ORIGINAL RESEARCH

Anatomical and Molecular Insights into Feasibility of Large-Amplicon PCR Targeting MSH2 Exon 12 in FFPE Rectal Tumors from Sri Lankan Patients

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Abstract

Objectives: To evaluate the feasibility of amplifying relatively a large (471 bp) MSH2 exon 12 fragments from formalin-fixed paraffin-embedded (FFPE) rectal tumors, and to optimize PCR conditions for large-amplicon recovery.

Methods: Archived FFPE rectal adenocarcinoma tissues (n = 12) and control rectal mucosa (n = 4) were analyzed. Tumor content was confirmed histologically, respecting anatomical boundaries of the rectum. DNA extraction was performed using a silica column-based method, and primer pairs specific for MSH2 exon 12 were optimized with gradient PCR. Strategies including increased $MgCl_2$, bovine serum albumin, DMSO, and pre-heating were applied to improve amplification from fragmented FFPE DNA. Amplicons were visualized via agarose gel electrophoresis, purified, and quantified.

Results: DNA yields varied widely (12.78–289.24 ng/µL), with most tumor samples showing adequate purity ratios. High-quality control DNA yielded consistent 471 bp products at annealing temperature of 64 °C. FFPE-derived DNA amplification was inconsistent, often producing faint or smeared products. Optimization improved yield for some samples but did not ensure reproducibility across all cases. Anatomical considerations including the rectum's complex lymphatic drainage and mesorectal compartment were noted as potentially relevant to mutation patterns.

Conclusion: While recovery of large-amplicon PCR products from FFPE rectal tumors remains challenging due to formalin-induced DNA damage, the study demonstrates methodological approaches to optimize yield in a real-world setting. Integrating molecular feasibility studies with rectal anatomical context may guide more precise surgical planning and targeted genetic screening in colorectal cancer management, particularly in resource-limited settings.

DOI: https://doi.org/10.4038/slaj.v9i2.288

Sri Lanka Anatomy Journal 2025; 9(II): 68–78

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Introduction

Colorectal cancer (CRC) remains a leading global malignancy, with rectal cancer comprising a distinct anatomical and molecular subset that poses unique challenges for diagnosis and treatment (1). Rectal cancer can be referred to as a malignant lesion located within 15 cm from the anal verge. The anatomic landmarks of rectum are important in tumor staging, diagnosis of rectal cancer and treatment strategies (2). The rectal wall comprises specific layers, including mucosa, submucosa, muscularis propria, and adventitia that determine local invasion pathways. Additionally, its venous drainage through both the portal and systemic circulations create dual metastatic routes. Lymphatic drainage is also segmentally organized, with upper rectal tumors draining primarily to inferior mesenteric nodes, and lower rectal lesions involving internal iliac nodes (3). These anatomical features are not only critical for accurate staging and surgical planning but also have molecular implications, as tumor microenvironments in different rectal segments may influence mutation patterns, including those in DNA mismatch repair genes such as MSH2.

In Sri Lanka, rectal cancer constitutes over two-thirds of CRC cases (4)., and its incidence has shown a steady increase. Despite this, molecular studies focusing specifically on rectal cancer in the local context remain limited, with diagnostics still largely dependent on imaging and histopathology (5). These modalities, while essential, fail to address the genomic underpinnings of tumor behavior or treatment response.

Mismatch repair (MMR) deficiency is a well-recognized molecular hallmark in a subset of colorectal cancers. Mismatch repair (MMR) proteins are crucial for correcting DNA replication errors. Failure of the system leads to an accumulation of mutations throughout the genome, particularly in repetitive regions known as microsatellites. This condition is known as microsatellite instability (MSI). This condition is driven by mutations in MMR genes such as MSH2, MLH1, MSH6, and PMS2(6). Among these, MSH2 is of clinical interest due to its high mutation frequency and established association contributing to genomic instability and cancer development in the rectal area. Globally, mutations in MSH2 are distributed across coding and non-coding regions. Extensive international research has shown variability in the frequency, type, and pathogenicity of MSH2 mutations across different populations, influenced by founder effects, environmental exposures, and genetic background (7,8, 9,10). However, Exon 12 of the MSH2 gene has been identified in multiple studies as a region with a higher frequency of mutations (11, 12,13) making it a relevant target for molecular screening. From a population genetics perspective, Sri Lanka's unique ancestry and migration history likely contribute to a distinctive spectrum of MMR mutations, which may differ significantly from those observed in Western populations or other Asian populations. The focus on the MSH2 gene exon 12 provides an insight into the presence of pathogenic mutations in Sri Lankan rectal cancer patients.

The use of formalin-fixed, paraffin-embedded (FFPE) tissues for molecular analysis is widespread, particularly in resource-

constrained settings where fresh tissue is rarely available (14). FFPE blocks are routinely archived in pathology laboratories and represent an invaluable biospecimen resource. In routine diagnostic settings, particularly in resource-limited countries such as Sri Lanka, FFPE blocks are often the only available material for retrospective or molecular studies.

However, the preservation of tissues in formaldehyde is not without complications. Although it maintains morphological detail, formalin fixation chemically alters nucleic acids through crosslinking and fragmentation. (14). Formalin-induced chemical modifications introduce artefactual mutations that closely mimic genuine biological mutational signatures (15). These modifications hinder polymerase chain reaction (PCR) amplification and compromise downstream applications such as Sanger sequencing.

Despite such technical limitations, targeted mutation screening from FFPE samples remains critical for expanding access to molecular diagnostics in under-resourced healthcare systems. Several studies have demonstrated the feasibility of amplification from FFPE DNA, (16,17) yet few have focused on longer targets such as MSH2 exon 12. Linking molecular pathology with anatomical context provides a deeper understanding of rectal carcinogenesis. The distribution of MMR gene mutations may not be uniform across the rectum; rather, local tissue oxygenation, blood flow, and microenvironmental differences could contribute to site-specific genetic alterations (2). By focusing on a well-defined anatomical segment and correlating these findings with a high-frequency mutation site in MSH2, this study bridges molecular diagnostics with surgical anatomy, highlighting potential implications for pre-operative planning, targeted surveillance, and prognostication. The present study sought to assess the feasibility of detecting mutations in MSH2 exon 12 from archived FFPE rectal tumor blocks using a 471 bp PCR amplicon and Sanger sequencing, under real-world clinical laboratory conditions.

This study bridges the fields of anatomical pathology and molecular oncology by interrogating the reliability of routinely stored rectal tumor tissues for mutation detection in a key MMR gene. By focusing on exon 12 of MSH2, this work aims to contribute to the evolving molecular understanding of rectal carcinogenesis in the Sri Lankan population while highlighting infrastructural and methodological challenges inherent to FFPE-based diagnostics.

Methods

This laboratory-based experimental study was conducted to evaluate the feasibility of detecting mutations within exon 12 of the MSH2 gene using DNA extracted from FFPE rectal tumor tissues. The study was conducted between 2024 and 2025 using archived histopathological specimens obtained from the National Cancer Institute Maharagama Sri Lanka.

A total of twelve FFPE blocks containing rectal adenocarcinoma tissues were selected

based on histopathological diagnosis, with an emphasis on ensuring sufficient tumor cellularity. Control FFPE tissues (n = 4) were selected from archived histologically confirmed non-neoplastic rectal mucosa obtained from diagnostic biopsies. All controls were verified by consultant histopathologists to exclude neoplastic changes prior to inclusion. Hematoxylin and eosin (H&E) stained slides corresponding to each block were reviewed by consultant histopathologists to verify tumor content and confirm tissue suitability for DNA extraction. Although all samples were histologically confirmed as moderately differentiated adenocarcinomas of the rectum, the precise anatomical sub-site of tumor origin within the rectum (upper, mid, or lower) was not documented in pathology records and therefore could not be incorporated into this analysis.

The Inclusion criteria comprised samples of histologically confirmed rectal cancer and Sigmo- rectal FFPE tissues with the availability of corresponding histological documentation. FFPE samples from any other area of colon, with no available histological/ microscopical analysis, polyps, post-chemotherapy/ post-radiotherapy, no documented records of chemotherapy / radiotherapy and with records of cancer recurrence were excluded from the studies.

All samples were de-identified before processing to maintain patient confidentiality. Ethical approval was obtained from The Faculty of Medicine, University of Colombo, Ethics review committee (MSC-24-014). Approval was also obtained from, the director, the National Cancer Institute, to obtain samples and access the laboratories.

DNA Extraction

Genomic DNA was extracted from FFPE tissue sections using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Germany), following an optimized protocol designed to minimize cross-linking artifacts and improve DNA recovery. For each sample, 5 µm-thick sections were cut using a microtome. Deparaffinization was carried out using two sequential 1 mL xylene washes followed by two washes in absolute ethanol to remove residual xylene. Column-based purification steps were performed according to manufacturer's protocol. DNA was eluted in two sequential aliquots of 25 µL and stored at −20 °C until further use.

DNA Quantification and Quality Assessment

Quantification and purity analysis of the extracted DNA were performed using a BioSpec-nano Spectrophotometer. 1 µL of DNA was loaded directly onto the micro-volume pedestal, and absorbance readings were recorded. DNA concentration was expressed in nanograms per microlitre (ng/µL), and the purity was evaluated using the A260/280 and A260/230 absorbance ratios.

Primer Design

A primer pair targeting exon 12 of the MSH2 gene was designed to amplify a 471 bp genomic region (NG_007110.2). Primer sequences were generated using NCBI Primer-BLAST and validated through BioEdit (v7.2.5) and IDT OligoAnalyzer to ensure

specificity, appropriate melting temperature (Tm), GC content, and absence of secondary structures such as hairpins, self-dimers, and cross-dimers. Primers were synthesized by SYNBIO Technologies. Primer sequences were Forward primer: 5'-GTTGAGTTTTAGGTGGGTTCCCT -3' and Reverse primer: 5'-AACGTTACCCCCAAAGGCC-3'

PCR Optimization

Initial PCR optimization was performed using high-quality genomic DNA extracted from peripheral blood. Reactions were set up in a 25 µL final volume containing Green Go Taq buffer (5X) 5 µL, MgCl2 (25Mm) 2.5 µL, dNTPs (10mM) 2.5 µL, Forward primer (2 µM) 2.5 µL, Reverse primer (2 µM) 2.5 µL, Taq polymerase (1U/ µL) 1 µL and 1.5 µL of blood genomic DNA were added. A PCR gradient was set up for the following thermal cycle. Initial denaturation 95 °C- 5 min, Denaturation 95 °C -1 min, Annealing 56–64 °C -1 min, Extension 72 °C -1 min, Final Extension 72 °C -5 min. The above conditions were set to complete 32 cycles. An optimized annealing temperature was identified based on sharp, specific band formation with minimal non-specific amplification on 2% agarose gel. Once conditions were validated using blood DNA, the protocol was adapted for FFPE-derived DNA through a series of optimization steps. These included adjusting the thermal cycle (annealing temperature of 64 °C for 1 min), increasing template input (up to 5 µL), raising $MgCl_2$ concentration (up to 3.5 mM), and supplementing reactions with 0.1 µg/µL BSA and DMSO. In addition, DNA was pre-treated at 95 °C for 10 minutes prior to amplification to reduce secondary structure interference. No template negative controls were included in all PCR runs to monitor for contamination. Amplified products were analyzed by gel electrophoresis and selected PCR products were subjected to Sanger sequencing using forward and reverse primers.

Gel Band Excision and Purification Using Low-Melting-Point Agarose

Due to insufficient PCR yield from FFPE-derived DNA for direct sequencing, selected amplicons were recovered from agarose gels using a low-melting-point (LMP) strategy. A 1.5% LMP agarose gel was prepared by dissolving 1.5 g of LMP agarose in 100 mL of 1X TAE buffer. The solution was heated until it was fully dissolved, cooled to ~60 °C, and stained with ethidium bromide (0.5 µg/mL) before casting. To maximize the recovery of low-concentration amplicons, PCR reactions from the same sample were pooled and loaded into a custom large-volume well. This well was created by taping adjacent comb teeth, allowing a single well to hold up to 30 µL. The pooled PCR product was electrophoresed on the LMP agarose gel, and the resulting bands were visualized under UV light using a transilluminator. Bands corresponding to the expected 471 bp MSH2 exon 12 fragments were excised using a sterile scalpel under limited UV exposure (≤10 seconds) to minimize DNA photodamage. Excised gel slices were purified using GFX silica spin column and purified DNA was recovered and quantified using NanoDrop spectrophotometer

Results

DNA Yield and Purity

DNA was successfully extracted from all 12 FFPE rectal tumor samples and four control FFPE tissues. Yields varied widely, with concentrations ranging from 12.78 to 289.24ng/µL (Table 1). Although many tumor samples had acceptable A260/280 ratios (~2.0), several controls showed deviations, with some biopsy-derived sample yielding an anomalously high ratio (4.49) indicating potential carryover of salts or organic solvents. Agarose gel electrophoresis of FFPE DNA revealed smearing patterns consistent with fragmentation, whereas control blood DNA displayed intact high-molecular-weight bands.

Table 1. DNA concentration and purity of sample and control FFPE blocks

Sample ID	Concentration (ng/µL)	A260/280 Ratio	A260/230 Ratio
S1	210.08	2.01	2.22
S2	158.37	2.03	2.70
S3	134.76	2.06	1.81
S4	87.00	1.93	2.80
S5	52.01	2.01	2.18
S6	84.27	1.87	3.99
S7	176.49	1.99	2.35
S8	119.90	1.98	3.39
S9	184.90	2.08	2.53
S10	289.24	2.05	2.43
S11	242.52	2.06	2.40
S12	102.53	2.10	2.35
C1	30.53	1.93	3.22
C2	32.88	2.36	0.82
C3	12.78	4.49	2.63
C4	35.42	2.43	1.19

(S- FFPE tumor tissue sample, C- FFPE non-neoplastic (control) tissue samples)

PCR Amplification with Control Blood derived DNA

Using high-quality blood-derived DNA, the MSH2 exon 12 primers consistently produced a clear single band of 471 bp under optimized conditions with MgCl2 2.5 mM, annealing temperature: 64°C, with no non-specific amplification or primer-dimer formation.

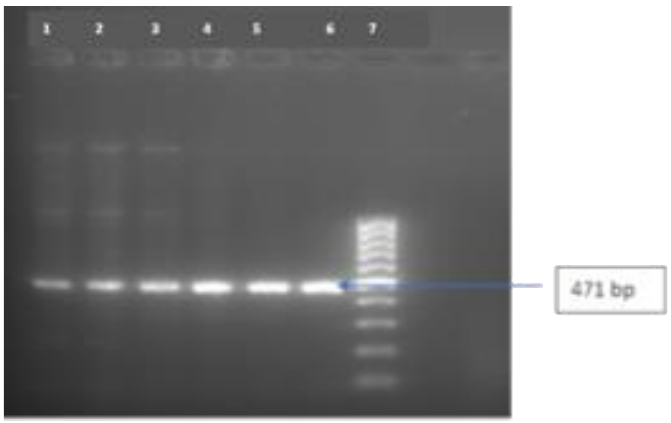

Figure 1: PCR Amplification 471bp of MSH2 exon 12 at 64 °C (lane 6). Lanes 1 to 6 represent PCR reactions performed at 56 °C, 57 °C, 58.8 °C, 60.6 °C, 62.4 °C, and 64 °C, respectively. Lane 7 contains the 100 bp DNA ladder.

PCR Optimization with FFPE DNA

On applying the optimized control DNA conditions to FFPE-derived DNA, amplification was inconsistent. Most reactions yielded faint, smeared products, or prominent primer-dimers. Optimization was carried out in at multiple levels in ana attempt to obtain consistent results, including optimization of template volume Figure 2 , concentration of MgCl2 (2.5 mM and 3.5 mM), Addition of BSA, DMSO and Pre-heating DNA at 95 °C for 10 minutes prior to PCR. These adjustments produced a slight improvement in band intensity for some samples (Figure 3) but

did not yield reproducible amplification across all samples.

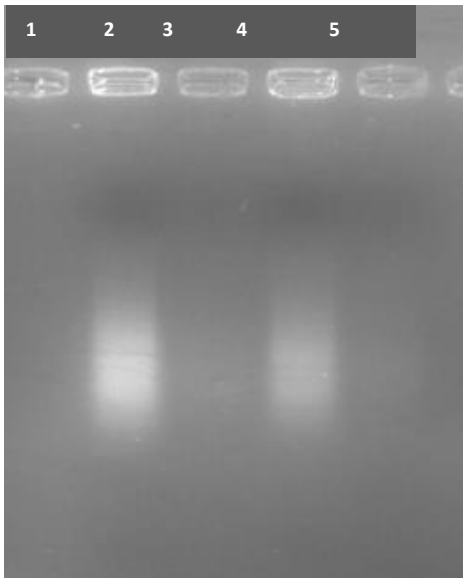

Figure 2: PCR products of FFPE-derived DNA showing fragmentation patterns. The sample produced a faint visible band within a smear after using 5 µL undiluted template as observed in Lane 2

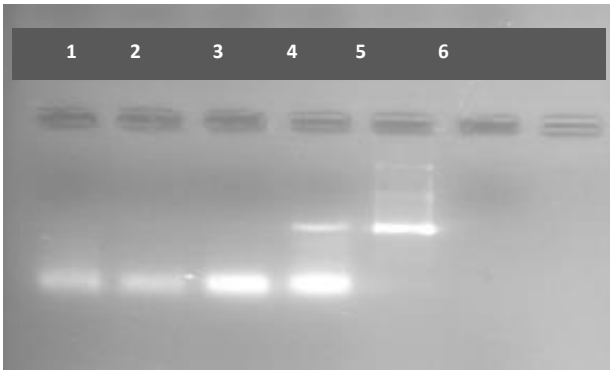

Figure 3: Successful PCR amplification using optimized 3.5 mM $MgCl_2$ with FFPE DNA. Lane 4 shows FFPE patient sample DNA with faint target band (~471 bp) indicating the successful PCR amplification. Lane 5: Blood-derived positive control for 471bp. Lanes 1–3: FFPE DNA samples showing only primer-dimer formation again showcasing the inconsistency.

Feasibility of Large Amplicon Recovery from FFPE DNA

For samples producing visible 471 bp bands, multiple PCR reactions were pooled and resolved on 1.5% low-melting-point agarose gels. Purified amplicons had yields between 13.89 –28.19 ng/µL, but the recovery rate was insufficient for consistent downstream analysis.

Discussion

This study evaluated the feasibility of amplifying and sequencing a 471 bp target within exon 12 of the MSH2 gene from FFPE-preserved rectal tumor DNA, with a focus on optimizing PCR conditions for large-amplicon generation. While FFPE samples are a readily accessible and cost-effective biospecimen source in Sri Lanka, formalin-induced DNA fragmentation, crosslinking, and base modification significantly impair amplification efficiency, particularly for fragments >200 bp. Our work demonstrates that these molecular alterations, compounded by prolonged fixation times in routine pathology workflows, remain a major technical barrier to obtaining intact large-amplicon templates.

Initial optimization with high-quality blood-derived DNA established a baseline for annealing temperature, $MgCl_2$ concentration, and template input, confirming that the selected primer pair could reliably amplify the intended 471 bp product under ideal conditions. But under refined conditions, amplification from FFPE DNA yielded

inconsistent results often with faint or smeared bands highlighting the unpredictable template integrity in archival material. Although conventional guidance recommends amplicon sizes 400 bp) using optimized reaction conditions (18,19,20). These findings are consistent with reports that while short amplicons (<200 bp) generally perform well, recovery of fragments in the 400–600 bp range is challenging (18,19).

All FFPE blocks used in the current project were initially prepared for routine histopathological diagnosis, primarily hematoxylin and eosin (H&E) staining, and not specifically for molecular purposes. As is common in such settings, tissues had been formalin-fixed for approximately 72 hours, a duration that, while sufficient for morphological preservation, is suboptimal for nucleic acid recovery due to extensive crosslinking and fragmentation. The recommended fixation time to 14–24 hours for molecular studies is the recommended fixation time. Standard formalin-fixation and paraffin-embedding procedures always result in significant fragmentation of nucleic acids. (17) However, since the available archive was used, the tissues were already subjected to longer fixation times that could have led to more severe DNA fragmentation, resulting in poor performance in downstream assays.

Additionally, previous studies have reported that DNA fragmentation in FFPE samples not only reduces target sequence availability but also produces short, random DNA debris (19). These fragments can inhibit DNA polymerase activity, thereby interfering with amplification even when partial templates are present. This is likely contributor to the smearing and weak products observed in this study. Optimization strategies such as increasing $MgCl_2$ concentration, inclusion of BSA and DMSO (21), and pre-heating DNA (16) are consistent with previously reported methods to overcome PCR inhibition and template fragmentation in FFPE-derived DNA. These modifications have been shown to improve amplification efficiency by reducing secondary structure interference and polymerase inhibition, although, as observed in our study, they cannot fully compensate for DNA fragmentation introduced during prolonged fixation.

Another recent study (22) reported that while FFPE samples remain valuable for retrospective studies, the extracted DNA consistently shows reduced integrity and fragment length, often impacting PCR success and genotyping reliability. In this study, although several tumor samples yielded relatively high concentrations and satisfactory purity, these did not consistently translate into successful PCR amplification or sequencing. This highlights the fact that high concentrations alone may not predict amplifiability, especially in FFPE tissues where DNA integrity is often severely compromised at the structural level.

Beyond the technical challenges, these findings also hold anatomical significance. Rectal cancers occur within a confined pelvic space where tumor growth patterns are influenced by fascial planes, mesorectal fat, and proximity to critical neurovascular bundles. Identifying MSH2 exon 12 mutations in such anatomically constrained environments may have implications for predicting patterns

of spread, particularly in relation to perirectal lymph node involvement and the risk of a positive circumferential resection margin. Furthermore, understanding molecular profiles in the context of precise anatomical location could guide more personalized surgical strategies, such as sphincter-preserving approaches or extended resections in genetically high-risk patients.

The results from this study reinforce the importance of tailoring both extraction and amplification workflows when large amplicons are required, including extended proteinase K digestion, the use of alternative polymerases with lesion-bypass capability, and consideration of nested or multiple primer strategies to mitigate template degradation effects. Also emphasizes the importance of pre-screening FFPE DNA for integrity before PCR.

From a clinical diagnostics perspective, this study highlights the utility of archived FFPE tissues as a cost-effective and widely available resource for molecular testing in Sri Lanka, laying the groundwork for local genetic studies using existing resources. From a translational perspective, the ability to recover large-amplicon sequences from FFPE tumor DNA has implications for comprehensive mutation scanning, as shorter targets may miss pathogenic variants outside high-frequency hotspots. However, in the present context, feasibility was constrained by inherent limitations in sample quality. Future work should investigate integrating DNA restoration steps, high-fidelity long-range enzymes, and pre-analytical fixation standardization to improve large-fragment recovery from FFPE material.

A limitation of the present study is the lack of precise documentation of tumor location within the rectum (upper, mid, or lower rectum). This study demonstrates methodological approaches to optimize large-amplicon PCR yield in FFPE rectal tumor DNA under real-world laboratory conditions. While anatomical considerations are relevant to rectal carcinogenesis, the present study did not document site-specific tumor location, and therefore molecular results could not be directly correlated with anatomy. Future investigations should integrate precise anatomical site data to strengthen the clinical relevance of molecular findings.

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