Multiomics

Efficient multianalyte analysis of mismatch repair pathways for colorectal cancer research

In this application note, we show:

- Efficient multianalyte analysis of DNA and RNA from a single sample
- Multiplex analysis of gene expression with Applied Biosystems™ TaqMan™ Assays
- Analysis of methylated DNA by Sanger sequencing and droplet-free dPCR
- Mechanistic insights into mismatch repair deficiencies using simple workflows

Introduction

Colorectal cancer (CRC) is one of the most common types of cancer worldwide [1]. Typically, CRC begins as a small, noncancerous polyp that can eventually develop into a cancerous tumor [2]. Several classification schemes based on the molecular profiles of different types of CRCs have been proposed, and in almost all of them, cancers with high levels of microsatellite instability (MSI) represent a distinct subclass of CRC [3,4]. MSI usually arises due to defects in nucleotide mismatch repair (MMR) processes that lead to expansion or contraction of repeats of simple single-nucleotide, di- or trinucleotide, or short oligonucleotide sequences that misalign during replication [5]. Deficiencies in the MMR pathway also produce mutations in coding regions of genes, leading to an increase in neoantigens in the tumor that may predict better response to immunotherapies. Therefore, understanding the dysregulation of mismatch repair pathways could be beneficial in researching an appropriate therapeutic intervention.

Several genes are known to be involved in MMR in humans. *MLH1* (MutL homolog 1) is one of the crucial genes for DNA MMR, and defects in this gene are often seen in CRC [6]. Along with other proteins, MLH1 forms a complex that recognizes and repairs mismatched bases during replication. When MLH1 activity is inhibited, these mismatches are not repaired, resulting in new mutations and MSI. The activity of MLH1 can be compromised by inactivating mutations in the coding region, mutations in promoters and enhancers that result in reduced expression, or methylation changes that can inactivate the gene [6,7]. Other genes that are important in the human MMR pathway include *MSH2*, *MSH6*, and *PMS2* [8].

We previously described a workflow that illustrated the analysis of methylated DNA by Sanger sequencing and by digital PCR (dPCR) [9]. In the work described here, we show how to incorporate multiplex gene expression into this workflow (Figure 1). Multiplex gene expression analysis increases the amount of information obtained from a single formalin-fixed, paraffin-embedded (FFPE) tissue section by facilitating the query of mRNA abundances. We designed a five-plex Applied Biosystems™ TaqMan™ Assay panel that queried levels of *MLH1*, *MSH2*, *MSH6*, and *PMS2* transcripts as well as an Applied Biosystems™ *PPIA* (Cyclophilin A) Endogenous Control (FAM™/MGB probe, non–primer-limited). We examined levels of expression of these genes in ten colorectal tumor FFPE samples and confirmed the results by droplet-free dPCR. We analyzed MSI in the same samples, used Sanger sequencing to detect methylated sequences in the *MLH1* promoter, and quantified the amount of methylation using dPCR. We found two samples that had decreased *MLH1* expression due to methylation in the *MLH1* promoter, which led to high levels of microsatellite instability. Together, these results illustrate efficient analysis of multiple different analytes from a single sample, facilitating important insights into the biology of that sample. Although this application note describes the analysis of MMR enzymes in CRC, in principle these methods could be adapted to other genes in other experimental paradigms.

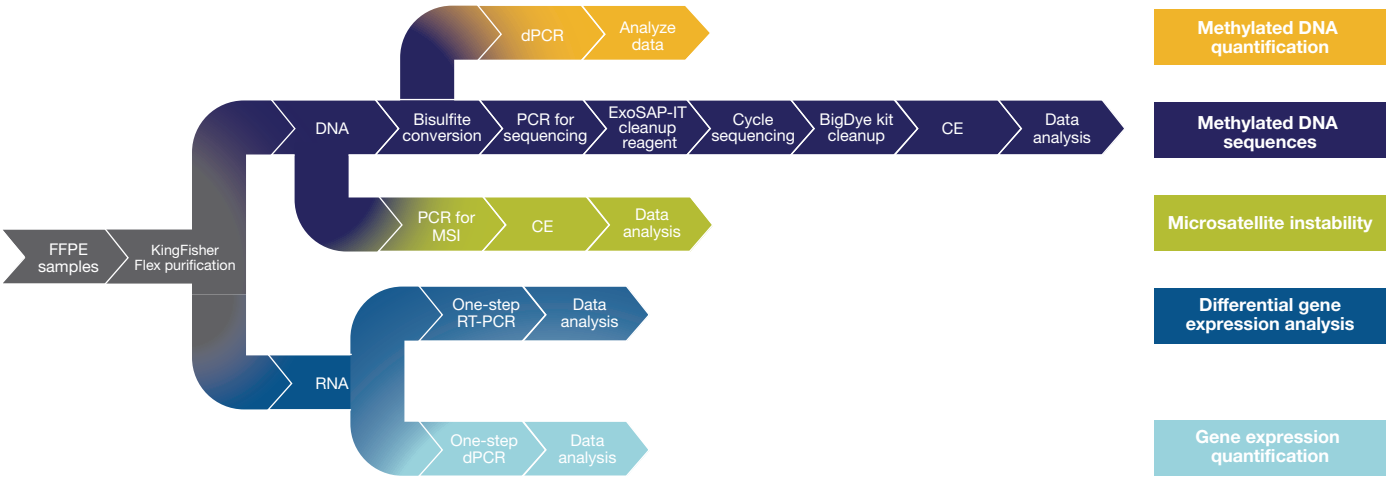


Figure 1. Multiple analyte extraction and analysis from colon tumor FFPE samples. The KingFisher Flex instrument and MagMAX FFPE DNA/RNA Ultra Kit facilitate purification of DNA and RNA from the same sample.

Results

Sample preparation

We purchased 10 different FFPE-preserved colorectal tumor samples. Each sample was a single 5 µm section mounted on a glass slide. Tissue was deparaffinized by incubating slides in xylene for 5 min, then washing with 100% ethanol 2 times for 5 min each, and air drying for 5–10 min. Tissue was removed from the slide with a razor blade and deposited into a microcentrifuge tube. The deparaffinized tissue was incubated with 110 µL protease solution from the Applied Biosystems™ MagMAX™ FFPE DNA/RNA Ultra Kit at 60°C for 1 hr with periodic vortexing, followed by a 60 min incubation at 90°C. The sample was loaded onto a Thermo Scientific™ KingFisher™ Flex Purification System and processed using the reagents in the MagMAX FFPE DNA/RNA Ultra Kit and the appropriate script for DNA purification [10]. When DNA purification was complete, the reserved sample fractions were reloaded onto the KingFisher Flex instrument and run with the reagents and script for sequential RNA purification [11]. DNA and RNA were quantified using an Invitrogen™ Qubit™ 4 Fluorometer. In both instances, the volume used for elution was 50 µL. The concentrations of recovered DNA and RNA are shown in Table 1.

Table 1. Concentrations of DNA and RNA recovered from FFPE sections.
Total volume of eluates was 50 µL.

Sample no.	DNA recovered (ng/µL)	RNA recovered (ng/µL)
CT10	21.0	38.1
CT11	7.30	49.3
CT12	1.54	13.7
CT13	1.40	13.3
CT14	2.90	4.91
CT15	21.8	72.0
CT16	7.50	52.0
CT17	2.43	14.4
CT18	2.80	8.44
CT19	5.85	18.6

MMR gene expression analysis

Mismatch repair deficiencies are commonly seen in CRC [12]. To determine expression of mismatch repair enzymes might be affected in these colon tumor samples, we designed a multiplex panel of TaqMan Assays to interrogate common mismatch repair genes (Table 2). We analyzed expression levels using 4 µL of colon tumor RNA (33.8–288 ng) or diluted Invitrogen™ Universal Human Reference RNA (UHR, 100 ng). RNA was combined with Applied Biosystems™ TaqPath™ DuraPlex™ 1-Step RT-qPCR Master Mix (no ROX™) and the multiplex assay in a total volume of 20 µL. PCR reactions were run on an Applied Biosystems™ QuantStudio™ 7 Pro Real-Time PCR System with the following profile: 20 min at 50°C for cDNA synthesis, 5 min at 95°C for enzyme activation, followed by 40 cycles of 5 sec at 95°C for denaturation and 60 sec at 60°C for annealing and extension. Data were analyzed using Applied Biosystems™ Design and Analysis 2.0 (DA2) Software and exported to Microsoft™ Excel™ software for further manipulation. Expression levels of the MMR gene transcripts were normalized using *PPIA* as an internal reference gene. All of the transcripts were detected in the different samples (Figure 2). However, *MLH1* was significantly lower in two of the samples (CT11 and CT15) and slightly lower in CT10 relative to the other samples.

Table 2. TaqMan Assays used to analyze gene expression.

Gene	TaqMan Assay ID	Probe dye	Quencher	Notes
MSH2	Hs00953527_m1	VIC™	NFQ-MGB	
MLH1	Hs00979919_m1	ABY™	NFQ-MGB	
PPIA	Hs99999904_m1	FAM™	NFQ-MGB	Reference gene
MSH6	Hs00943000_m1	JUN™	NFQ-MGB	
PMS2	Hs00241052_m1_qsy	Cy®5	QSY™ dye	

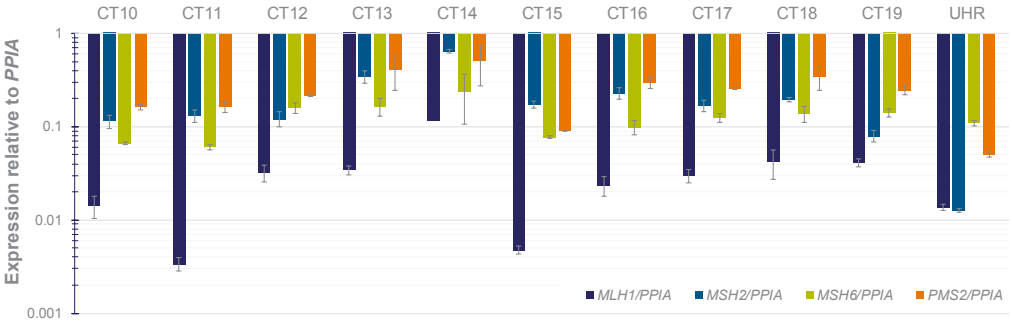


Figure 2. Multiplex gene expression analysis of RNA from colon tumor FFPE sections. Each sample was analyzed in triplicate using the 5-plex TaqMan Assay. The ratio of expression of each gene relative to *PPIA* is shown. Note that although there is some variation in the levels of *MSH2*, *MSH6*, and *PMS2*, *MLH1* is significantly decreased in samples CT11 and CT15, and slightly decreased in CT10. Error bars represent standard deviations of triplicate measurements. The expression levels in the UHR control sample are provided for completeness; they are unrelated to the biology in the other samples. UHR: universal human reference positive control.

We confirmed these results by analyzing mRNA levels using droplet-free dPCR. For this, we used 4 µL of RNA (33.8–288 ng) and Applied Biosystems™ Absolute Q™ 1-Step RT-dPCR Master Mix (4X) with the same multiplex assay described above, in a total volume of 10 µL. Samples were loaded onto a MAP16 plate from the Applied Biosystems™ Absolute Q™ MAP16 Plate Kit and Master Mix as described in the user guide. PCR conditions were 20 min at 50°C for cDNA synthesis, then 10 min at 95°C for enzyme activation, followed by 40 cycles of 5 sec at 95°C for denaturation and 15 sec at 60°C for annealing and extension.

dPCR was performed on the Applied Biosystems™ Absolute Q™ Digital PCR System. Data were analyzed using the Applied Biosystems™ QuantStudio™ Absolute Q™ Digital PCR System Software and exported to Microsoft Excel software for further manipulation. To facilitate comparison between qPCR and dPCR results, each sample's measurement was normalized to the average for all samples (Figure 3). Note that although the absolute magnitudes of the measurements differ slightly between the two platforms, the expression levels between samples exhibit strong correlation. In particular, the measured level of *MLH1* was significantly lower in samples CT11 and CT15 relative to the other samples. These results confirmed that expression of *MLH1* was decreased in two of the colon tumor samples.

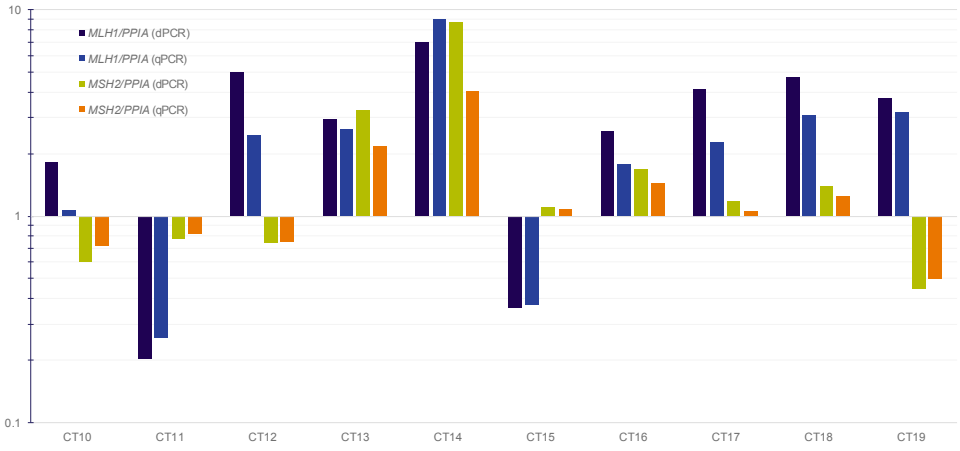


Figure 3. Agreement of dPCR and qPCR quantification of *MLH1* and *MSH2* expression. The colon tumor samples were analyzed with the multiplex TaqMan Assay by dPCR (dark blue and green) and qPCR (light blue and orange). For this comparison, the *MLH1*/*PPIA* (blue) or *MSH2*/*PPIA* (green and orange) ratio of each sample was normalized to the average across all ratios for that gene and platform. For example, the *MSH2*/*PPIA* ratio in CT15 was approximately the same as the average of all samples. Note that the expression ratios of *MLH1* in CT11 and CT15 were much lower than the average as measured by dPCR and qPCR.

MSI analysis

To assess the functionality of the MMR pathway, we analyzed the level of MSI using a panel of assays that targets relevant markers. For this, we used the Applied Biosystems™ TrueMark™ MSI Assay. The assay analyzes 13 microsatellite loci, including the Bethesda panel of markers [13-15], by fragment analysis using capillary electrophoresis (CE). In addition, the assay includes two highly variable short tandem repeat (STR) sequences that can be used to track sample identity. By building upon an extensive database of normal samples, the analysis software does not require side-by-side analysis of normal, non-tumor tissue to make MSI calls. This simplified workflow enables scientists to conserve material and quickly identify the microsatellite instability status of samples.

One microliter of isolated gDNA (1.4–21.8 ng) from the FFPE extractions, along with Applied Biosystems™ Control DNA (gDNA from CEPH Individual 1347-02), was analyzed using the protocol and reagents provided in the TrueMark MSI Assay kit. PCR products were run on the Applied Biosystems™ SeqStudio™ 8 Flex Genetic Analyzer with a 50 cm capillary and Applied Biosystems™ POP-7™ Polymer for 3500/SeqStudio™ Flex. Results were analyzed using Applied Biosystems™ TrueMark™ MSI Analysis Software v1.0.1. MSI status was called by the software and exported as a table (Figure 4). Most of the samples showed no instability (MSS). However, CT11 and CT15, the two samples that had decreased *MLH1* expression, showed high levels of instability (MSI-High) across most or all microsatellite loci. Interestingly, the CT10 sample showed instability at one locus (MSI-Low). Although the expression levels of *MLH1* in CT10 were not as low as in CT11 and CT15, Figures 2 and 3 show that they were reduced relative to the other samples.

TrueMark™ MSI Analysis Software Batch Report

Date of Report: 2025-02-11 14:26:10

Description	Value
Batch ID	ColonTum_MSI_0250109_20250109_132329-20250110103643
Analysis Software version	TrueMark™ MSI Analysis Software 1.0.1
Instrument ID	
Instrument Operator	
Minimum peak height	200
Minimum Unstable Rate to call MSI-High	30%
Minimum Unstable Rate to call MSI-Low	5%

Specimen ID	Overall call	Unstable	Stable	No call	Total	Review Flag	Well Pair Status	Well	Review Status	Edited?	Comments
Blank	No Call	0	0	13	13	Warning		D05	New	No	
CEPH	MSS	0	13	0	13	OK		C05	New	No	
CT10	MSI-Low	1	12	0	13	Warning		A04	New	Yes	
CT11	MSI-High	12	1	0	13	OK		B04	New	No	
CT12	MSS	0	13	0	13	OK		C04	New	No	
CT13	MSS	0	13	0	13	OK		D04	New	No	
CT14	MSS	0	13	0	13	OK		E04	New	No	
CT15	MSI-High	13	0	0	13	OK		F04	New	No	
CT16	MSS	0	13	0	13	OK		G04	New	Yes	
CT17	MSS	0	13	0	13	OK		H04	New	Yes	
CT18	MSS	0	13	0	13	OK		A05	New	Yes	
CT19	MSS	0	12	0	12	Warning		B05	New	Yes	
NEG	MSS	0	13	0	13	Warning		E05	New	No	
NEG2	No Call	0	0	13	13	Warning		F05	New	No	

Figure 4. Microsatellite status of colon tumor DNA samples. The report is the table exported from TrueMark MSI Analysis Software. Note that CT11 and CT15 had high levels of instability, and CT10 had low instability (one locus out of 13 was unstable). The other colon tumor samples had stable microsatellite sequences. “Warning” in the Review Flag column indicates the data fail to cross the threshold brightness for detection. The status in the Edited? column means calls were made based on manual evaluation of the fragment profile. CEPH and NEG: normal human DNA control sample; NEG2: no–DNA template control; Blank: no–amplification control.

Table 3. Primers used for Sanger sequencing of bisulfite-converted *MLH1* DNA to examine methylation state. M13 forward and reverse tails are shown in red.

Primer name	Sequence	Amplicon length	Chromosome	Start site (Feb 2009 GRCh37 hg19)	End site (Feb 2009 GRCh37 hg19)
MLH1_MSP_F	TGTAAAACGACGGCCAGTTATTTTTGTTTTTATTGGTTGGAT	160	3	37034702	37034882
MLH1_MSP_R	CAGGAAACAGCTATGACCTAAATACCAATCAAATTTCTCAACTCTA				

Sequencing of methylated DNA

Methylation at promoter sequences is typically associated with suppressing expression of the associated gene [16]. To correlate MSI status with methylation of *MLH1*, we examined the methylation status of a portion of the *MLH1* promoter by Sanger sequencing. Sanger sequencing is ideal for focusing on a region because many potential methylation sites can be examined in a single reaction.

We analyzed methylated Cs in the *MLH1* promoter covering the region ch3:37034702–37034882 (Table 3) following methods described by Benhamida et al. [17]. Primers were designed using Applied Biosystems™ Methyl Primer Express™ Software v1.0 and synthesized with M13 forward or reverse tails. Extracted FFPE DNA (20 µL) was treated using the Thermo Scientific™ EpiJET™ Bisulfite Conversion Kit according to the recommended protocol. To conserve the converted DNA, the eluted samples were not quantified. Amplicons for sequencing were generated using 5 µL of bisulfite-converted DNA, the M13-tailed forward and reverse primers, and Applied Biosystems™ AmpliTaq Gold™ 360 DNA Polymerase, in a total volume of 20 µL. Due to the reduced sequence complexity of the bisulfite-converted DNA, annealing temperatures for PCR were optimized with control DNA. Following PCR, the reactions were treated with Applied Biosystems™ ExoSAP-IT™ Express PCR Product Cleanup Reagent to remove excess primers. For cycle sequencing, 4 µL of PCR product was added to Applied Biosystems™ BigDye™ Terminator v3.1 Ready Reaction Mix and either the forward or reverse M13 sequencing primer. The reactions were run for 40 cycles and prepared for CE with the Applied Biosystems™ BigDye XTerminator™ PCR Purification Kit. Sequencing was performed on the SeqStudio 8 Flex instrument with a 50 cm capillary array and POP-7 polymer. Data were analyzed using Applied Biosystems™ InnoviGene™ Suite software.

Sanger sequencing simplifies the process of distinguishing between unprotected, unmethylated Cs—which are converted to Ts in the sequencing trace—and protected, methylated Cs. In the normal CEPH gDNA sample, all the Cs in the amplicon were converted to Ts, indicating that none of the Cs were methylated (Figure 5). Similarly, most of the Cs in the colon tumor samples analyzed were converted to Ts, demonstrating that there was no methylation over this region (data not shown). However, the two samples showing high microsatellite instability, CT11 (Figure 5) and CT15 (not shown), demonstrated mixed Cs and Ts at CpGs that were potential methylation sites. These data suggest that these two samples had at least some methylation of the *MLH1* promoter.

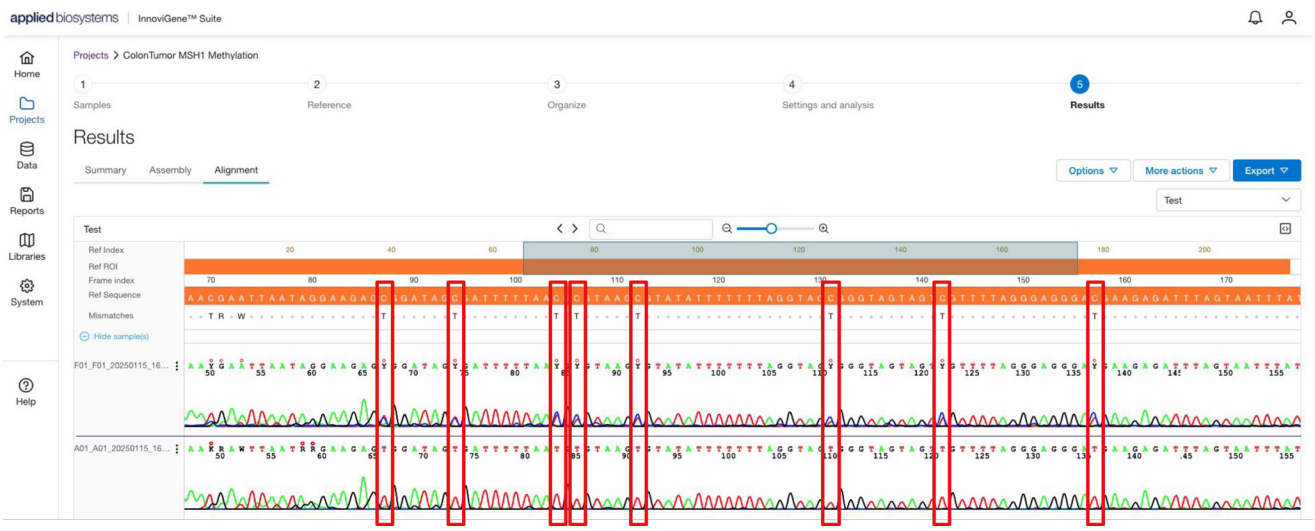


Figure 5. InnoviGene Suite analysis of Sanger sequencing of a portion of the *MLH1* promoter. The reference sequence shown (Ref Sequence, orange highlight) is based on bisulfite-converted, fully methylated sequences. Mismatches between samples and the reference are shown below the reference sequence. The CT11 sample is from a colon tumor; note that all of the potentially methylated CpGs have a mixed base call (red boxes), indicating at least partial methylation at those positions. The sequence of the normal CEPH DNA sample (bottom) has only Ts at all CpG positions, indicating a lack of methylation at these sites. For InnoviGene Suite software details, see thermofisher.com/order/catalog/product/A40001760.

dPCR of methylated DNA

The sequencing results presented here suggest that in CT11 and CT15, there was a mixture of methylated and unmethylated sequences. To confirm that the mixed base calls were accurate and to quantify the amount of heterogeneity, we performed droplet-free dPCR. We designed a custom TaqMan genotyping assay to query the second CpG in the *MLH1* amplicon (Figure 6A), with the FAM probe designed to detect T (unmethylated at that base) and the VIC probe designed to detect C (methylated). Since the bisulfite-converted DNA contained uracil, we used an RT-dPCR master mix that lacked uracil glycosylase. Uracil glycosylase is typically included in a standard PCR to help with error correction by cleaving DNA when a uracil is present; however, it would degrade bisulfite-converted DNA and must be excluded when analyzing bisulfite-converted DNA.

dPCR was performed using Absolute Q 1-Step RT-dPCR Master Mix (4X) (lacks uracil-DNA glycosylase), 4 µL of bisulfite-converted DNA, and a 1X custom TaqMan genotyping assay in a total volume of 10 µL. The reaction mix was loaded onto the QuantStudio Absolute Q MAP16 Plate, run on the QuantStudio Absolute Q Digital PCR System (10 min at 95°C, then 40 cycles of 5 sec at 96°C for denaturing, 10 sec at 58°C for annealing, and 20 sec of extension at 60°C), and analyzed using Absolute Q Digital PCR Software.

The results of this assay showed clear discrimination between FAM and VIC fluorophore signals (Figure 6B). A control sample that was fully methylated (FMG) had a very high fraction of fully methylated sequence (95.8%, Figure 6C). Conversely, the sample that completely lacked methylation (0MG) had very low levels of methylation (0.76%). Further, the normal CEPH control also lacked significant methylation (0.18%). Most of the colon tumor samples that had normal *MLH1* expression and were microsatellite-stable showed minimally methylated DNA at this position (Figure 6B and C). However, the two samples that had decreased *MLH1* expression and were MSI-High had significant methylation (CT11: 57.9%; CT15: 24.3%). The fractional methylation amounts may reflect tumor heterogeneity. Importantly, these values correlated with the presence of mixed peaks detected in the Sanger sequencing traces described above.

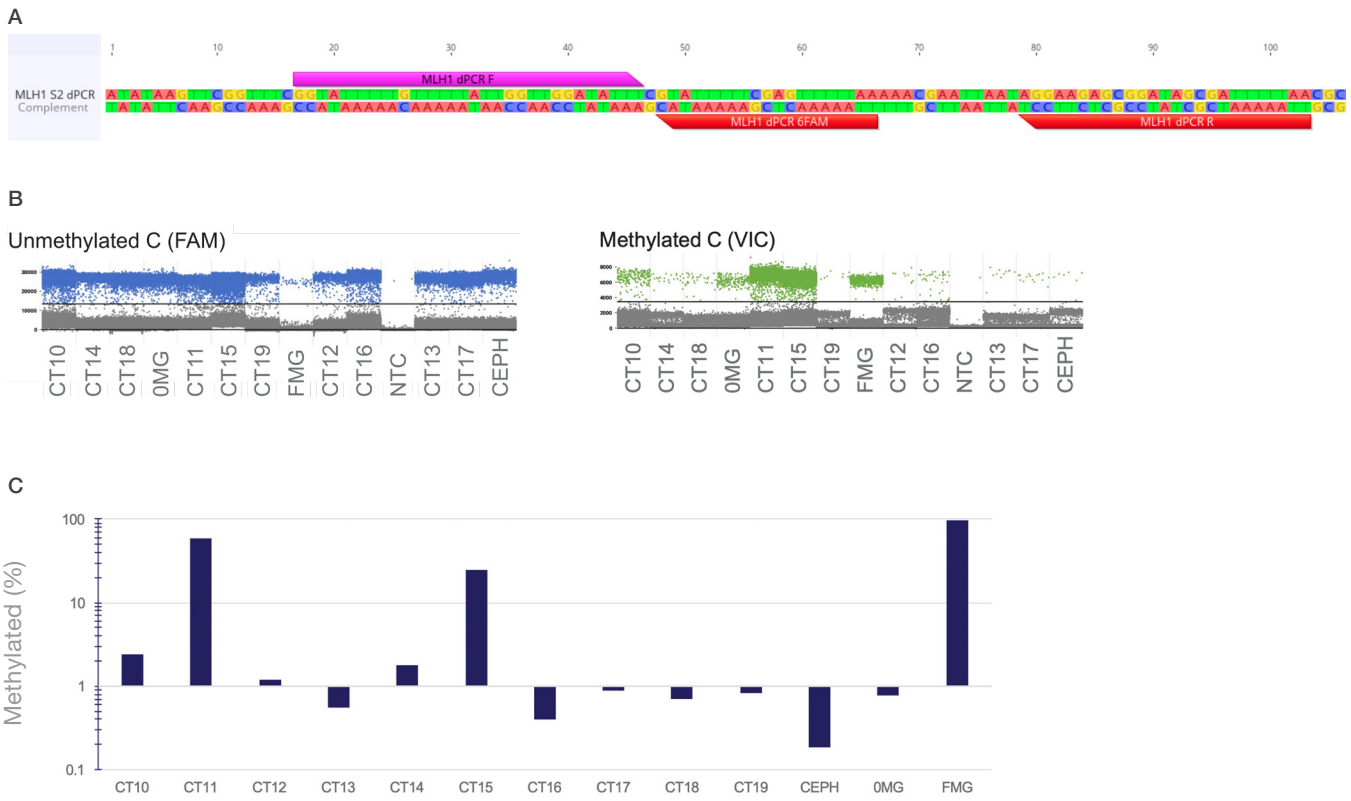


Figure 6. dPCR quantification of methylation of the *MLH1* promoter. (A) Positions of primers and probes used for this assay. Note that the target is based on a fully methylated, bisulfite-converted sequence. **(B)** Results from bisulfite-converted colon tumor DNA samples showing unmethylated (FAM fluorophore) and methylated (VIC fluorophore) populations of partitions. **(C)** Quantification of results, with percent methylated calculated as (number of VIC fluorophore–positive)/(number of VIC fluorophore–positive + number of FAM fluorophore–positive). Note that CT11 and CT15 had a large percentage methylated at the position queried by the assay (57.9% and 24.3%, respectively). 0MG: unmethylated control; FMG: fully methylated control; CEPH: normal human genomic DNA; NTC: no-template control.

Summary

Here we have illustrated how insights into biological mechanisms can be obtained by efficient multianalyte analysis of tumor samples. Out of ten colon tumor samples, we found two that had high levels of MSI. These same two tumor samples had reduced levels of transcripts encoding the mismatch repair enzyme MLH1, presumably due to significant methylation at the *MLH1* promoter that downregulates expression of the gene. The other tumor samples had stable microsatellites, average expression of *MLH1*, and no methylation of the *MLH1* locus. Notably, the automated extraction of nucleic acids from a single FFPE sample facilitated these analyses.

These results allowed us to segregate the samples into two groups: one in which methylation of the *MLH1* promoter caused downregulation of expression of the enzyme, leading to genomic instability, and one in which the MMR pathway was intact, and thus the cause of the tumor was dysregulation other than via genomic instability. The different classifications may suggest and facilitate research into different intervention strategies. While the work described here focuses on mismatch repair enzymes in colon cancer, in principle the methods could be adapted for other genes in other experimental paradigms.

Thermo Fisher Scientific provides multiple solutions for effective nucleic acid analysis. The KingFisher platforms use magnetic beads to extract DNA and RNA from the same FFPE lysate. The extensive catalog of predesigned TaqMan Assays can be used to build a multiplex gene expression panel; alternatively, custom assays can be designed if needed.

The QuantStudio family of qPCR instruments and the Absolute Q dPCR instrument have been optimized for sensitive detection of the fluorophores conjugated to TaqMan Assays in multiplex reactions. The TrueMark MSI Assay Kit facilitates analysis of genome instability in genomic DNA. The EpiJET Bisulfite Conversion Kit simplifies production of DNA for methylation analysis, while BigDye Terminator and BigDye XTerminator kits and SeqStudio 8 Flex CE instruments remain the trusted standards for Sanger sequencing analysis of DNA. The InnoviGene Suite of Sanger sequencing analysis tools helps enable the detection of mismatches, including those that indicate methylation differences. Each of these systems uses sample-to-answer workflows that are economical and simple and provide results in about a day. When used together, they can quickly generate important insights that can help guide further research.

ThermoFisher SCIENTIFIC

Ordering information

Description	Cat. No.
MagMAX FFPE DNA/RNA Ultra Kit	A31881
TrueMark MSI Assay	A45295
AmpliTaq Gold 360 Master Mix	4398876
ExoSAP-IT Express PCR Product Cleanup	75001.40.UL
BigDye Terminator v3.1 Cycle Sequencing Kit	4337454
BigDye XTerminator Purification Kit	4376486
Methyl Primer Express Software v1.0	4376041
EpiJET Bisulfite Conversion Kit	K1461
Custom TaqMan SNP Genotyping Assay, non-human	4332076
Absolute Q 1-Step RT-dPCR Master Mix	A55146
QuantStudio Absolute Q MAP16 Plate Kit	A52865
TaqMan Gene Expression Assays	thermofisher.com/taqman
TaqPath DuraPlex 1-Step RT-qPCR Master Mix, no ROX dye	A58671
Quant-iT dsDNA Assay Kit, High-Sensitivity (HS)	Q33120
Quant-iT RNA Assay Kit, Broad-Range (BR)	Q10213
KingFisher Apex Purification System, with 96 DW head	5400930
ProFlex PCR System, 96-well	4484075
SeqStudio 8 Flex Genetic Analyzer	A53627
InnoviGene Suite, sequencing software	A40001760
QuantStudio Absolute Q Digital PCR System, desktop	A52864
QuantStudio 7 Pro Real-Time PCR System, 96-well, 0.2 mL	A43162
Qubit 4 Fluorometer, with WiFi	Q33238

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