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Original Research

Performance Evaluation of a Colorectal Cancer-Associated MassARRAY-Based Hotspot Genotyping Assay for *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* Using Solid Tumor Specimens

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Abstract

Background: Accurate identification of actionable somatic variants is essential for therapeutic stratification in colorectal cancer (CRC). While next-generation sequencing (NGS) enables comprehensive genomic profiling, targeted approaches may provide faster and more practical alternatives for routine diagnostics. **Methods:** This study evaluated the analytical performance of the Agena Bioscience iPLEX® High Sensitivity (HS) Colon Panel using MassARRAY MALDI-TOF technology for detection of hotspot variants in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* from formalin-fixed paraffin-embedded (FFPE) specimens. A total of 60 unique clinical and reference samples were analyzed, targeting 26 single-nucleotide variants and compared with an orthogonal targeted NGS assay. Limit of detection (LOD) was assessed using serial dilutions of reference materials, and intra- and inter-run reproducibility was evaluated across multiple runs. Analytical performance metrics including positive and negative percent agreement, predictive values, and error rates were calculated. **Results:** The assay demonstrated complete concordance with NGS across all evaluated variants, requiring only 20 ng of DNA input, compared to 80–120 ng for a successful NGS run. LOD studies showed reliable detection of multiple variants at approximately 5% variant allele frequency, with *BRAF* p.V600E detectable near 1%. Intra- and inter-run analyses achieved 100% concordance, confirming assay reproducibility. Aggregated performance metrics demonstrated high sensitivity and specificity across a heterogeneous sample set. **Conclusions:** These findings establish the iPLEX® HS Colon Panel as a reliable platform for rapid detection of clinically actionable hotspot mutations. This study represents analytical validation using mixed FFPE tumor specimens; evaluation in larger colorectal cancer cohort, is warranted.

Keywords: colorectal cancer; molecular diagnostic techniques; formalin-fixed paraffin-embedded tissue; somatic mutation; mass spectroscopy

1. Introduction

Colorectal cancer (CRC) represents a significant global health burden, ranking as the third most commonly diagnosed malignancy and the second leading cause of cancer-related mortality worldwide based on GLOBOCAN 2022 estimates [1]. According to recent estimates, approximately 1.93 million new cases and 904,000 deaths from CRC occurred in 2021, with projections indicating a continued rise due to lifestyle factors such as diet, physical inactivity, obesity, and environmental influences [1,2,3,4]. In the United States, the American Cancer Society projected approximately 153,000–155,000 new CRC diagnoses and ~53,000 deaths in recent years (with 2026 estimates at ~55,000 deaths) [5]. Alarmingly, CRC incidence among adults under 50 years old has increased by 1–2% annually since the mid-1990s [6]. This rise in early-onset CRC, now accounting for approximately 10–20% of cases, particularly among individuals younger than 55 years, highlights the need for improved early detection and more personalized management strategies [7]. Survival rates decline sharply with disease progression, falling from over 90% in localized disease to approximately 13–15% in metastatic CRC [8]. Advances in screening modalities, including fecal immunochemical tests (FIT), colonoscopy, and emerging blood-based biomarkers, have contributed to declining overall mortality rates of approximately 1.5% per year among older adults [9]. However, significant disparities in screening access and outcomes persist in underserved populations and resource-limited settings.

The molecular landscape of CRC is characterized by heterogeneous genetic alterations that drive tumorigenesis and influence therapeutic responses. Key somatic mutations include those in *KRAS* (prevalence ~35–45%), *NRAS* (~3–6%), *BRAF* (~5–12%, predominantly V600E), and *PIK3CA* (~10–20%), which are critical for prognostication and guiding targeted therapies [10,11,12,13,14]. For instance, *KRAS* and *NRAS* mutations confer resistance to anti-*EGFR* monoclonal antibodies like cetuximab

and panitumumab, limiting their use to *RAS* wild-type tumors, while *BRAF* V600E mutations identify patients eligible for BRAF/MEK inhibitor combinations such as encorafenib plus cetuximab (with or without binimetinib), which have shown improved progression-free survival in clinical trials [15,16,17]. *PIK3CA* mutations, often co-occurring with RAS alterations, are associated with poorer prognosis and potential responsiveness to PI3K pathway inhibitors [11]. Recent advances in CRC diagnostics emphasize multi-omics integration, including microsatellite instability (MSI) testing for immunotherapy eligibility (e.g., pembrolizumab in MSI-high tumors) [18], and circulating tumor DNA (ctDNA) for non-invasive monitoring of minimal residual disease and treatment response [19]. Artificial intelligence (AI)-enhanced endoscopy and imaging have also improved polyp detection rates, while organoid models facilitate personalized drug screening [20,21]. Despite these innovations, formalin-fixed paraffin-embedded (FFPE) tissue analysis remains essential for somatic variant detection in advanced CRC, where rapid and accurate profiling is crucial for timely therapeutic decisions.

Contemporary molecular diagnostic approaches for CRC vary in scope, sensitivity, and practicality, each with inherent strengths and limitations. Next-generation sequencing (NGS) panels, considered the gold standard, provide comprehensive genomic profiling, enabling detection of rare variants, copy number alterations, and fusion events with high throughput and scalability; however, they are resource-intensive, with costs often exceeding $1000 per test, turnaround times of 1–2 weeks, high DNA input requirements (>50 ng), and complex bioinformatics analysis that can introduce interpretive errors or artifacts [22]. In contrast, targeted assays like quantitative PCR (qPCR) or immunohistochemistry (IHC) offer rapid, cost-effective hotspot detection but are limited in multiplexing, sensitivity for low-frequency variants, and breadth, often requiring multiple tests per sample. Liquid biopsies via ctDNA analysis enhance non-invasive, real-time monitoring but exhibit variable sensitivity (especially in early-stage disease), higher costs, and challenges in standardization. The Agena Bioscience MassARRAY system with the iPLEX® High Sensitivity (HS) Colon Panel mitigates these issues by targeting over 107 variants across five key genes (*BRAF*, *EGFR*, *KRAS*, *NRAS*, *PIK3CA*) with minimal FFPE DNA input (20 ng from 10 µL), reduced hands-on time, and results within 8–10 hours *via* matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) technology, achieving sensitivity down to 1% variant allele frequency in FFPE samples. This targeted approach prioritizes clinically actionable mutations, reduces costs, and simplifies interpretation compared to broad NGS, making it particularly suitable for routine diagnostics in advanced CRC where speed is essential for therapies like anti-*EGFR* agents or *BRAF* inhibitors. The present study evaluates this panel in 60 FFPE solid tumor specimens, focusing on 26 high-impact somatic variants, and compares its performance to a validated NGS platform to assess concordance and clinical utility in identifying variants in *PIK3CA*, *NRAS*, *BRAF*, and *KRAS*.

2. Material and Methods

2.1 Sample Information

This retrospective study was conducted in accordance with the Association for Molecular Pathology (AMP) and College of American Pathologists (CAP) guidelines for clinical assay validation and the Declaration of Helsinki. Institutional Review Board approval was obtained from Augusta University, GA, USA. All protected health information was removed, and samples and data were coded prior to analysis. Inclusion criteria required adequate FFPE DNA quality and availability of prior orthogonal sequencing data. Samples with insufficient DNA quality, incomplete documentation, or hematologic malignancies were excluded.

A total of 60 unique samples were processed across seven runs (Fig. 1). Fifty-one unique patient samples were derived from banked DNA extracted from FFPE tissues, including 41 solid tumor samples and 10 samples from individuals with no history of malignancy (true negative controls). The cohort comprised 31 females (median age: 61 years) and 20 males (median age: 68 years). Patient samples were analyzed in triplicate to assess inter- and intra-run reproducibility. Also run some replicates and reference materials.

Assay accuracy was evaluated using nine CAP proficiency testing samples containing known single nucleotide variants (SNVs). Reference controls included the AcroMetrix Oncology Hotspot Control (969056, Thermo Fisher Scientific, Fremont, CA, USA), containing over 500 COSMIC mutations across 53 genes; three genes encompassing six variants were evaluated (**Supplementary Table 1**). An additional 12 confirmed wild-type samples, distinct from true negative controls, were processed in duplicate (n = 24) to generate a baseline spectral profile in the Typer software (v5.0.2) within the Agena Biosciences MassARRAY system (Agena Bioscience, San Diego, CA, USA), which was applied during analysis to subtract wild-type–associated peaks and enhance detection of low variant allele frequency mutations.

All samples had been previously sequenced on the Illumina NextSeq 550 Dx using an orthogonal targeted oncogene panel, and archived sequencing data were retrieved for comparative analysis.

2.2 Sample Processing and Quality Assessment

Tumor cellularity was evaluated on hematoxylin and eosin (H&E)–stained sections by a board-certified pathologist, and only specimens containing a minimum of 20% tumor content were deemed suitable for molecular analysis. For each qualifying case, two to five unstained 5-µm FFPE sections of corresponding H&E slide were macrodis-

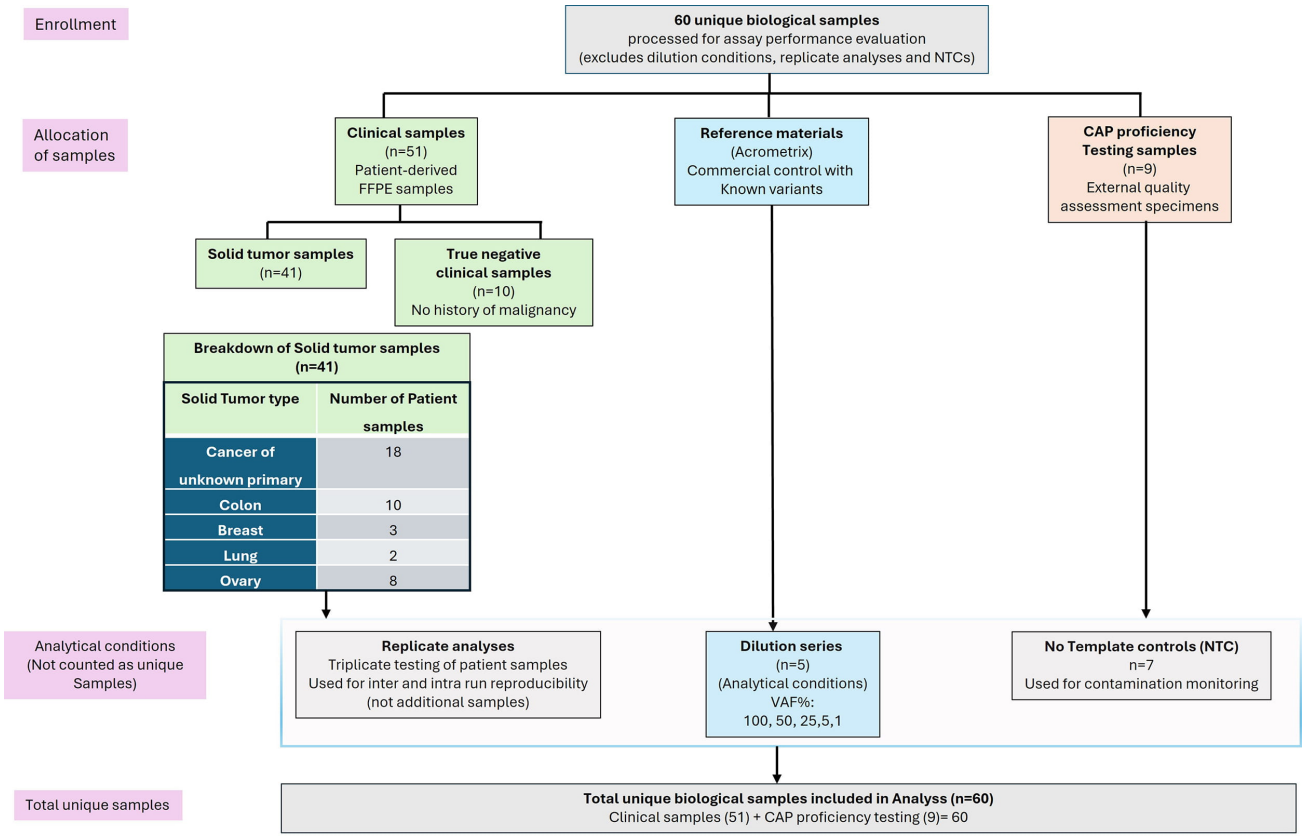

Fig. 1. Flow diagram of sample inclusion. A total of 65 unique biological samples were analyzed, including 51 clinical samples (41 tumors, 10 true-negatives), 9 College of American Pathologists (CAP) proficiency samples, and 5 reference materials. Tumor types included cancers of unknown primary (n = 18), colon (n = 10), ovarian (n = 8), breast (n = 3), and lung (n = 2). Dilution series, replicate analyses, and no-template controls (n = 7) were treated as analytical conditions and not counted as independent samples. FFPE, formalin-fixed paraffin-embedded; VAF, variant allele frequency.

sected to enrich tumor cellularity. Unstained sections were processed immediately following cutting to minimize oxidative DNA damage associated with prolonged storage of exposed tissue sections.

Paraffin removal and genomic DNA isolation were performed using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany; catalog no. 56404) in strict accordance with the manufacturer's protocol. Briefly, deparaffinization was achieved through xylene treatment followed by ethanol washing steps to ensure complete paraffin removal prior to tissue lysis. Proteinase K digestion was carried out at 56 °C for a minimum of 1 hour, followed by an incubation step at 90 °C for 1 hour to reverse formalin-induced DNA crosslinks, a critical step for maximising DNA yield and integrity from FFPE-derived material. DNA was eluted in a final volume of 30–50 µL of the kit-supplied elution buffer.

Extracted DNA underwent quality assessment using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), with samples required to demonstrate an absorbance ratio (A260/A280) between 1.7 and 2.2 to proceed to downstream analysis; samples falling outside this range were excluded. Double-stranded DNA concentration was subsequently quantified fluorometrically using the Qubit 1× High Sensitivity dsDNA Assay Kit (Thermo Fisher Scientific; catalog no. Q33231), which selectively quantifies double-stranded DNA and is therefore less susceptible to overestimation from single-stranded degradation fragments, a known limitation of spectrophotometric quantification alone in FFPE-derived samples. DNA samples meeting all quality control criteria were stored at –20 °C until further analysis.

2.3 Targeted PCR Amplification

Genomic regions encompassing clinically relevant hotspot mutations in *BRAF*, *NRAS*, *KRAS*, and *PIK3CA* were selectively amplified using the iPLEX® HS Colon Panel RUO kit (Catalog no# 13332D, Agena Bioscience, San Diego, CA, USA) in a multiplex PCR format. An overview of the iPLEX® HS assay workflow is presented in Fig. 2, and the complete list of targeted genes and variants is provided in **Supplementary Table 2**. Prior to amplification, dsDNA were normalized to a minimum concentration of 2 ng/µL with a total input volume of 10 µL

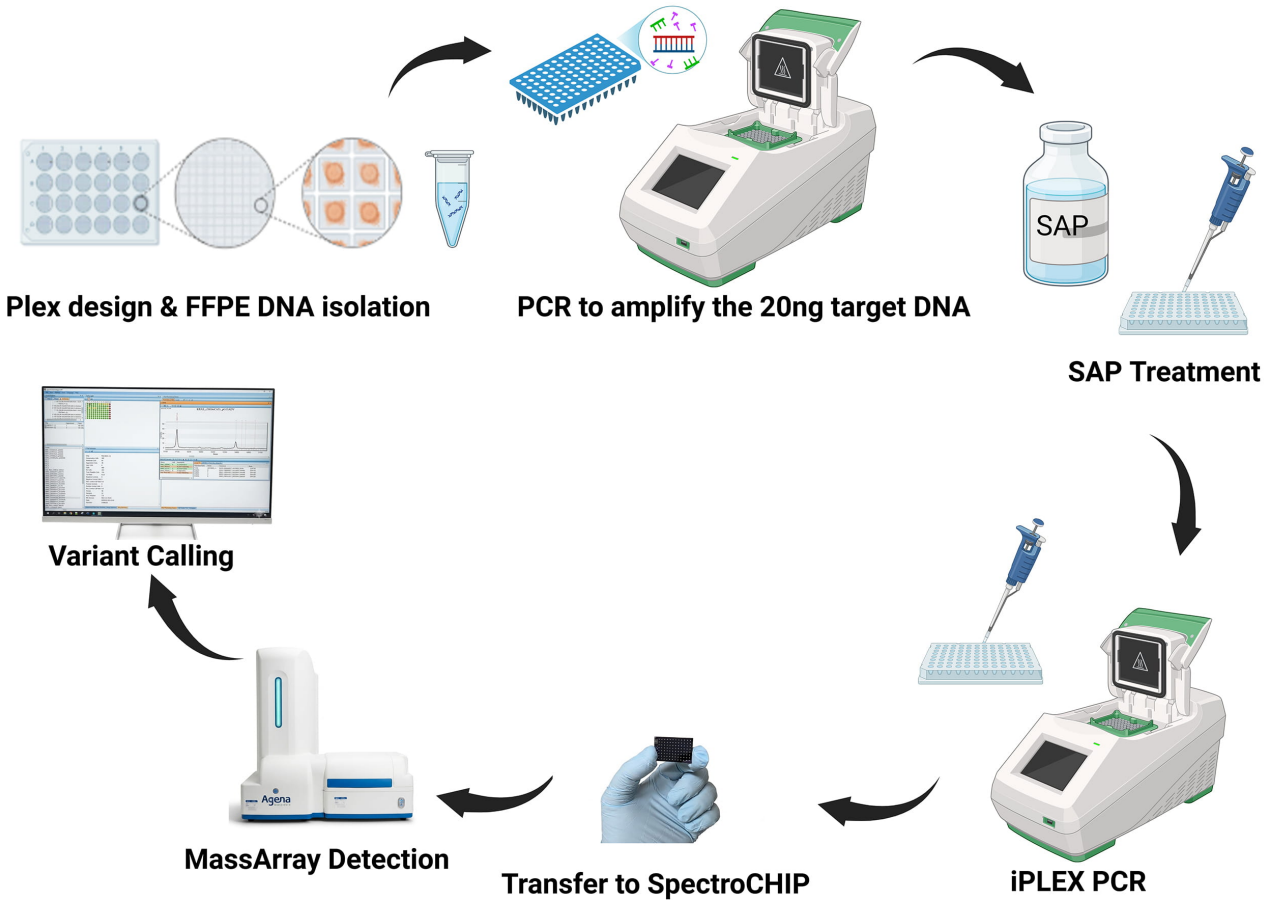

Fig. 2. Workflow of the iPlex® High Sensitivity (HS) Colon Assay, showing the steps from plex design to variant analysis using MassARRAY TYPER software. Created in BioRender. VASHISHT, A. (2026) https://BioRender.com/74bdls3.

to ensure adequate template availability. PCR reactions were assembled using reagents supplied in the iPLEX® HS Colon Panel V2 kit (Agena Bioscience, San Diego, CA, USA), including DNA polymerase, locus-specific primer pools, and deoxynucleotide triphosphates. Thermal cycling parameters were applied as recommended by the manufacturer. Following amplification, residual unincorporated dNTPs were enzymatically inactivated using Shrimp Alkaline Phosphatase, thereby preventing interference with subsequent extension reactions.

2.4 Single-Base Extension and Sample Preparation for Mass Spectrometry

Detection of sequence variants was accomplished using the iPLEX® HS single-base extension chemistry, which is specifically optimized for the identification of low variant allele frequency (VAF) mutations. Extension primers were designed to anneal immediately upstream of each targeted variant position and were extended by a single mass-modified dideoxy nucleotide terminator catalyzed by the iPLEX® extension enzyme. The wild-type–signal suppression chemistry intrinsic to the iPLEX® HS assay minimizes background from wild-type alleles, thereby improving sensitivity in heterogeneous tumor specimens. Following the extension reaction, products were desalted using a proprietary resin to remove excess salts and reaction by products that could adversely affect mass spectrometric analysis. Desalted samples were subsequently diluted with nuclease-free water to achieve optimal analyte concentration. Final reaction products were dispensed onto a SpectroCHIP Array for downstream mass spectrometry–based detection.

2.5 Mass Spectrometric Analysis and Data Acquisition

SpectroCHIP arrays were analyzed on the MassARRAY Chip Analyzer (Agena Bioscience, San Diego, CA, USA), which utilizes matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to discriminate allele-specific mass signatures of extended oligonucleotide products. Each well was subjected to a predefined number of laser shots, and only spectra meeting manufacturer-established quality metrics with a minimum of five acceptable acquisitions per well were retained for analysis. Data acquisition was performed within an operational mass window of 4500–9000 Da, with a nominal mass resolution of 16 Da and an average resolving power of approximately 850 m/Δm at full width at half maximum.

Table 1. Analytical performance metrics of the colon panel: sensitivity, specificity, accuracy and predictive values.

Metric	Abbreviation	Formula	Value%	Raw values (n/N)
Positive percent agreement (Sensitivity)	PPA	TP / (TP + FN) × 100	100	55/55
Negative percent agreement (Specificity)	NPA	TN / (TN + FP) × 100	100	10/10
Positive predictive value	PPV	TP / (TP + FP) × 100	100	55/55
Negative predictive value	NPV	TN / (TN + FN) × 100	100	10/10
False negative rate	FNR	FN / (FN + TP) × 100	0	0/55
False positive rate	FPR	FP / (FP + TN) × 100	0	0/10
Accuracy	-	(TP + TN) / (TP + TN + FP + FN) × 100	100	(55 + 10)/(55 + 10 + 0 + 0)

A total of 26 variants were analyzed across 55 positive cases and 10 true negative cases.

TP, true positives; TN, true negatives; FP, false positives; FN, false negatives.

2.6 Variant Calling, Quality Control, and Data Output

Variant calling was performed using the proprietary Agena Caller algorithm implemented in the MassARRAY Typer software suite. Genotype determination incorporated multiple spectral features, including peak intensity, mass accuracy, extension-to-unextended primer ratios, and overall spectral quality. Calls were categorized based on confidence metrics as either high-confidence ("conservative") or low-confidence ("low probability"), with model-based auto clustering applied to refine cluster separation and improve call reliability. Quality assurance measures included the incorporation of one no-template control (NTC) per 96-well plate; plates exhibiting amplification or signal in NTC wells were excluded from downstream analysis.

Assay performance across plates was visually assessed using a color-coded 96-well plate map reflecting genotyping success rates: dark green (≥85%), light green (50–<85%), yellow (>15–<50%), and red (<15%). Final data processing was conducted using the TYPER Analyzer software, which quantifies peak heights and signal-to-noise ratios to generate validated genotyping outputs. Somatic variant reports encompassing all detected variant calls were produced, and finalized datasets were exported in comma-separated values (.csv) format for downstream analysis.

2.7 Orthogonal NGS Concordance Analysis

Samples analyzed using the iPLEX® assay had been previously characterized by an orthogonal targeted NGS assay and were reused in this study to assess inter-platform concordance. For the orthogonal targeted NGS assay, qualified genomic DNA underwent library construction using a capture-based enrichment kit (Illumina, San Diego, CA, USA; catalog no. 20028214) according to the manufacturer's instructions. DNA fragmentation to an average insert size of approximately 130 bp was performed using a Covaris ultrasonicator (S220, Woburn, MA, USA), followed by end repair, 3′ adenylation, and ligation of indexed adapters. Adapter-ligated fragments were amplified using universal primers containing unique dual indices (UP-index). Target enrichment was achieved through hybrid capture using gene-specific probe sets, followed by post-capture PCR amplification, purification, and quantification of double-stranded DNA using the Qubit 1X High Sensitivity dsDNA Assay (Thermo Fisher Scientific, Waltham, MA, USA; catalog no. Q33231). Libraries were normalized using bead-based methods and sequenced on the Illumina NextSeq 550 Dx platform with V2 reagent kits, in accordance with the manufacturer's sequencing guidelines. Sequencing reads generated in FASTQ format were processed through the Qiagen Cloud Connect (QCC) clinical analysis pipeline (Qiagen, Germantown, MD, USA) to generate variant call format (VCF) files. Variant interpretation was performed using Qiagen Clinical Insight–Interpret (QCI-I) software (Qiagen, Germantown, MD, USA). Variant filtering criteria included a minimum VAF threshold of >5% and a minimum sequencing depth of 250×. Detected variants were classified into evidence-based tiers using curated literature, clinical databases, and expert-reviewed annotations within QCI-I, in accordance with the Association for Molecular Pathology (AMP) guidelines for somatic variant interpretation.

3. Results

3.1 Variant Detection and Analytical Performance of iPlex® HS Colon Panel

In a cohort of 60 samples, the colon panel exhibited full analytical concordance with NGS while requiring markedly lower DNA input and delivering a reduced turnaround time. This study interrogated four target genes encompassing 26 SNVs (Fig. 3). All four genes were consistently detected across samples using the MassARRAY platform, and all 26 SNVs previously characterized by an orthogonal NGS assay were accurately identified, demonstrating high-fidelity variant calling. To assess the clinical applicability of the panel, key analytical performance metrics, including clinical sensitivity (positive percent agreement, PPA), specificity (negative percent agreement, NPA), precision (positive predictive value, PPV), negative predictive value (NPV), false-negative rate (FNR), and false-positive rate (FPR) were calculated. These metrics, summarized in Table 1, underscore the robust performance and diagnostic reliability of the panel across a genomically diverse sample set.

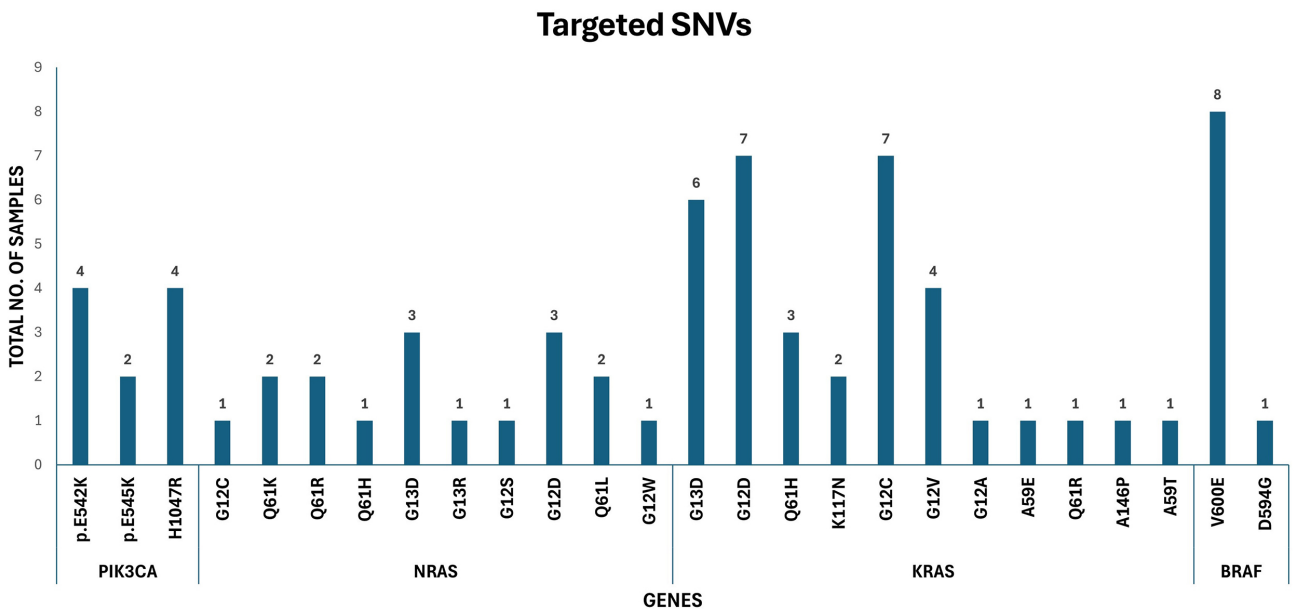

Fig. 3. Targeted single nucleotide variants (SNVs) across 4 genes analyzed using the iPLEX® HS Colon Panel. All variants detected on an orthogonal next-generation sequencing (NGS) panel were successfully identified by the MassARRAY platform, demonstrating high concordance.

3.2 Determination of Limit of Detection (LOD)

For LOD studies, the target SNVs were analyzed using commercial reference materials with serial dilutions. SNV LOD was assessed with AcroMetrix Oncology Hotspot Control (VAF 5–35%), tested undiluted (100%, 20 ng) and diluted to 50% (10 ng), 25% (5 ng), 5% (1 ng) and 1% (0.2 ng) with wild-type DNA to determine the minimum detectable VAF. Wild-type DNA was added to maintain a consistent 100% final input across all dilutions.

For the *NRAS* p.Q61R, *PIK3CA* variants p.E542K and p.H1047R, *KRAS* variants p.Q61H and p.G12D the target VAF ranged from 5% to 15%, while for *BRAF* p.V600E, the target VAF ranged from 15% to 35%, as specified by the supplier's instructions. Here, a 100% DNA input corresponds to the undiluted control with a VAF of 15–35%.

The assay successfully detected all 4 genes and 10 associated variants at the 100% DNA input, 50% and 25% dilutions (except for *PIK3CA* p.H1047R which was only detected at 100%). *KRAS* p.G12D; p.Q61H and *BRAF* p. V600E; D594G were detected down to 5% dilution (1ng DNA input). However, *BRAF* p. V600E was the only variant found at 1% dilution (0.2ng DNA input) (Fig. 4). This evaluation demonstrates that while the iPlex® HS Colon assay exhibits robust sensitivity across a range of dilutions for most variants, its analysis of specific variants at the lowest (1%) concentration defines the practical LOD for those targets.

Additionally, a representative example of the spectral output from the MassARRAY system for the LOD study featuring *BRAF* p. V600E is presented in Fig. 5.

3.3 Intra- and Inter-Run Reproducibility

A total of four distinct samples were subjected to both inter-run and intra-run analyses to evaluate the reproducibility of the iPlex® HS Colon assay. In the intra-run assay, designed to assess consistency within the run, three samples each tested in triplicate were processed in the same run (Fig. 6a). Conversely, the inter-run analysis, aimed at verifying precision across multiple runs, utilized 2 unique samples (in 3 different runs) to ensure reliability (Fig. 6b). Across all 4 samples, the expected variants *PIK3CA* p.E542K; p.H1047R, *BRAF* p.V600E and *KRAS* p.G13D; p.Q61H were consistently detected with 100% concordance. This alignment underscores the high reproducibility and reliability of the iPlex® HS Colon assay in identifying targeted genomic variants across both inter- and intra-run conditions.

4. Discussion

Precision molecular profiling has become a cornerstone of clinical management in metastatic CRC, where detection of actionable somatic alterations informs targeted therapy selection and prognostication. A critical yet underexplored gap in existing literature concerns the real-world analytical performance of targeted mass spectrometric genotyping platforms in a genuine diagnostic setting—specifically, the performance of the iPLEX® HS Colon Panel applied to clinically derived, routine FFPE specimens rather than idealized research-grade samples. To our knowledge, the present study is among the first to systematically evaluate this platform under conditions that closely mirror routine diagnostic practice, including the use of

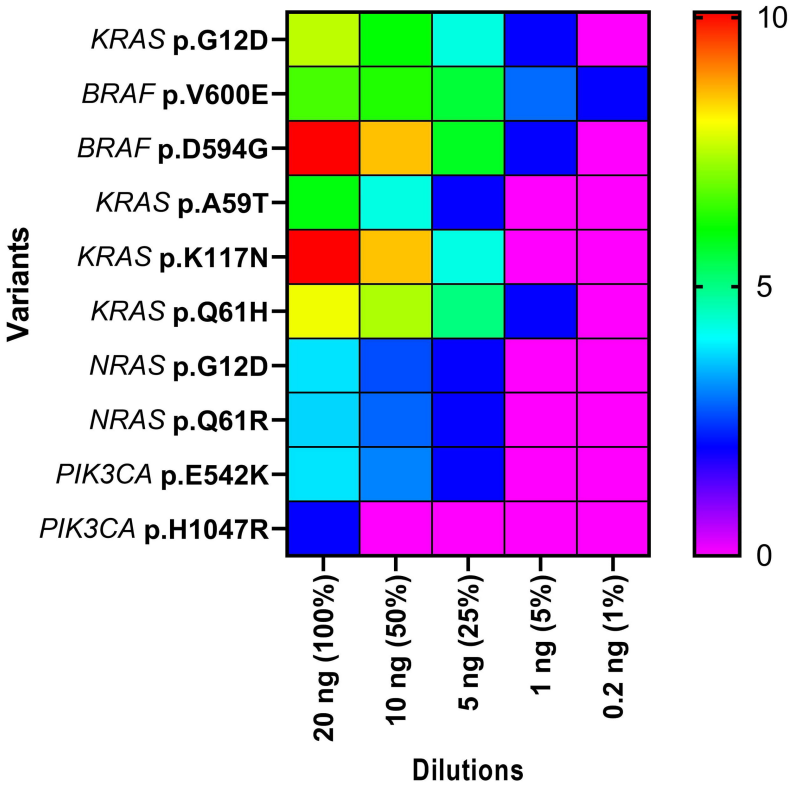

Fig. 4. Heatmap representation of VAF across serial DNA input dilutions. Heatmap shows observed variant allele frequencies for representative *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* mutations measured across decreasing DNA input amounts (20 ng, 10 ng, 5 ng, 1 ng, and 0.2 ng). Rows correspond to individual variants and columns represent input DNA quantities. Color intensity reflects variant allele frequency (%), with darker colors indicating higher VAFs and lighter colors indicating lower or absent signal. Consistent detection of variants at higher DNA inputs is observed, with progressive signal attenuation at lower inputs. Several high-abundance variants remain detectable near the assay limit of detection, demonstrating assay sensitivity and performance across a range of input DNA quantities. Variant allele frequencies reported as below the assay reporting threshold (<2%) were assigned a nominal value of 1.99%, and values exceeding the upper reporting limit (>10%) were capped at 10.01% for visualization purposes only.

banked clinical FFPE tissue, stringent quality thresholds, and direct head-to-head comparison with a validated orthogonal NGS assay. This contextual novelty is important, as performance characteristics established in controlled research environments do not necessarily translate to the heterogeneous specimen quality and pre-analytical variability encountered in routine pathology workflows. Our analytical validation of the Agena Bioscience iPLEX® HS Colon Panel using MassARRAY MALDI-TOF technology reveals robust performance for detecting clinically relevant hotspot mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* from FFPE solid tumor samples. The assay demonstrated complete concordance with an orthogonal targeted NGS platform across 26 SNVs in a cohort of 60 specimens, confirming high fidelity of variant calling and reproducibility across inter- and intra-run conditions. These results highlight the ability of focused mass spectrometric genotyping to accurately capture established CRC biomarkers that directly impact treatment decisions.

Previous studies comparing mass spectrometric genotyping platforms to broad genomic assays have reported strong overall concordance, supporting MALDI-TOF as a viable alternative for hotspot detection. A recent work using a custom MassARRAY assay applied to 93 CRC tissue specimens reported 99% concordance with orthogonal methods (Sanger sequencing and NGS confirmation of discordant calls), with *KRAS* mutations found in ~49% of cases and *NRAS/BRAF* alterations in ~3% of cases overall (n = 93) [23]. In comparison, our assay demonstrated complete analytical concordance (100%) with a validated targeted NGS platform, reinforcing the high specificity and accuracy of MALDI-TOF-based genotyping. Similarly, in a cohort of 160 CRC patients (106 with distant metastasis), Su-Jin Shin and colleagues [24] applied the MassARRAY platform to plasma ctDNA and detected tumor-specific KRAS mutations in 39.6% of metastatic cases, with sensitivity increasing from ~40% to ~58% upon selective mutant allele amplification, demonstrating reasonable detection rates for low-frequency ctDNA variants in liquid biopsy. While direct comparison to tissue-based assays is not one-to-one, our tissue-based iPLEX® HS Colon Panel showed reliable detection down to ~5% variant allele frequency for multiple

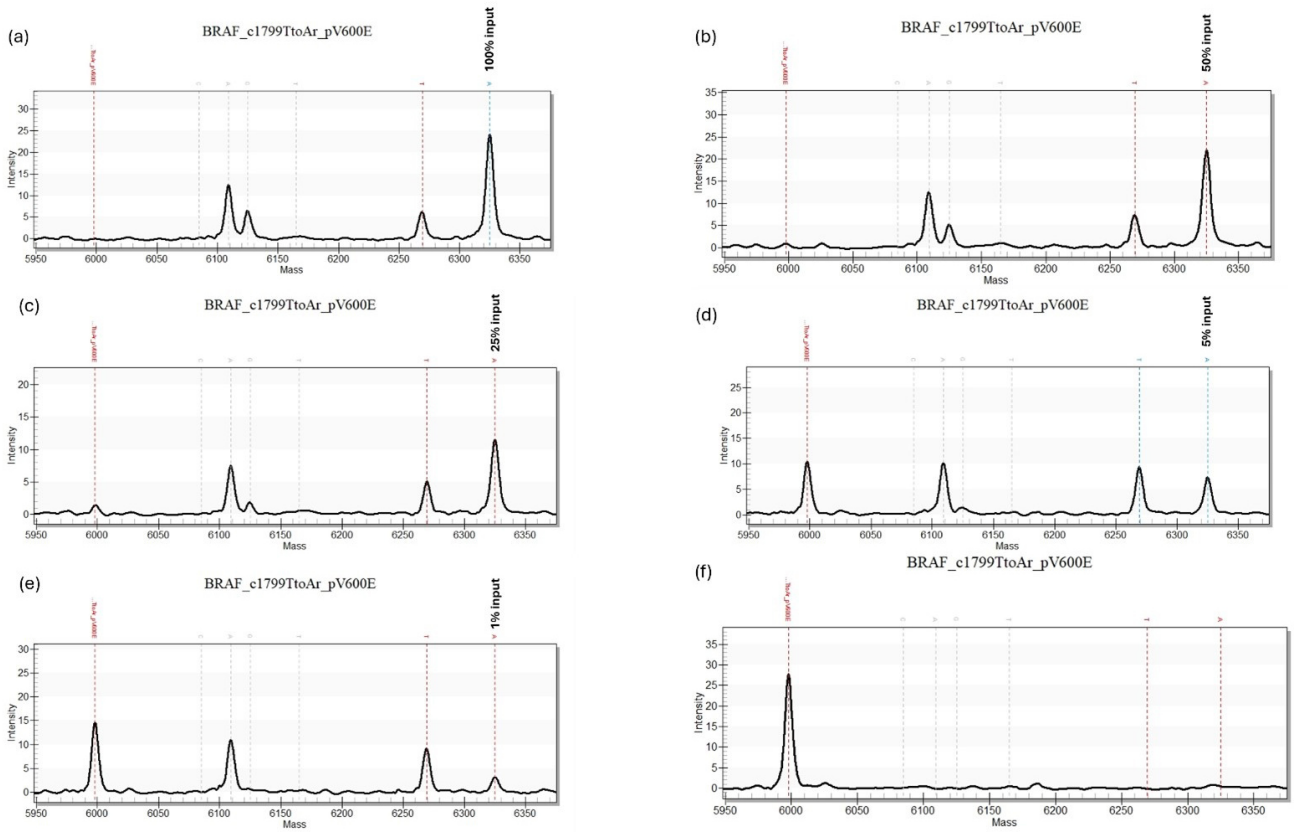

Fig. 5. Spectral graphs illustrate the observed limit of detection (LOD) results for *BRAF* p.V600E at decreasing mutant allele input levels. Panels (a–f) correspond to 100%, 50%, 25%, 5%, 1%, and no input, respectively. Distinct mass peaks corresponding to mutant and wild-type extension products are observed at their expected m/z positions, with progressive reduction in mutant peak intensity as the variant allele fraction decreases. The *BRAF* p.V600E mutant signal remains detectable down to low input levels, demonstrating the high analytical sensitivity and specificity of the MassARRAY platform for hotspot mutation detection. Intensity is plotted against mass (m/z), and dashed vertical lines indicate expected mass positions of extension products.

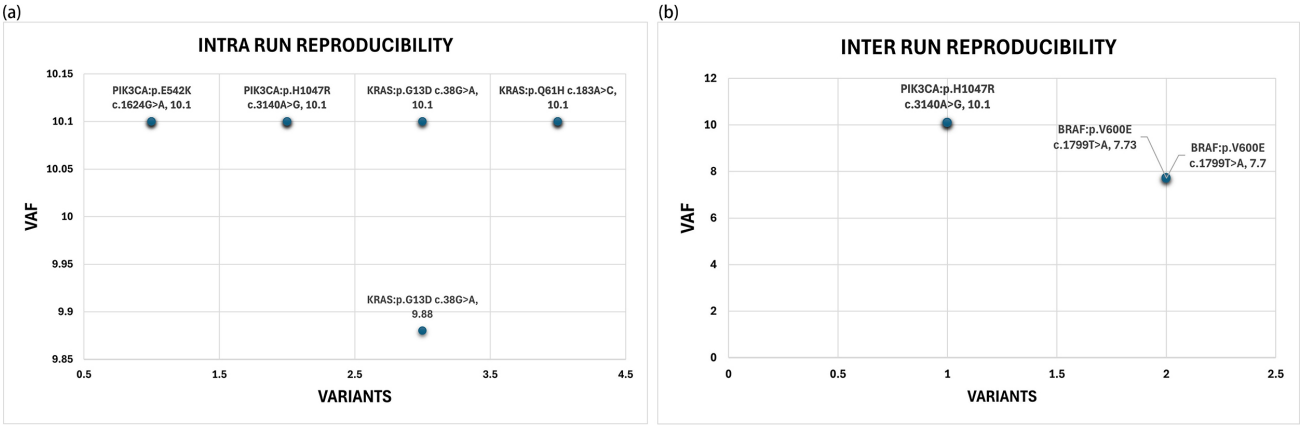

Fig. 6. Intra- and Inter-Run Reproducibility of VAF Measurements. (a) Dot plot showing variant allele frequencies for representative *PIK3CA* and *KRAS* mutations measured in technical triplicates within a single sequencing run and (b) in separate runs. Each dot represents an individual replicate measurement for the indicated variant, and dots are grouped by variant on the x-axis. VAF is shown on the y-axis. Variants exhibiting high allele frequencies, including *PIK3CA* p.E542K, *PIK3CA* p.H1047R, *KRAS* p.G13D, *KRAS* p.Q61H and *BRAF* p.V600E demonstrate tight clustering of replicate measurements, indicating minimal intra-run variability and high assay precision. VAFs reported as exceeding the upper reporting limit (>10%) were assigned a nominal value of 10.01% for visualization purposes only. No additional normalization or scaling was applied.

hotspot mutations and 1% for *BRAF* V600E, highlighting that for FFPE specimens, our approach achieves comparable or improved analytical sensitivity relative to broader, less controlled sample conditions like plasma [24]. Furthermore, the superior analytical sensitivity observed for *BRAF* p.V600E, detectable down to 1% VAF compared with approximately 5% for most other panel variants, reflects the convergence of a highly favorable mass differential between the c.1799T>A mutant and wild-type extension products and particularly efficient wild-type allele suppression at this locus within the iPLEX® HS chemistry. Importantly, this differential does not reflect a systemic limitation for non-BRAF targets; rather, all remaining panel variants were reliably detected at 5% VAF, a threshold that meets or exceeds the clinically established and guideline-endorsed detection requirement for RAS and RAF mutation testing in metastatic CRC, at which level therapeutic decisions regarding anti-*EGFR* eligibility and *BRAF/MEK* inhibitor selection are made. The enhanced sensitivity for *BRAF* p.V600E therefore represents an additional analytical advantage at a clinically critical locus, rather than compensating for deficiency elsewhere.

Furthermore, clinical evidence supports that low-frequency *RAS* and *BRAF* mutations, present at VAFs less than 5–10%, can confer resistance to anti-*EGFR* therapy and correlate with poorer outcomes in CRC, underscoring the necessity for assays with high analytical sensitivity to avoid false-negative calls that could misguide therapeutic decisions (NGS studies show detection of 23% of mutations in the 2–20% VAF range) [25]. In this context, the ability of the iPLEX® HS assay to detect select variants down to 1–5% VAF in FFPE DNA demonstrates clinically relevant sensitivity that aligns with detection thresholds where therapeutic resistance may be biologically meaningful. On another note, it is important to distinguish between the two complementary components of this analytical validation: concordance with the orthogonal NGS platform, which was conducted within the NGS reporting threshold of >5% VAF and therefore validates assay performance for clinically actionable variant detection at that range; and LOD studies using certified reference materials with known variant allele frequencies, which provide controlled analytical evidence for assay sensitivity in the 1–5% VAF range. The use of certified reference standards for LOD assessment is the accepted and standard methodology for this purpose, as clinical specimens with independently verified sub-5% VAFs are not routinely available. Together, these two components provide complementary evidence for the analytical performance of the platform across the clinically relevant VAF spectrum.

Importantly, while broad genomic profiling by NGS provides expansive genomic coverage, including detection of rare variants, copy number alterations, and structural rearrangements, the majority of clinically actionable decisions in CRC currently revolve around a defined set of hotspot mutations (e.g., *RAS*, *BRAF*) that are reliably captured with targeted approaches [26]. In this regard, focused assays such as the iPLEX® HS Colon Panel can deliver actionable results faster and at lower cost, which may be particularly beneficial in settings where rapid treatment stratification is essential or where access to comprehensive NGS is limited. Of particular clinical relevance is the substantially lower DNA input requirement of the iPLEX® HS Colon Panel—as little as 20 ng from a 10 µL volume—compared with the 80–120 ng typically required for a successful NGS library preparation run. This difference carries meaningful practical implications in scenarios where tissue availability is inherently constrained, such as endoscopic biopsies of small or anatomically inaccessible lesions, core needle biopsies yielding scant material, or re-biopsy specimens obtained at disease progression. In such cases, the residual DNA quantity after initial histopathological assessment may fall below the threshold required for NGS-based profiling, yet remain sufficient for MALDI-TOF–based genotyping. This low-input capability therefore extends molecular testing access to a subset of patients who would otherwise be rendered untestable by standard NGS workflows, thereby broadening the clinical reach of precision oncology in CRC.

The present study primarily focused on analytical validation of hotspot SNV detection using the iPLEX® HS Colon assay. While complete concordance with orthogonal NGS was observed for the evaluated variants, quantitative VAF estimation on the MassARRAY platform is optimized within an approximate 2–10% range, with results reported categorically as <2% or >10% outside this window. Although this constrains precise allelic quantification at very low or high frequencies, mutation presence rather than exact VAF magnitude typically informs therapeutic stratification in colorectal cancer. In addition, this validation was performed on a defined cohort of FFPE specimens, and larger prospective studies incorporating diverse sample types and clinical outcome correlations will further strengthen the clinical utility framework of this approach.

5. Limitations

Several limitations of the present study warrant careful consideration when interpreting its findings. First, although complete concordance with an orthogonal targeted NGS assay was observed, the study cohort comprised 60 specimens, a sample size that, while sufficient for preliminary analytical validation, may not be fully representative of the broader spectrum of tumor heterogeneity, rare variant co-mutations, or low-prevalence subclonal alterations encountered in routine clinical practice. In addition, colon tumor was underrepresented, which limits the generalizability of performance metrics for this indication.

The absence of discordant calls, while analytically encouraging, should be interpreted cautiously as this study mandated a minimum tumor cellularity of ≥20% and ac-

ceptable DNA quality. These thresholds are standard for molecular diagnostic validation, but specimens in the real world scenarios with borderline tumor content such as those compromised by extensive necrosis, mucinous differentiation, or suboptimal fixation may present additional analytical challenges. Prospective evaluation across a broader specimen quality spectrum will be necessary to fully characterize platform performance in unselected clinical populations.

As with all targeted genotyping platforms, the iPLEX® HS Colon Panel interrogates a predefined set of variants; the panel's design, however, encompasses an exhaustive catalogue of all clinically documented hotspot substitutions across codons 12, 13, 59, 61, 117, and 146 of *KRAS* and *NRAS*, key *BRAF* and *PIK3CA* hotspots, and *EGFR* p.S492R, thereby capturing the full spectrum of currently actionable mutations in CRC. Detection of alterations entirely outside this scope such as structural rearrangements or copy number variations is beyond the intended use of the assay and would appropriately require reflex to broader genomic profiling where clinically indicated.

Additionally, while reproducibility was demonstrated within a single laboratory across multiple intra- and inter-run conditions, multicenter validation studies would be necessary to assess the portability and translational applicability of the assay across institutions with differing instrumentation, operator expertise, and tissue processing protocols.

Finally, the real-world diagnostic utility of the panel was evaluated in a retrospective setting using banked FFPE specimens with previously confirmed variant status, which may not fully reflect the analytical challenges encountered with prospectively collected clinical samples exhibiting a wider range of pre-analytical variables, including variable fixation times, tissue age, and processing artifacts. These factors are known to introduce DNA fragmentation and chemical modifications (e.g., cytosine deamination) that may differentially affect assay performance and should be systematically evaluated in future prospective studies.

6. Conclusion

In conclusion, the iPLEX® HS Colon Panel demonstrated strong analytical performance, with complete concordance to NGS, high reproducibility, and sensitive detection of variants from low-input FFPE DNA. These results support its use as a rapid and reliable targeted genotyping assay; however, further validation in larger, tumor-specific cohorts, particularly colorectal cancer is warranted to fully establish its clinical utility.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization, VV, AKM & RK; methodology, VV, AV & AKM; software, VV, AV & AKM; validation, VV, AV & AKM; analysis, VV, AV; resources, RK; data curation, VV, AV, PKA, JF, JW & AKM; writing—original draft preparation, VV & AV; writing—review and editing, PKA, JF, JW and RK; visualization, VV and AV; supervision, RK; project administration, RK. All authors have read and agreed to the final version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Institutional Review Board (IRB #00000150, HAC IRB #611298, 4 April 2018) at Augusta University, GA, USA. Patient consent was waived due to retrospective nature of the study. Only left-over DNA was used. The study was conducted in accordance with the Declaration of Helsinki.

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Conflicts of Interest

The authors received financial support from Agena Biosciences for this study. The authors declare that the funder had no role in the study design, data collection, analysis, interpretation, or manuscript preparation, and that the work was conducted independently of any commercial interests. The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL53019.

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