Evaluation of Illumina 5-base sequencing for integrated methylation and variant analysis in FFPE samples and its potential for tumor-informed MRD applications

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Introduction

- Formalin-fixed, paraffin-embedded (FFPE) tissue represents an abundant and clinically valuable source of genomic material, yet nucleic acid degradation and chemical modifications inherent to FFPE processing pose significant challenges to downstream analyses.
- The limited quantity of extracted DNA further restricts the ability to perform multiple assays for comprehensive biomarker evaluation.
- The Illumina 5-base workflow integrates genetic and epigenetic information in a single assay, enabling simultaneous whole-genome variant calling and methylation detection from a single sample prep.
- We present an assessment of 5-base compatibility with FFPE samples.
- We explore whether genomic variants and aberrant methylation patterns identified in CRC FFPE tissue samples can be detected at low frequency in matched cfDNA samples.

5-Base enables integrated high-accuracy methylation and variant detection

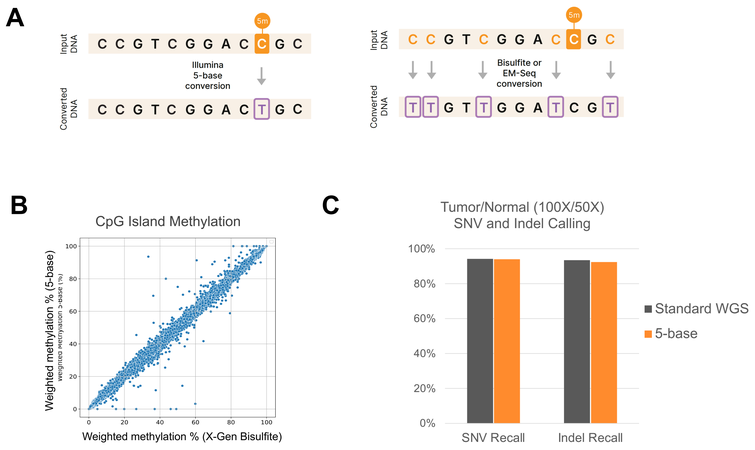
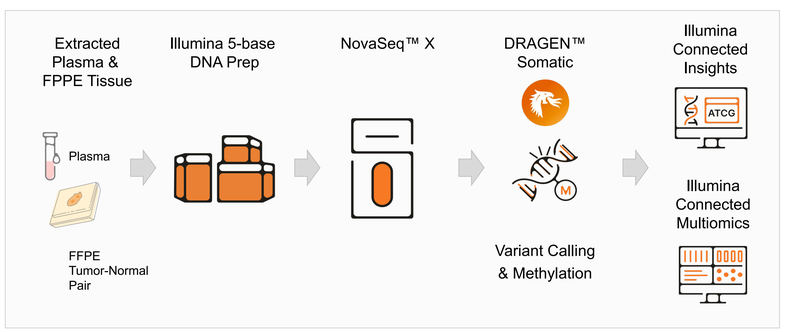


Figure 1 (A) Illumina 5-base chemistry directly converts methylated cytosine to thymine, resulting in libraries that enable detection of all 5 bases: A, T, C, G and methylated-C. (B) 5-base methylation measurements across CpG islands are highly concordant with traditional methyl-seq technologies. (C) DRAGEN algorithms paired with Illumina 5-base chemistry enables high sensitivity somatic SNV and Indel calling, approaching WGS PCR-Free performance. Somatic Tumor-Normal SNV and Indel sensitivity for a tumor/normal cell line with high confidence truth variants.

Methods

FFPE colorectal tissue and plasma was obtained from 12 individuals with colorectal cancer. FFPE tissue was macro-dissected to separate tumor from adjacent normal and DNA was extracted. 50 ng FFPE DNA and 10 ng cfDNA was processed with Illumina 5-base DNA Prep, using FFPE-optimized Covaris shearing conditions[1] with minor modifications to the workflow. Libraries were sequenced on NovaSeq™ X at 150X - 200X coverage. Secondary analysis was processed using 5-base aware DRAGEN™ Somatic v4.4.6, generating simultaneous variant calling and methylation results. Interpretation of variants was performed with Illumina Connected Insights (ICI). Differential methylation analysis was performed with Illumina Connected Multiomics (ICM).



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5-base application is compatible with FFPE sample type

Genomic and epigenomic variant detection accuracy was evaluated using genomic DNA (gDNA) from fresh frozen tissue or FFPE inputs processed through whole-genome sequencing (WGS) and Illumina 5-base workflows. All sample types generated high-complexity libraries with mapping rates exceeding 85%, independent of FFPE DNA quality (ΔCq 1–4). Methylation annotations were concordant across technical replicates, with minimal introduction of false-positive or false-negative methylation conversion events in FFPE. Somatic variant detection sensitivity was >80% for FFPE samples prepared with Illumina 5-base prep, with minimal reduction observed for 5-base compared to WGS (≤ 3%).

High accuracy methylation and variant calling in FFPE

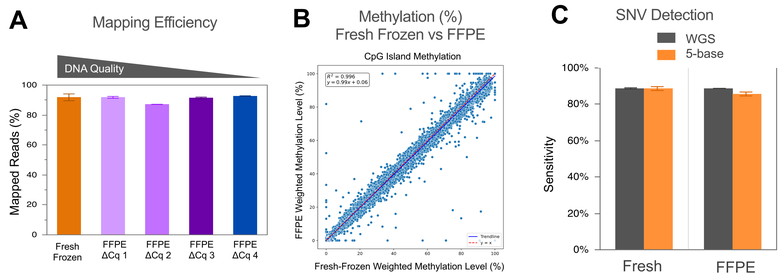

Figure 2 (A) Illumina 5-base libraries show high mapping rate across FFPE samples ranging in DNA quality score (ΔCq 1-4) consisting of colon, small bowel and lung tissue types. (B) A fresh-frozen cancer cell line, with high confidence truth variants, was formalin-fixed and paraffin-embedded to mimic FFPE processing. Methyl measurements and (C) SNV detection are concordant between fresh frozen and FFPE.

5-base detects SNVs and DMRs associated with CRC

Illumina 5-base was used to obtain genomic and epigenomic signatures of colorectal cancer (CRC) for a cohort of 12 FFPE samples consisting of tumor and adjacent normal tissue from individuals diagnosed with colorectal cancer. Tumor-Normal variant analysis identified cancer-associated variants specific to the tumor, across the 12 CRC samples. Genome-wide methylation profiles for CRC Tumor vs Normal FFPE were analyzed to identify differentially methylated regions (DMRs) across the 2 cohorts. We identified hypermethylated DMRs and hypomethylated DMRs in the cancer cohort relative to the adjacent normal tissue cohort.

Dual-omic analysis identifies DMRs and cancer-related variants in CRC FFPE tissue

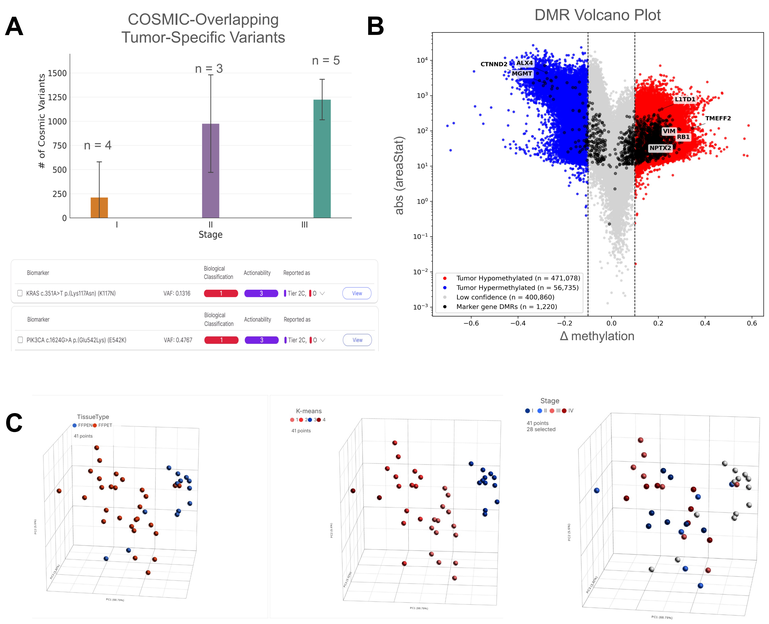

Figure 3 (A) Number of COSMIC overlapping variants identified in pair-wise tumor vs normal somatic variant detection for each CRC FFPE sample pair was determined using DRAGEN™ plus Illumina Connected Insights. Screenshot shows example of 2 detected small variants classified as oncogenic in a tumor sample. (B) Volcano plot of DMRs detected in cohort analysis of 12 CRC FFPE tissue versus 12 adjacent normal using Illumina Connected Multiomics. (C) PCA plot clustering CRC and adjacent normal FFPE based on methylation profile using downselected DMRs.

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5-base application for molecular residual disease research

Detection of molecular residual disease (MRD) in solid tumors using liquid biopsy is an emerging approach for cancer disease monitoring based on detection of low abundance cancer signatures from circulating tumor DNA. Detection of aberrant methylation and genomic variants simultaneously with a single assay has potential to enhance sensitivity over a single-omic analyses. When evaluated in a tumor-informed MRD use case, 5-base successfully captured tumor fingerprint mutations from FFPE that were also detected in matched cfDNA, while additionally identifying cancer-informative methylation signatures.

5-Base captures tumor fingerprint mutations and methylation

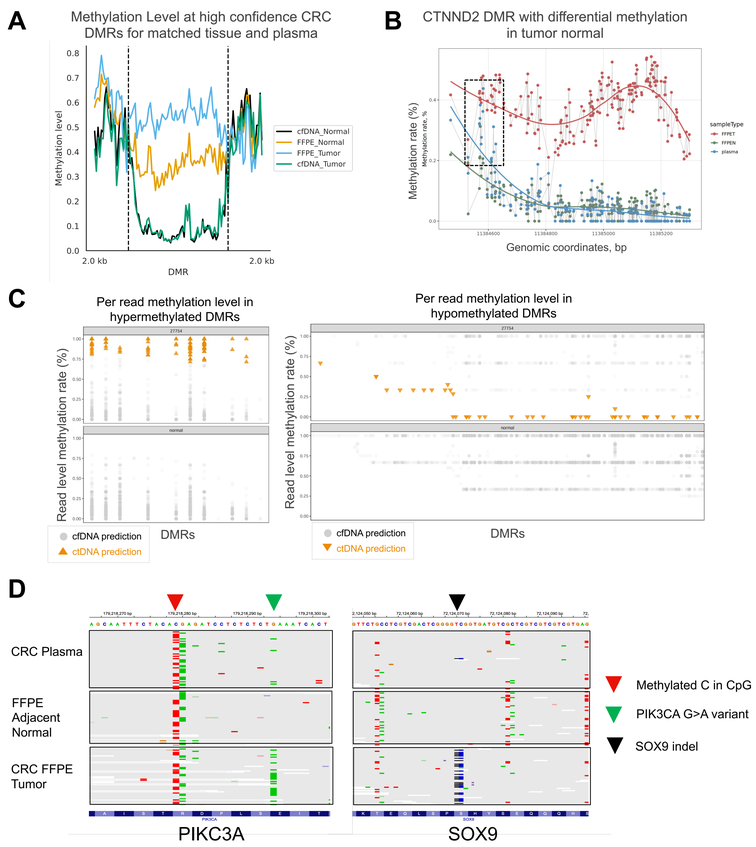

Figure 4 The methylation patterns in tumor and matched plasma were evaluated for a subset of CRC DMRs. (A) Average %methylation observed across ~60 CRC hypermethylated DMRs. 5-base detects hypermethylation in tumor CRC tissue compared to adjacent normal tissue. Hypermethylation signal from ctDNA is not distinguishable from healthy plasma when looking at DMRs in aggregate. (B) Focusing on an individual DMR reveals methylation-based evidence of ctDNA in plasma at a prognostic CRC promoter CTNND2. (C) Read-level analysis shows evidence of ctDNA detected in plasma at 13 hypermethylated DMRs and 148 hypomethylated DMRs. Orange triangles represent reads that reflect the expected methylation level for ctDNA fragments; gray circles are reads that reflect the expectation for cfDNA fragments. (D) IGV tracks of matched FFPE CRC tumor, normal, and plasma demonstrate tumor-informed methylation and small variants can be observed in the plasma at PIK3CA gene and SOX9 gene.

Summary

These results highlight the potential of using Illumina 5-base to obtain multiomic, clinically actionable profiles from FFPE samples. By unifying methylation and variant detection in a single workflow, 5-base could empower more comprehensive molecular profiling and supports development of high sensitivity applications spanning oncology research and translational biomarker discovery.

References

1. https://support.illumina.com/downloads/ilmn-ffpe-dna-prep-exome-enrichment-product-documentation.html
2. Oladapo OB, Moussa MR. Colorectal Cancer Biomarker Identification via Joint DNA-Methylation and Transcriptomics Analysis Workflow. Genes. 2025; 16(6):620. https://doi.org/10.3390/genes16060620

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