

Detection of Tumor-Associated Methylation and Mutation Signatures for Early Colorectal Cancer Diagnosis

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INTRODUCTION

- Colorectal Cancer (CRC) is the third leading cause of cancer-related death, accounting for nearly 153,020 new cases diagnosed and 52,550 deaths in 2023^{1,2}. Overall, the CRC mortality rate has declined by about 2% per year during the most recent decade (2010-2019), due to the effective screening techniques.
- Liquid biopsy-based cell free DNA (cfDNA) in blood plasma holds tremendous potential for early cancer detection³. However, it is technologically challenging to achieve clinically meaningful high sensitivity and specificity in asymptomatic individuals due to significant lower tumor cfDNA fractions⁴.
- The extensive analysis of cfDNA beyond the mutations detection to encompass methylation analysis contribute to improve sensitivity and specificity for early CRC screening, diagnostic accuracy of analytical platforms, and diagnostic power to detect tumors.
- Herein, we developed targeted next-generation sequencing (NGS)-based assays for assessing multiple DNA methylated and mutated regions in both low-quantity (plasma cfDNA) and low-quality formalin-fixed paraffin-embedded (FFPE) tissue for CRC detection. We assessed the usefulness of integrating genomic and epigenomic signatures in cfDNA to detect CRC.

METHOD

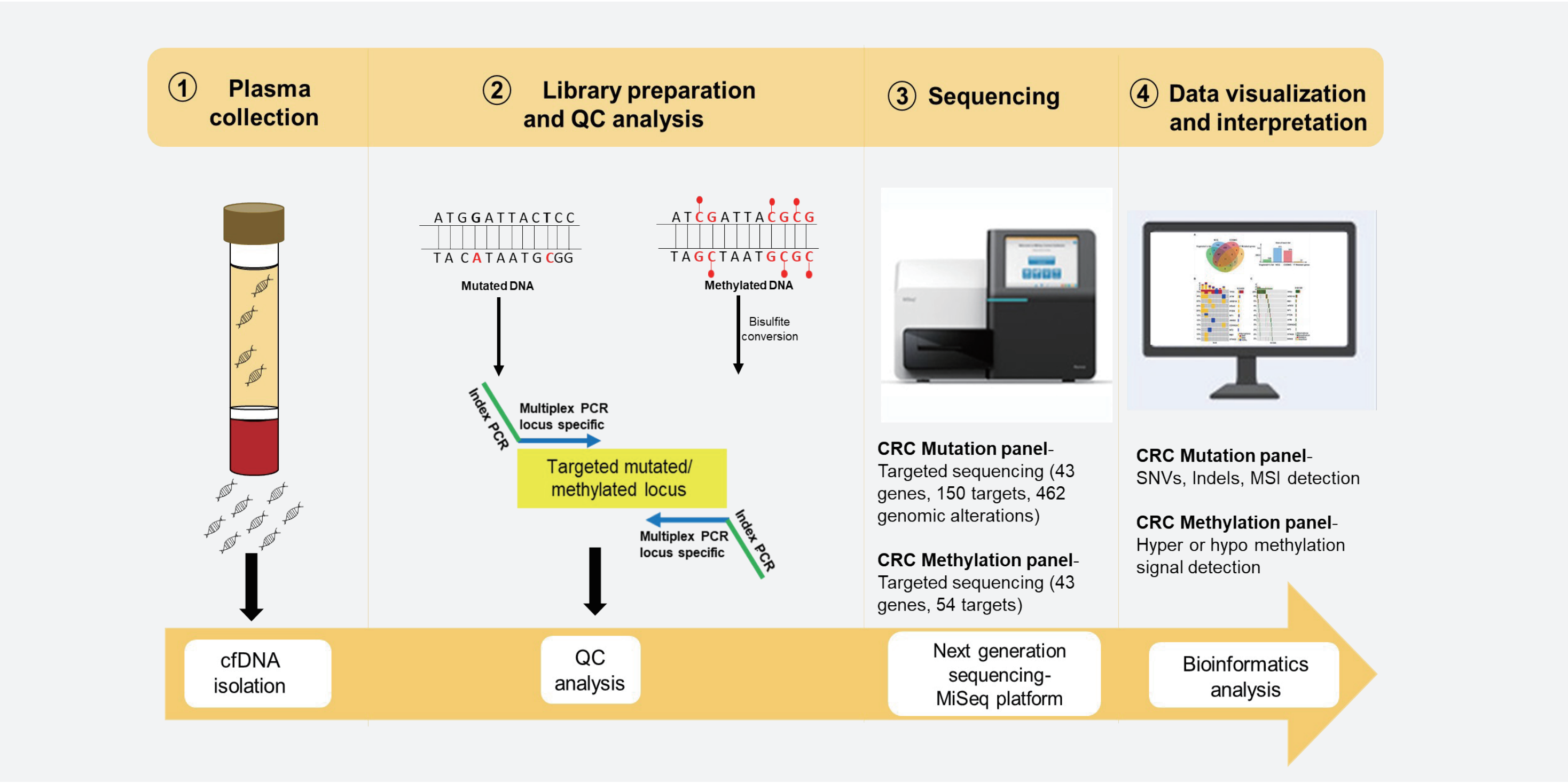


Fig. 1: Overview of multiplex OptiSeq™ CRC NGS mutation and methylation panel protocol.

RESULTS

I. Analytical Performance of OptiSeq™ CRC Methylation Panel

OptiSeq™ CRC NGS methylation detection panel shown that its analytical sensitivity was around 0.5%.

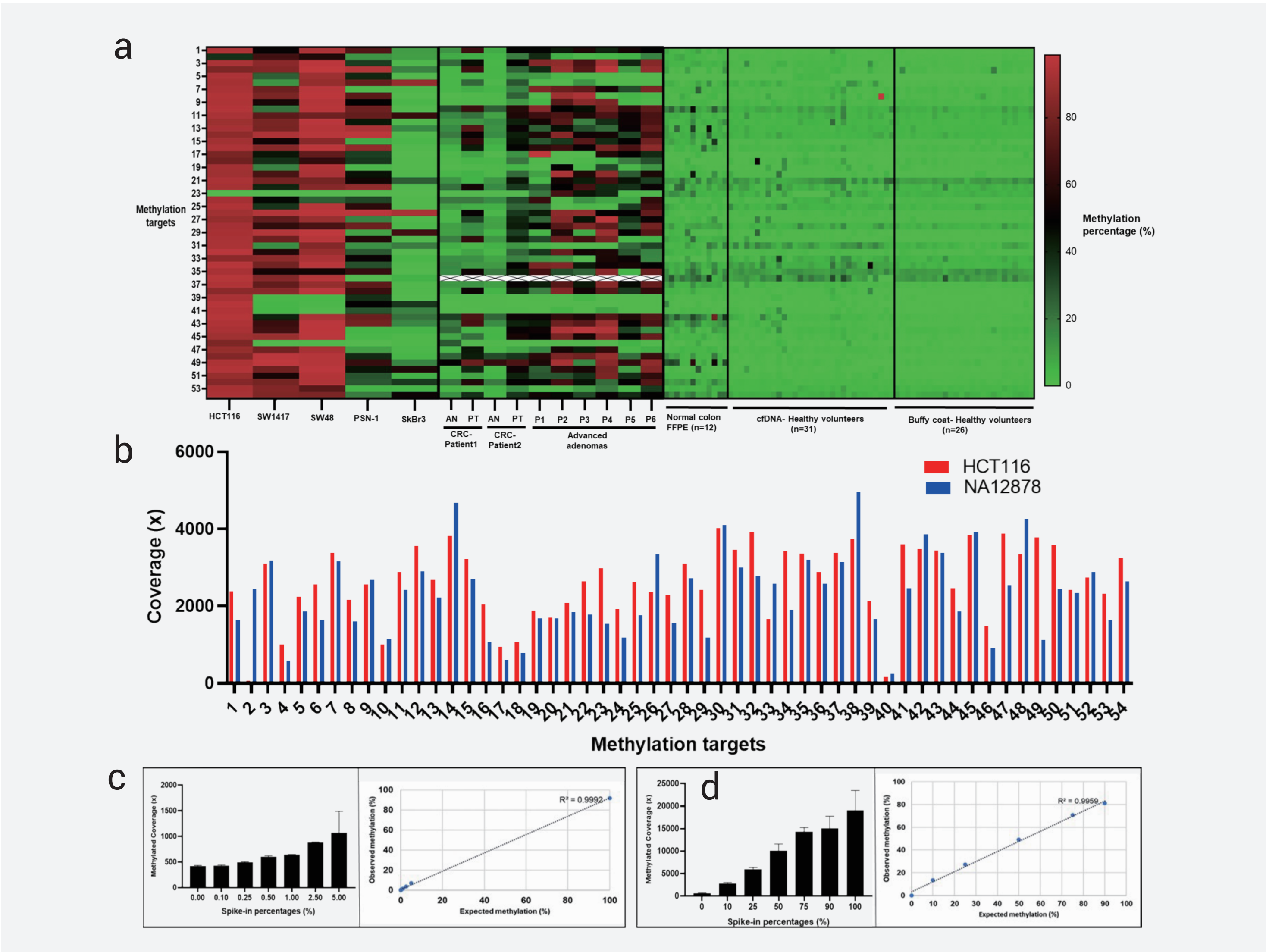


Fig. 2: Multiplex bisulfite sequencing data for OptiSeq™ Colorectal Cancer NGS methylation detection panel. (a) Heatmap showing the methylation pattern in the target regions of interest between CRC (HCT116, SW1417 and SW48), non-CRC (PSN-1 and SkBr3), CRC FFPE specimens, advanced adenomas, healthy volunteers cfDNA and matching buffy coat DNA. All amplicons show statistically significant methylation differences between cancer and normal ($p < 0.05$). AN-Adjacent Normal, PT-Primary tumor, P-Patient. (b) Bar graph showing the range of sequencing coverage for individual amplicons in the CRC methylation panel. (c) Left fig.-analytical sensitivity of the methylation assay was assessed using methylated DNA spike-in at 0%, 0.1%, 0.25%, 0.5%, 1%, 2.5% and 5% expected levels of methylation, across 3 replicates and two independent sequencing runs. Right fig.-Correlation plot between observed methylation (%) (y-axis) against expected methylation (%) (x-axis). (d) Left fig.-PCR bias was assessed using methylated DNA spike-in at 0%, 10%, 25%, 50%, 75%, 95% and 100% expected levels of methylation, across 3 replicates. Right fig.- Correlation plot between observed methylation (%) (y-axis) against expected methylation (%) (x-axis) showing no PCR bias.

II. Analytical Performance of OptiSeq™ CRC Mutation Panel

OptiSeq™ CRC mutation panel data indicted that its LOD around 0.25% VAF.

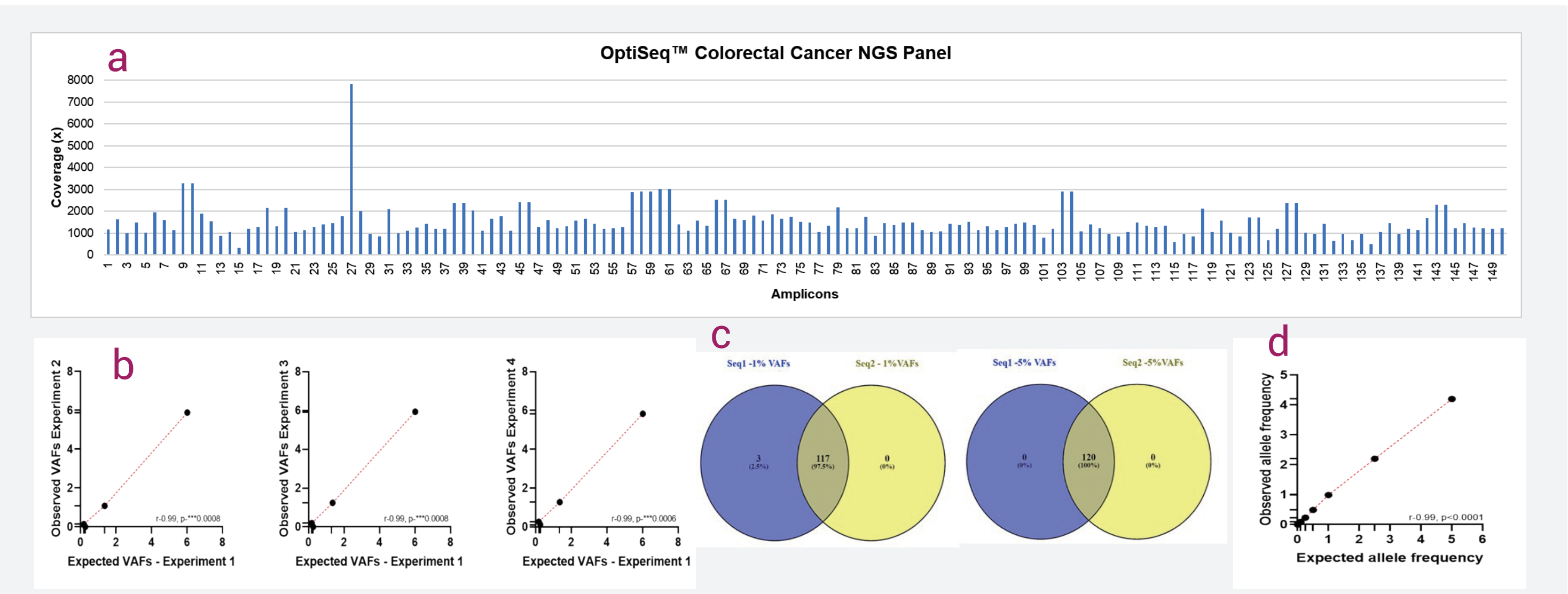


Fig. 3: Panel performance of OptiSeq™ CRC NGS mutation detection panel (a) Bar graph showing the range of sequencing coverage for individual amplicons in the CRC mutation panel (b) Correlation between variant allele frequencies (VAFs) obtained in the reproducibility and repeatability of the assay for reported variants in multiplex I cfDNA reference from Horizon Discovery. (c) Venn diagram displays common variants observed in four different library preparations (d) Analytical sensitivity of the panel was assessed using Thermo Fisher's Acrometrix ctDNA oncology DNA frequency ladder. Observed VAFs (%) (y-axis) is plotted against expected VAFs (%) (x-axis). All five genomic alterations (Table 1) demonstrated acceptable precision in four different experiments.

Table 1: Precision of the OptiSeq™ CRC NGS mutation detection panel

| Gene | AA mut | CDS mut | Number Positive / Number Expected | | PPV (95% CI) | | NPV (95% CI) |
|--------|--------|---------|-----------------------------------|-------|-------------------|-----------------|-------------------|
| | | | 1%VAF | 5%VAF | 1%VAF | 5%VAF | |
| NRAS | Q61K | 181C>A | 23/24 | 24/24 | 95.8% (79.8-99.3) | 100% (86.2-100) | 100% (96.9-100) |
| NRAS | A59T | 175G>A | 22/24 | 24/24 | 91.7% (74.2-97.7) | 100% (86.2-100) | 100% (96.9-100) |
| PIK3CA | E545K | 1633G>A | 24/24 | 24/24 | 100% (86.2-100) | 100% (86.2-100) | 100% (96.9-100) |
| EGFR | L858R | 2573T>G | 24/24 | 24/24 | 100% (86.2-100) | 100% (86.2-100) | 100% (96.9-100) |
| KRAS | G12D | 35G>A | 24/24 | 24/24 | 100% (86.2-100) | 100% (86.2-100) | 97.5% (92.9-99.1) |

III. Clinical Performance

For the OptiSeq™ CRC NGS methylation panel, the sensitivity was 50% and specificity was 100% for cfDNA and FFPE samples, and 100% sensitivity and specificity for advanced adenoma FFPE samples (Table 2). For the OptiSeq™ CRC NGS mutation panel, the sensitivity was 88% and specificity was 100% for CRC cfDNA and FFPE samples (Table 3). However, when both panels were used for patient samples testing, the preliminary data showed that their sensitivity and specificity were 100% for CRC (Table 4). The assay needs to be tested on more CRC patient samples for validation.

Table 2: Clinical performance of the OptiSeq™ CRC NGS methylation detection panel

| Sample Status | Positive | Negative | OptiSeq™ CRC NGS Methylation Panel | |
|-----------------------|----------|----------|------------------------------------|---------------------------|
| | | | Sensitivity (%) | Specificity (%) |
| CRC-cfDNA and FFPE | Positive | 3 | 50% (95% CI: 13.9-86.0) | 100% (95% CI: 89.5-100.0) |
| | Negative | 0 | | |
| | Total | 3 | | |
| Advanced Adenoma-FFPE | Positive | 6 | 100% (95% CI: 51.6-100.0) | 100% (95% CI: 56.0-100.0) |
| | Negative | 0 | | |
| | Total | 6 | | |

Table 3: Clinical performance of the OptiSeq™ CRC NGS mutation detection panel

| Sample Status | Positive | Negative | OptiSeq™ CRC NGS Mutation Panel | |
|---------------|----------|----------|---------------------------------|-------------------------|
| | | | Sensitivity (%) | Specificity (%) |
| Positive | 22 | 3 | 88% (95% CI: 67.7-96.8) | 100% (95% CI: 89.5-100) |
| Negative | 0 | 42 | | |
| Total | 22 | 45 | | |

Table 4: Integrated clinical performance of the OptiSeq™ CRC NGS mutation and methylation detection panel

| Sample Status | OptiSeq™ CRC NGS Mutation and Methylation Panel | | Sensitivity (%) | Specificity (%) |
|---------------|-------------------------------------------------|----------|-------------------------|--------------------------|
| | Positive | Negative | | |
| Positive | 6 | 0 | 100% (95% CI: 51.6-100) | 100% (95% CI: 65.1-93.3) |
| Negative | 0 | 10 | | |
| Total | 6 | 10 | | |

CONCLUSIONS

- Our targeted NGS panels evaluate DNA methylation levels of individual CpG sites across multiple regions and clinically actionable variants including single nucleotide variants (SNVs), insertions and deletions (Indels), microsatellite instability (MSI), and using low input clinical material from cfDNA and FFPE.
- The OptiSeq™ CRC NGS methylation panel showed a clear and significant differences in the methylation level between normal and cancer cell lines, CRC patients and healthy volunteer cfDNA and FFPE samples demonstrating that assay provides accurate methylation data for monitoring epigenetic biomarkers in clinical samples. The post-sequencing quality control step showed that the OptiSeq™ CRC NGS methylation panel can confidently distinguish small changes in the methylation level between 0% and 5% and can be used to screen advanced adenoma cases.
- The performance metrics for OptiSeq™ CRC NGS mutation panel showed the high correlation for VAFs ranged from 5% to as low as 0.25%, demonstrating the assay's high analytical sensitivity.
- The integrated genomic and epigenomic cfDNA assay provides clinically significant 100% sensitivity and specificity for CRC detection.

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