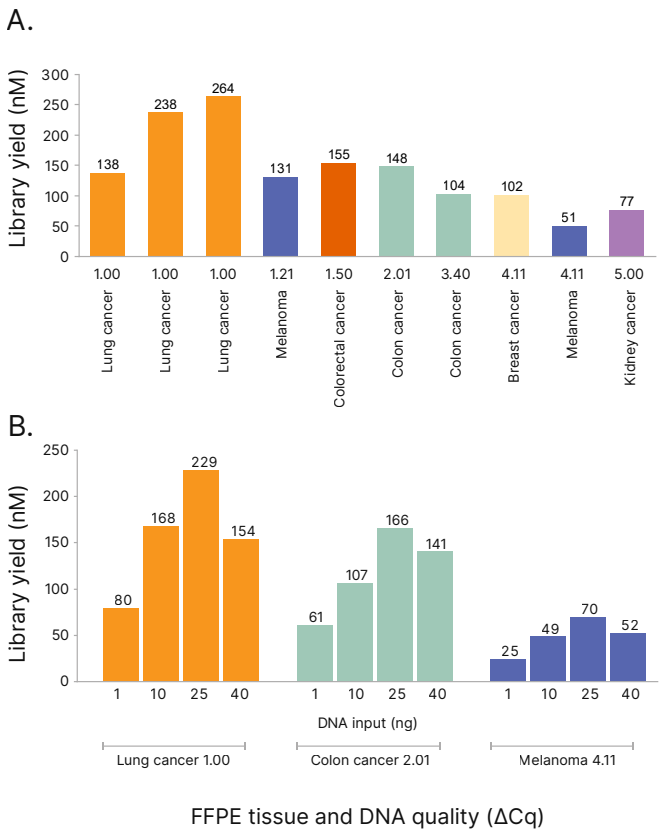

Figure 3: Library yield across FFPE DNA of varying input amounts and quality with Illumina 5-Base DNA Prep

Qubit DNA quantification of library yield from (A) 10 FFPE tumor samples with 40 ng input, and (B) three FFPE tumor samples tested with 40 ng, 25 ng, 10 ng, and 1 ng inputs (Table 1) using Illumina 5-Base DNA Prep with protocol modifications (Table 2).

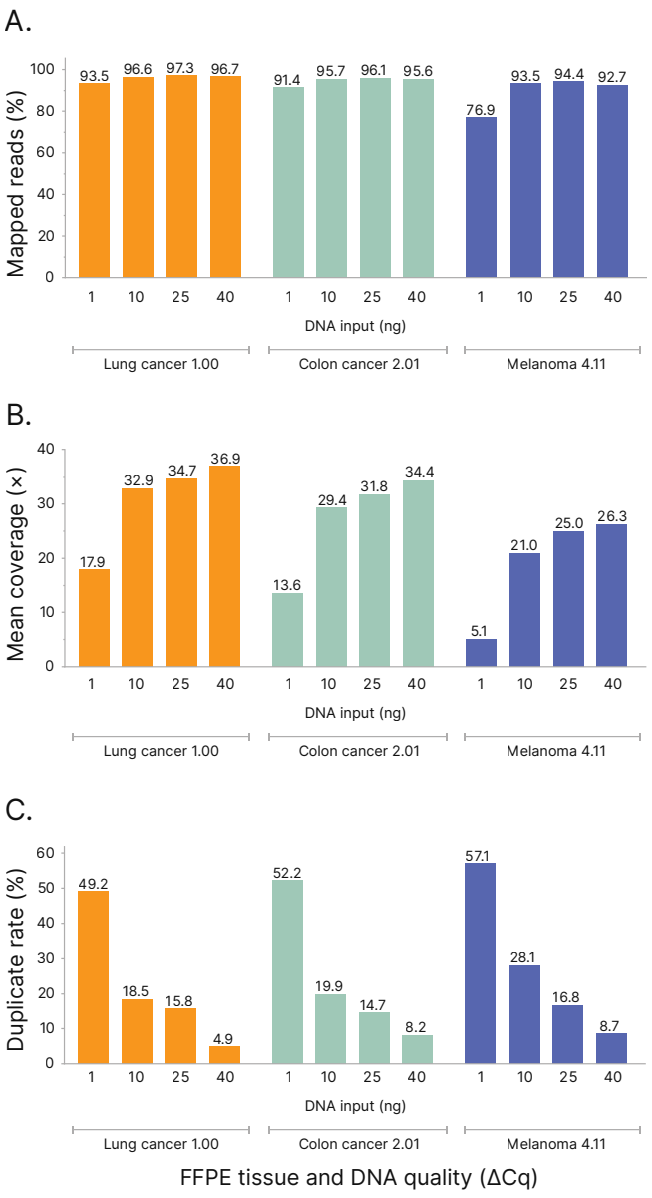

Figure 4: Robust sequencing performance across FFPE DNA input amounts with Illumina 5-Base DNA Prep

DRAGEN Somatic library metrics for three FFPE tumor samples with 40 ng, 25 ng, 10 ng, and 1 ng inputs (Table 1) using Illumina 5-Base DNA Prep with protocol modifications (Table 2) show (A) high mapping rates, (B) 29×–37× mean coverage, and (C) low duplicate rates for higher quality FFPE DNA at inputs > 10 ng. Lower quality FFPE samples (ΔCq ≥ 3) and lower input levels may require more sequencing depth to achieve 30× coverage. Libraries were sequenced at 2 × 151 bp and downsampled to 500M clusters.

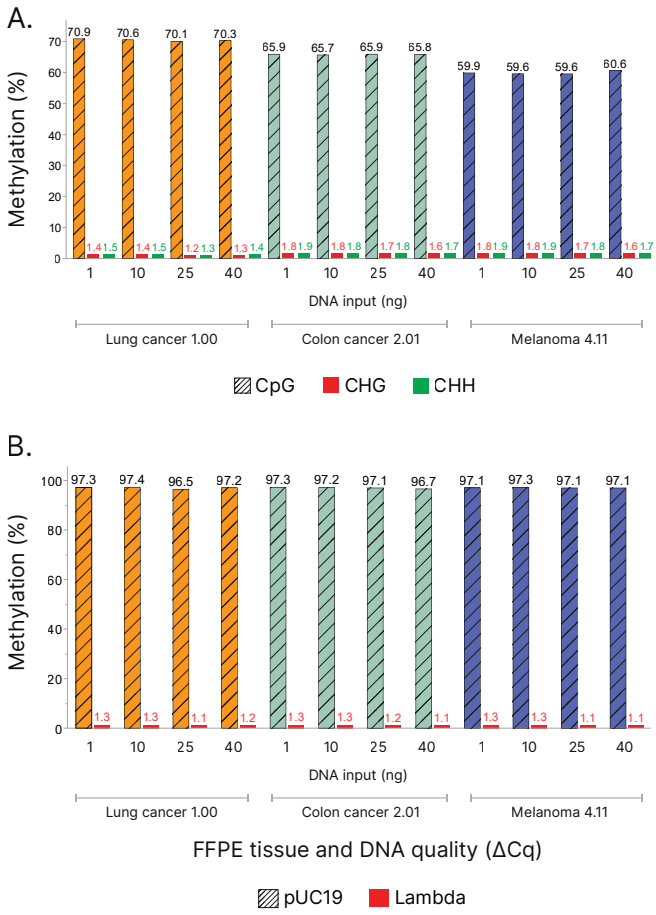

Figure 5: Robust methylation detection across FFPE DNA input amounts with Illumina 5-Base DNA Prep

Methylation metrics for three FFPE tumor samples with 40 ng, 25 ng, 10 ng, and 1 ng inputs (Table 1) using Illumina 5-Base DNA Prep with protocol modifications (Table 2) show (A) percent methylation at cytosines in CpG, CHG, and CHH context is consistent across input levels. (B) Lambda and pUC19 classified reads from the genomic DNA methylation control (GDMC) show high true positive and low false positive rates across DNA inputs.

Somatic variant detection for FFPE comparable to results with fresh frozen samples or to WGS alone

Illumina 5-Base DNA Prep using matched tumor–normal cell lines, treated as fresh frozen or as FFPE, generated high-sensitivity somatic variant calling for single nucleotide variants (SNVs) and insertion-deletions (indels) (Figure 6). SNV and indel sensitivity for 5-base libraries at 100×/40× coverage is within ~2% of the sensitivity of nonconverted WGS libraries at equivalent sequencing depth. With increased sequencing depth of 150×/50× coverage, 5-base sensitivity is equivalent to WGS. While formalin fixation can introduce DNA damage impacting precision, 5-base precision on FFPE samples is > 90% (Figure 6).

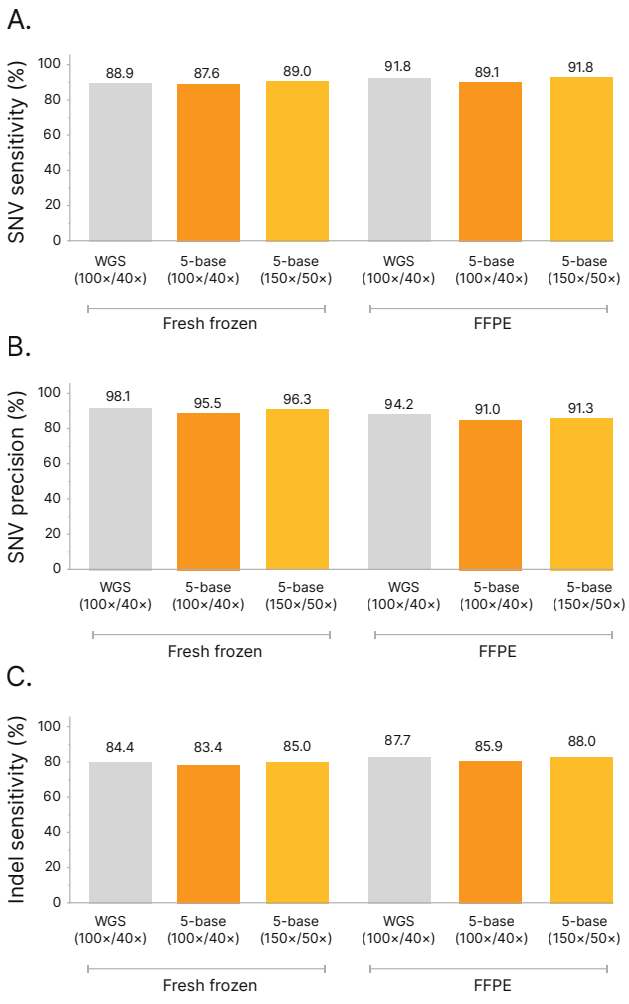

Figure 6: Tumor–normal somatic variant calling for fresh frozen and FFPE-treated cell lines using Illumina 5-Base DNA Prep

(A) Sensitivity and (B) precision of single nucleotide variant (SNV) calling and (C) sensitivity of insertion-deletion (indel) calling for the HCC1395 breast cancer cell line and matched normal HCC1395BL cell line, processed either as fresh frozen or FFPE treated. Libraries were prepared using Illumina 5-Base DNA Prep, with two replicate libraries prepared without the Illumina methylation conversion enzyme as WGS-only controls (gray). Data was downsampled to 100× (orange) or 150× coverage (yellow) (2.4B or 4B paired-end reads) for tumor samples and to 40× (orange) or 50× coverage (yellow) (950M or 1.4B paired-end reads) for normal samples. SEQC2 v1.2.2 truth set for HCC1395 was used.

Summary

Illumina 5-Base DNA Prep combines genome and methylome sequencing into one streamlined workflow and is compatible with FFPE samples. Gentle conversion chemistry preserves DNA integrity, allowing for dual analysis of FFPE DNA of varying quality. For limited sample inputs, simple assay modifications enable robust results. With Illumina 5-Base DNA Prep, cancer researchers can access high-accuracy somatic variant calling and methylation detection from valuable archived tumor tissue.

Learn more

Illumina 5-Base DNA Prep

Illumina 5-base solution

Genome and methylome sequencing

References

1. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis*. 2010;31(1):27-36. doi:10.1093/carcin/bgp220
2. Illumina. Illumina 5-Base DNA Prep data sheet. illumina.com/ content/dam/illumina/gcs/assembled-assets/marketing-literature/ illumina-5-base-dna-prep-data-sheet-m-gl-03689/illumina-5-base-dna-prep-data-sheet-m-gl-03689.pdf. Published 2025. Accessed June 18, 2026.

illumina®

1.800.809.4566 toll-free (US) | +1.858.202.4566 tel
techsupport@illumina.com | www.illumina.com

M-GL-04497 v1.0