ORIGINAL RESEARCH

Optimization of PCR Conditions for BAT-25 and BAT-26 Amplification Using Commercial DNA Extraction Kits: A Methodological Approach from Sri Lanka

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Abstract

Objective: Reliable amplification of microsatellite loci from formalin-fixed paraffin-embedded (FFPE) tissues remains a technical challenge due to DNA fragmentation and chemical modification. This study aimed to systematically optimize polymerase chain reaction (PCR) conditions for robust amplification of the BAT-25 and BAT-26 mononucleotide loci using FFPE-derived DNA from colonic tissues. The investigation was designed strictly as a methodological optimization study and did not assess microsatellite instability (MSI) status.

Materials and methods: Genomic DNA was extracted from anonymized FFPE colonic tissue sections obtained from colon carcinoma cases and non-neoplastic controls using the QIAamp® DNA FFPE Tissue Kit. DNA yield and purity were evaluated spectrophotometrically. Critical PCR parameters, including annealing temperature, thermal cycling profile, and template DNA input, were systematically optimized for BAT-25, BAT-26, and the β-actin housekeeping gene. Amplification products were evaluated using 2% agarose gel electrophoresis to assess specificity and reproducibility.

Results: Optimization experiments identified PCR conditions that consistently produced clear and specific amplification of BAT-25 and BAT-26 loci from fragmented FFPE-derived DNA. An annealing temperature of 55.8 °C combined with an extended initial denaturation step yielded reproducible amplicons of expected sizes. The optimized protocol minimized nonspecific amplification and primer-dimer formation, demonstrating stable performance across independent samples.

Conclusion: This study establishes a reproducible and technically validated PCR framework for amplification of BAT-25 and BAT-26 loci using FFPE-derived DNA. The optimized conditions provide a practical methodological foundation for future investigations requiring reliable amplification of microsatellite markers from archival tissue specimens.

Keywords: PCR optimization; FFPE DNA; BAT-25; BAT-26; Colorectal tissue; Methodological Validation

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Introduction

In the 21st century, cancer has emerged as a major global health challenge, imposing a significant social and economic burden worldwide. Cancers account for approximately one in six deaths (16.8%) globally and nearly one in four deaths (22.8%) from noncommunicable diseases (NCDs)(1). Among these, colorectal cancer (CRC) is the third most diagnosed malignancy, representing about 10% of all cancer cases worldwide (2). In Sri Lanka, data from the National Cancer Control Program (NCCP) registry reported 1564 CRC cases among males and 1519 CRC among females in 2022(3).

Colorectal carcinogenesis results from the accumulation of genetic alterations in tumor suppressor genes, proto-oncogenes, and genes responsible for DNA repair mechanisms (4). CRC occurs more frequently in the proximal (right-sided) colon, accounting for approximately 70–85% of cases (5). Three principal molecular pathways have been described in the development of CRC: chromosomal instability (CIN), DNA mismatch repair (MMR) deficiency and the CpG island methylator phenotype (CIMP)(6).

Mismatch repair proteins are nuclear enzymes responsible for correcting base–base mismatches and insertion–deletion errors that arise during DNA replication in proliferating cells (7,8). These proteins function through the formation of heterodimer complexes that recognize and remove abnormal DNA segments (7,8). Loss or dysfunction of MMR proteins leads to deficient mismatch repair (dMMR), allowing replication errors to accumulate, particularly within short repetitive DNA sequences known as microsatellites (7,8). This phenomenon, termed microsatellite instability (MSI), is a well-recognized molecular mechanism in colorectal carcinogenesis (7,8). MSI is observed in more than 90% of Lynch syndrome–associated CRCs and in approximately 15% of sporadic CRC cases, making it an important pathway in the development of CRC (7,8). CRC with MSI have a comparable good prognosis compared to colorectal tumors without MSI and they may differ in the response to chemotherapeutics where overall, survival rates are better in patients with microsatellite unstable neoplasms (7,9). With the detection of MSI, it also helps in naval checkpoint inhibitor (CI) therapies for CRC patients (10).

In microsatellite instability (MSI) analysis, a standardized panel of DNA markers is typically amplified to evaluate length variations in microsatellite regions. This reference panel was proposed by the National Institutes of Health (NIH) in 1997 and includes five markers: BAT-25 and BAT-26 (mononucleotide repeats), and D5S346, D2S123, and D17S250 (dinucleotide repeats) (7).

MSI arises from variations in the length of short tandemly repeated DNA sequences caused by insertion–deletion errors during DNA replication. These alterations are conventionally assessed by comparing tumor DNA with corresponding normal DNA from the same individual (11). Among the recommended markers, BAT-25 and BAT-26 are widely used mononucleotide loci due to their high sensitivity to MMR deficiency and their quasi-monomorphic nature, which exhibits minimal size variation in normal populations (11,12). The BAT-26 locus consists of a polyadenine tract of approximately 26 repeats located within the fifth intron of the MSH2 gene, whereas BAT-25 contains a polythymine tract of approximately 25 repeats situated within intron 16 of the c-kit proto-oncogene on chromosome 4q12(11,12. Because these loci demonstrate limited polymorphism in normal tissue, changes in repeat length—particularly shortening of the mononucleotide tract—serve as highly sensitive indicators of replication errors in MMR-deficient cells(13). Their stability in microsatellite-stable (MSS) tissues makes them particularly suitable for methodological optimization studies focusing on amplification reliability. Given these characteristics, BAT-25 and BAT-26 are frequently selected for protocol development studies aimed at establishing robust PCR conditions prior to downstream MSI characterization.

Formalin-fixed, paraffin-embedded (FFPE) blocks are derived from human tissues obtained through biopsy or surgical procedures for routine histopathological examination and diagnosis (14).

These tissues are typically fixed in 10% neutral buffered formalin, processed through graded alcohols, and embedded in paraffin wax. FFPE blocks are widely preserved as archival material and represent an invaluable resource for retrospective research (14). Thin sections prepared from FFPE tissues are routinely used for histopathological staining as well as for nucleic acid extraction in molecular investigations (15). Importantly, FFPE blocks can be stored at room temperature for extended periods, substantially reducing long-term storage costs compared with frozen specimens (15).

Formalin remains the most used fixative in diagnostic histopathology due to its excellent preservation of tissue morphology. However, it introduces significant molecular alterations by creating crosslinks between nucleic acids and proteins, leading to fragmentation and chemical modification of DNA and RNA (16). These changes pose challenges for downstream molecular applications, as fixation duration and processing conditions are associated with reduced PCR yield and difficulty in amplifying longer DNA fragments (17). DNA degradation in FFPE samples can therefore influence the performance of several molecular techniques, including quantitative PCR, multiplex PCR, and next-generation sequencing (18).

Polymerase chain reaction (PCR) remains one of the most practical and accessible molecular techniques applicable to FFPE-derived DNA (19). Nevertheless, PCR efficiency is affected by multiple variables, including the type and duration of fixation, DNA extraction methodology, amplicon length, template DNA concentration, and the need for careful optimization of amplification conditions (19). Despite these limitations, FFPE biospecimens continue to play a crucial role in genomic research and clinical molecular studies because their accessibility and availability often outweigh challenges related to DNA quality, provided that appropriate methodological optimization is performed (20).

DNA extracted from FFPE tissues is frequently fragmented and chemically modified due to formalin-induced crosslinking, which can inhibit polymerase activity and reduce amplification efficiency. Therefore, optimization of PCR conditions is essential when working with archival specimens.

In Sri Lanka and other resource-limited settings, access to fresh or frozen tissue for molecular analysis is often limited, whereas FFPE archives are readily available in routine pathology laboratories. Establishing reliable PCR-based protocols using FFPE-derived DNA is therefore a practical prerequisite for future molecular investigations involving colorectal cancer. However, variations in fixation practices, DNA quality, and laboratory infrastructure necessitate local validation and optimization of amplification conditions before such methods can be applied consistently. This investigation was designed as a methodological optimization study focusing exclusively on PCR amplification feasibility of BAT-25 and BAT-26 loci using FFPE-derived DNA. The study does not evaluate microsatellite instability status, prevalence, or diagnostic performance. The present work was undertaken as a pilot effort to optimize PCR conditions using a commercially available DNA extraction system and FFPE specimens obtained from Sri Lankan clinical settings. Standardizing this protocol is expected to support reproducible molecular workflows and provide a technical foundation for subsequent large-scale studies in similar low-resource environments.

Methods

This investigation was conducted as a pilot methodological feasibility study designed exclusively to optimize PCR amplification conditions for BAT-25 and BAT-26 loci using FFPE-derived DNA. The study did not aim to determine microsatellite instability status, disease prevalence or diagnostic performance. Anonymized archival FFPE tissues were obtained from the Department of Histopathology of the National Cancer Institute and Colombo South Teaching Hospital, Sri Lanka. Ethical approval was granted by the Ethics Review Committee, Faculty of Medicine, University of Colombo (Ref: EC-24-131). Institutional permission was obtained

prior to sample retrieval. All specimens were anonymized before laboratory analysis.

FFPE blocks from histologically confirmed colon carcinoma cases were selected following review of pathology reports certified by consultant histopathologists. On-neoplastic colon tissues from patients diagnosed with non-malignant lower gastrointestinal conditions (e.g., microscopic colitis, nonspecific colitis, inflammatory bowel disease) were used as comparison samples to evaluate amplification performance across varying tissue characteristics. These samples were used solely to assess technical robustness of PCR amplification from FFPE-derived DNA.

FFPE tissue blocks were obtained from the Department of Pathology archives. Malignant samples consisted of histologically confirmed colon adenocarcinoma tissues resected during surgery, including specimens from right colectomy, hemicolectomy, transverse colon resection, and sigmoid colectomy (segmental sigmoid resections). Control FFPE samples were derived from non-malignant colon biopsies obtained during diagnostic endoscopic examinations. These tissues demonstrated no histological evidence of carcinoma and included non-neoplastic mucosa and benign lesions (e.g., polyps/adenomatous changes where applicable). Samples representing multiple anatomical regions of the colon were included to reflect routine diagnostic material. Rectal cancers were not included in this study.

Four sections of 8 µm thickness were cut from each FFPE block using a microtome under contamination-controlled conditions. Genomic DNA was extracted using the QIAamp® DNA FFPE Tissue Kit (Qiagen, Germany), selected due to its suitability for fragmented FFPE DNA recovery. Extraction was performed according to manufacturer protocols optimized for reversal of formalin crosslinks. DNA was eluted in ATE buffer and stored at −20 °C until analysis. DNA concentration and purity were measured spectrophotometrically at 260 nm using a BioSpec Nano (Shimadzu corporation A115746, Japan) instrument. Yield variability was expected due to FFPE-associated fragmentation and fixation-induced damage.

DNA input for PCR was standardized to approximately 80 ng per reaction to ensure reproducible amplification while minimizing inhibitor carryover.

Only two mononucleotide microsatellite markers, BAT-25 and BAT-26, included in the Bethesda panel, were selected for this study due to their suitability for amplification from degraded FFPE-derived DNA. Forward and reverse primer sequences were obtained from previously published literature and verified in silico using the Primer-BLAST tool of the National Center for Biotechnology Information to confirm primer specificity and appropriate amplicon size. For BAT 25, as forward primer 5’-TCGCCTCCAAGAATGTAAGT -3’ and 5’-TCTGCATTTTAACTATGGCTC -3’was used as reverse primer. For BAT 26, 5’-TGACTACTTTTGACTTCAGCC-3’ was used as forward primer and 5’-AACCATTCAACATTTTTAACCC -3’ was used as reverse primer (21).

PCR amplification conditions were systematically optimized to establish reproducible amplification from FFPE-derived DNA. Optimization focused primarily on annealing temperature determination, template DNA input, and reaction component balance.

A gradient PCR approach was employed using annealing temperatures of 50 °C, 52.2 °C, 54.6 °C, 55.8 °C, 58 °C, and 60 °C to identify the optimal temperature yielding specific and consistent amplification for both BAT-25 and BAT-26 markers.

PCR reactions were carried out in a final volume of 25 µL containing nuclease-free water, 5 µL of (5X) PCR buffer, 2.5 µL of dNTP mix (10 mM), 2.5 µL of $MgCl_2$ (25 mM), 2.5 µL each of forward and reverse primers (2 µM), 1.0 µL of Taq DNA polymerase (5 U/µL), and in each reaction approximately 80 ng/3µl of FFPE DNA was also included as DNA template. Thermal cycling was performed in Heal Force Touch Thermal Cycler T960 under these conditions: initial denaturation at

95 °C for 10 minutes, 35 cycles of denaturation at 95 °C for 20 seconds, annealing at 56°C for 20 seconds, extension at 72 °C for 40 seconds, final extension at 72 °C for 5 minutes and final hold at 4 °C. PCR products (5 µL) were resolved on 2% agarose gels containing ethidium bromide and electrophoresed at 70 V for 60 minutes alongside a molecular weight DNA ladder. Amplification products were visualized under UV illumination using a gel documentation system. Successful amplification was defined by the presence of a single, discrete band of expected size, absence of non-specific bands or primer-dimer formation, and reproducibility across samples.

Results:

DNA yield and purity

FFPE DNA was extracted using QIAamp® DNA FFPE tissue kit DNA successfully. Yields varied widely, with concentrations ranging from 17.5 to 247.4 ng/µL . Although many tumor samples had acceptable A260/280 ratios (~.1.8), several FFPE tissues showed an isolated extreme absorbance ratio. This was observed and determined to represent a spectrophotometric artifact. This value was excluded from interpretation due to lack of biochemical plausibility and known limitations of absorbance-based quantification in highly fragmented FFPE-derived DNA. With 0.8% agarose gel electrophoresis of FFPE DNA revealed smearing patterns consistent with fragmentation.

Optimization of annealing temperature

A temperature-gradient PCR (50 °C–60 °C) was performed to determine the optimal annealing temperature for BAT-25 and BAT-26 primers. PCR products were resolved on 2% agarose gel electrophoresis. The strongest and most specific amplification for both loci was observed at 55.8 °C, which was therefore selected as the optimized annealing temperature (Figure 1A and Figure 1B).

Optimization of PCR thermal profile

Two thermal cycling conditions were evaluated to improve amplification efficiency from FFPE-derived DNA. Thermal Profile I failed to produce detectable amplification, whereas Thermal Profile II (Table 1) yielded clear and specific bands for both BAT-25 and BAT-26 loci (Figure 2). Therefore, Thermal Profile II was selected as the optimized cycling condition.

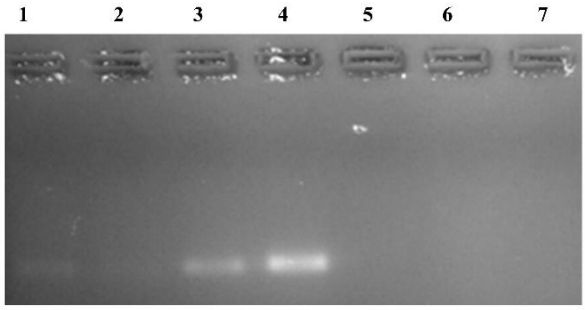

Figure 1A: Gradient PCR optimization for the BAT-25 marker using FFPE-derived DNA. Lanes 1–6 correspond to annealing temperatures of 50.0 °C, 52.2 °C, 54.6 °C, 55.8 °C, 58.0 °C, and 60.0 °C, respectively. Lane 7: no-template negative control. The most specific and intense amplification was obtained at 55.8 °C.

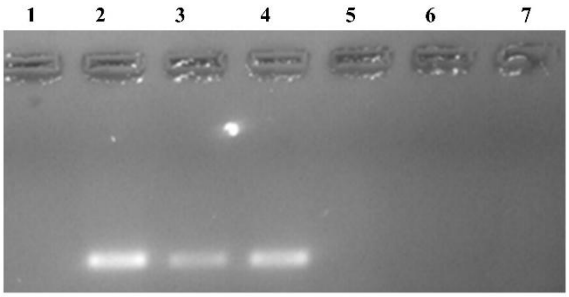

Figure 1B: Gradient PCR optimization for the BAT-26 marker using FFPE-derived DNA. Lanes 1–6 correspond to annealing temperatures of 50.0 °C, 52.2 °C, 54.6 °C, 55.8 °C, 58.0 °C, and 60.0 °C, respectively. Lane 7: no-template negative control. Optimal amplification was observed at 55.8 °C.

Table 1: Optimized PCR thermal cycling conditions for BAT-25 and BAT-26 amplification

Step	Thermal Profile II
Initial denaturation	95 °C – 10 min
Denaturation	95 °C – 20 s
Annealing	56 °C – 20 s
Extension	72 °C – 40 s
Final extension	72 °C – 5 min
Final hold	4 °C – 20 min

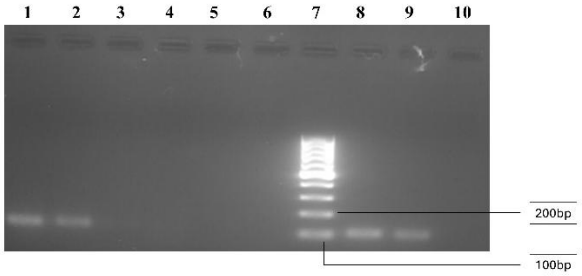

Figure 2: PCR amplification of BAT-25 and BAT-26 loci under optimized Thermal Profile II conditions. Lane 1: BAT-25 (P6); Lane 2: BAT-25 (H1); Lane 3: BAT-25 negative control; Lanes 4–6: empty wells; Lane 7: 100-bp DNA ladder; Lane 8: BAT-26 (P6); Lane 9: BAT-26 (H1); Lane 10: BAT-26 negative control. P = colon cancer sample; H = non-malignant colon sample

Optimization of FFPE DNA template input

Two DNA template inputs were evaluated to determine the optimal amount of FFPE-derived DNA required for reliable amplification (Table 2). A lower template volume (0.5 µL; 27 ng/µL) resulted in failed amplification, nonspecific binding, and primer-dimer formation. Increasing the template input to 3 µL (~80 ng total DNA) produced clear and specific amplification of BAT-25, BAT-26, and the internal control (β-actin). The optimized template input was subsequently validated using randomly selected colon cancer and non-neoplastic colon tissue samples, demonstrating reproducible amplification under the established conditions (Figures 3 and Figure 4).

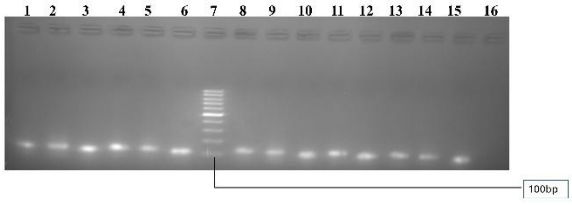

Figure 3: PCR amplification of BAT-25, BAT-26, and β-actin in randomly selected non-malignant FFPE samples using the optimized DNA input (~80 ng per reaction). Lane 7: 100-bp DNA ladder.

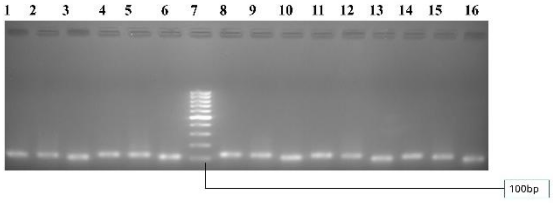

Figure 4: PCR amplification of BAT-25, BAT-26, and β-actin in randomly selected colon cancer FFPE samples under optimized conditions, demonstrating reproducible amplification from FFPE-derived DNA. Lane 7: 100-bp DNA ladder

Table 2: PCR Reaction composition used for template optimization

Reagent	Concentration	Volume (µL)
PCR Buffer	5X	5.0
MgCl2	25mM	2.5
dNTP	10mM	2.5
Forward Primer	2µm	2.5
Reverse Primer	2µm	2.5
Taq polymerase	5U/µL	1.0
Nuclease free water		6.0
DNA template	80 ng/3µL	3.0

Discussion

This study focused on the technical optimization of PCR amplification for two mononucleotide microsatellite loci, BAT-25 and BAT-26, using DNA extracted from formalin-fixed paraffin-embedded (FFPE) colorectal tissue. FFPE specimens represent an essential resource for retrospective molecular analysis because they preserve tissue morphology; however, formalin fixation introduces DNA fragmentation, cross-linking, and chemical modifications that can significantly impair PCR performance (22).

The Nanodrop purity ratios, together with smearing observed on agarose gels, confirmed that the

extracted DNA was highly fragmented, a finding consistent with the known effects of formalin-induced nucleic acid damage and prolonged storage. Because DNA derived from FFPE tissue is typically degraded and present in low effective template length, amplification efficiency depends strongly on PCR parameter optimization (23). In this study, amplification failure, nonspecific bands, and primer-dimer formation were initially observed under non-optimized conditions, highlighting the sensitivity of FFPE-derived templates to annealing temperature, denaturation conditions, and template concentration. These challenges necessitated systematic optimization of the PCR thermal profile. A key experimental finding was the failure of amplification under Thermal Profile I compared with the reproducible amplification achieved under Thermal Profile II. The absence of visible products under the initial profile likely reflects insufficient denaturation of cross-linked FFPE DNA (24). Formalin-induced protein–DNA complexes reduce strand accessibility, requiring more stringent or prolonged denaturation to achieve effective template separation. The extended initial denaturation incorporated in Thermal Profile II plausibly enhanced strand dissociation, allowing improved primer binding and polymerase access, resulting in stable amplification. This observation underscores the importance of tailoring denaturation conditions specifically for chemically modified FFPE DNA rather than applying standard PCR settings developed for high-quality genomic DNA.

Template input was another critical determinant of amplification success. Low DNA input resulted in inconsistent amplification and nonspecific products, whereas increasing the template volume produced sharp, reproducible bands without evidence of primer-dimer formation. This finding reflects the balance required when working with fragmented DNA: insufficient template reduces the availability of intact target regions, while optimized input increases the probability of amplifying short, preserved fragments. Primer selection also contributed to assay performance. The BAT-25 and BAT-26 primer sequences were obtained from previously published microsatellite studies and subsequently verified using NCBI Primer-BLAST to confirm locus specificity and reduce the likelihood of nonspecific amplification. Successful amplification under optimized conditions supports the compatibility of these established primer sets with degraded FFPE-derived DNA, demonstrating their suitability for laboratories working with archival material. Importantly, this investigation was limited strictly to optimization of PCR amplification conditions. No fragment analysis, allelic size determination, or tumor–normal comparison was performed. Therefore, microsatellite instability status was not assessed, and the study does not represent a diagnostic or prevalence analysis. Instead, the work establishes methodological parameters required before such downstream analyses can be reliably undertaken. The limited sample size and inclusion of heterogeneous control tissues were appropriate for technical optimization objectives, where the goal is to evaluate amplification behavior across variable DNA quality rather than to perform biological comparison. These data are therefore not intended to support conclusions regarding MSI frequency or clinicopathological associations but rather to define workable laboratory conditions.

Overall, the findings highlight that successful amplification of microsatellite loci from FFPE material depends primarily on adapting PCR conditions to the biochemical constraints of fixation-damaged DNA. Careful adjustment of denaturation time, annealing temperature, and template concentration can markedly improve amplification reliability, providing a necessary methodological foundation for future molecular applications using archival tissue.

Conclusion

This study demonstrates the technical feasibility, stability, and reproducibility of PCR amplification of BAT-25 and BAT-26 loci from FFPE-derived DNA following targeted optimization of thermal cycling conditions and template input. The work establishes practical laboratory parameters required for reliable amplification of fragmented archival DNA. The investigation was methodological in scope and did not evaluate

microsatellite instability status. These findings therefore provide foundational optimization data intended to support future studies specifically designed for MSI detection using validated analytical approaches such as fragment analysis.

Availability of data and materials: Data available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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