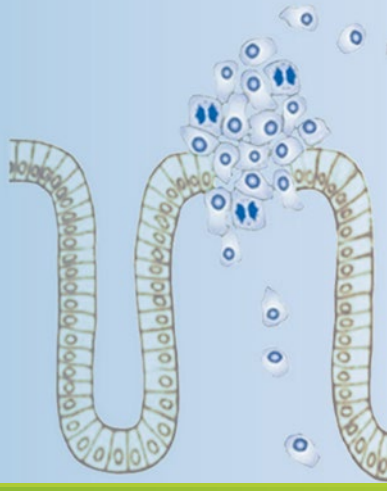
Methods in Molecular Biology 1765

Springer Protocols



Jean-François Beaulieu *Editor*

Colorectal Cancer

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For further volumes:
http://www.springernature.com/series/7651

Colorectal Cancer

Methods and Protocols

Edited by

Jean-François Beaulieu

Laboratory of Intestinal Physiopathology, Department of Anatomy and Cell Biology, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, QC, Canada

Humana Press

Editor
Jean-François Beaulieu
Laboratory of Intestinal Physiopathology
Department of Anatomy and Cell Biology
Faculty of Medicine and Health Science
Université de Sherbrooke
Sherbrooke, QC, Canada

ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-7764-2 ISBN 978-1-4939-7765-9 (eBook)
https://doi.org/10.1007/978-1-4939-7765-9

Library of Congress Control Number: 2018936904

Printed on acid-free paper

This Humana Press imprint is published by the registered company Springer Science+Business Media, LLC part of Springer Nature.
The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.

Dedication

I would like to thank my former mentors, Raymond Calvert, Daniel Ménard, Robert Tanguay, and Andrea Quaroni, who initiated me to the world of cell and molecular biology and its applications to the field of intestinal physiopathology.

Jean-François Beaulieu

Preface

Colorectal cancer is a complex disease and still one of the leading causes of cancer-related deaths worldwide. A better understanding of the molecular basis underlying colorectal cancer progression, from adenomas to metastasis, is crucial to establishing new noninvasive efficient tests for the detection of early lesions as well as for advancing the development of new therapeutic approaches for preventing advanced disease.

This edition of the book *Colorectal Cancer: Methods and Protocols* covers the most recent developments in the study of the mechanisms, diagnostics, screening methods, and therapeutics of colorectal cancer. The chapters were written by the scientists who established and or adapted these procedures and assays in the field of intestinal physiopathology. The first part (Chaps. 1–10) examines current approaches and techniques in use for the discovery of the molecular mechanisms underlying colorectal cancer development and progression. The second part (Chaps. 11–17) is devoted to the development of innovative tools for the diagnosis and detection of cancer lesions at early stages using cell and molecular biology approaches. Finally, the third part (Chaps. 18–22) presents recent advances in the treatment of colorectal tumors and approaches for the identification of novel therapeutic molecules. It is hoped that this book will be of great use to scientists in the field of colorectal cancer.

I would like to thank all the contributors for making this book possible.

Sherbrooke, QC, Canada *Jean-François Beaulieu*

Contents

Contributors

Farid E. Ahmed • *GEM Tox Labs, Institute for Research in Biotechnology, Greenville, NC, USA*

Nancy C. Ahmed • *GEM Tox Labs, Institute for Research in Biotechnology, Greenville, NC, USA*

Paula Álvarez-Chaver • *Proteomics Unit, Structural Determination, Proteomics and Genomics Service, CACTI, University of Vigo, Vigo, Spain*

Dominique Arsenault • *Department of Pediatrics, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, QC, Canada; Department of Immunology Division, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, QC, Canada*

Jean-François Beaulieu • *Laboratory of Intestinal Physiopathology, Department of Anatomy and Cell Biology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada*

Yannick D. Benoit • *Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada*

Susan H. Berry • *Gastrointestinal Research Group, School of Medicine, Medical Sciences and Nutrition, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK*

Rahul Bhome • *Cancer Research UK Centre, University of Southampton Cancer Sciences Division, Southampton University Hospital NHS Trust, Southampton, UK; University Department of Surgery, Southampton University Hospital NHS Trust, Southampton, UK*

François-Michel Boisvert • *Department of Anatomy and Cell Biology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada*

Chris Bonnerup • *Department of Physics, East Carolina University, Greenville, NC, USA*

Salah Boudjadi • *Cancer Molecular Pathology Section, Laboratory of Pathology, National Cancer Institute, Bethesda, MD, USA*

Danielle M. Burgenske • *Center for Cancer Cell Biology, Van Andel Research Institute, Grand Rapids, MI, USA*

Daniele Calistri • *Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Forlì-Cesena, Italy*

Julie C. Carrier • *Department of Medicine, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, QC, Canada*

Walid Chababi • *Department of Anatomy and Cell Biology, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, QC, Canada*

Anaïs Chauvin • *Department of Anatomy and Cell Biology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada*

Martine Chevillard-Briet • *Laboratoire de Biologie Cellulaire et Moléculaire du Contrôle de la Prolifération (LBCMCP), Centre de Biologie Intégrative (CBI), Université de Toulouse, CNRS, UPS, Toulouse, France*

Loretta De Chiara • *Department of Biochemistry, Genetics and Immunology, Faculty of Biology, University of Vigo, Vigo, Spain*

Fiona Clegg • *Gastrointestinal Research Group, School of Medicine, Medical Sciences and Nutrition, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK*

Katja Dahlgaard • *Department of Science and Environment, Roskilde University, Roskilde, Denmark*

Danijel Djukovic • *Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, WA, USA*
Christophe Dubois • *Aix Marseille University, INSERM UMR-S1076, C2VN, Marseille, France*
Claire M. Dubois • *Department of Pediatrics, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, QC, Canada; Department of Immunology Division, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, QC, Canada*
Katherine Emo • *Cancer Research UK Centre, University of Southampton Cancer Sciences Division, Southampton University Hospital NHS Trust, Southampton, UK*
Fabrice Escaffit • *Laboratoire de Biologie Cellulaire et Moléculaire du Contrôle de la Prolifé ration (LBCMCP), Centre de Biologie Intégrative (CBI), Université de Toulouse, CNRS, UPS, Toulouse, France*
Mostafa M. Gouda • *Department of Nutrition and Food Science, National Research Center, Cairo, Egypt*
Alex Gregorieff • *Lunenfeld-Tanenbaum Research Institute, Sinai Health System, Toronto, Canada; Department of Pathology, McGill University, Montreal, Canada; Cancer Research Program of the Research Institute of McGill University Health Centre, Montreal, Canada*
Sang-Bae Han • *College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea*
Richard Hansen • *Department of Paediatric Gastroenterology, Royal Hospital for Children, Glasgow, UK*
Elizabeth Herring • *Laboratory of Intestinal Physiopathology, Department of Anatomy and Cell Biology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada*
Calley L. Hirsch • *Lunenfeld-Tanenbaum Research Institute, Sinai Health System, Toronto, Canada*
Georgina L. Hold • *Gastrointestinal Research Group, School of Medicine, Medical Sciences and Nutrition, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK*
Sanne ten Hoorn • *Laboratory for Experimental Oncology and Radiobiology (LEXOR), Center for Experimental and Molecular Medicine (CEMM), Academic Medical Center and Cancer Center Amsterdam, Amsterdam, The Netherlands*
Steve Jean • *Department of Anatomy and Cell Biology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada*
Joan de Jong • *Laboratory for Experimental Oncology and Radiobiology (LEXOR), Center for Experimental and Molecular Medicine (CEMM), Academic Medical Center and Cancer Center Amsterdam, Amsterdam, The Netherlands*
Shigeru Kanaoka • *Department of Gastroenterology, Hamamatsu Medical Center, Hamamatsu, Japan*
Jong Soon Kang • *Korea Research Institute of Bioscience and Biotechnology, Cheongju, Chungbuk, Republic of Korea*
Lianne Koens • *Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands*
Annie Lauzier • *Department of Anatomy and Cell Biology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada*
Chang Woo Lee • *Korea Research Institute of Bioscience and Biotechnology, Cheongju, Chungbuk, Republic of Korea*

Myeong Youl Lee • *Korea Research Institute of Bioscience and Biotechnology, Cheongju, Chungbuk, Republic of Korea*
Suk Jong Lee • *College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea*
Patrick S. Lin • *Division of Hematology/Oncology, Department of Internal Medicine, University of California Davis Comprehensive Cancer Center, Sacramento, CA, USA*
Ian Y. Luk • *Olivia Newton-John Cancer Research Institute, Melbourne, VIC, Australia*
Jeffrey P. MacKeigan • *Center for Cancer Cell Biology, Van Andel Research Institute, Grand Rapids, MI, USA; College of Human Medicine, Michigan State University, Grand Rapids, MI, USA*
Giulia De Maio • *Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Forlì-Cesena, Italy*
John M. Mariadason • *Olivia Newton-John Cancer Research Institute, Melbourne, VIC, Australia*
Vicenta Soledad Martínez-Zorzano • *Department of Biochemistry, Genetics and Immunology, Faculty of Biology, University of Vigo, Vigo, Spain*
Stephen McManus • *Department of Anatomy and Cell Biology, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, QC, Canada*
Diane Mege • *Aix Marseille University, INSERM UMR-S1076, C2VN, Marseille, France*
Massimiliano Mellone • *Cancer Research UK Centre, University of Southampton Cancer Sciences Division, Southampton University Hospital NHS Trust, Southampton, UK*
Maura Menghi • *Diatech Pharmacogenetics, Jesi, Ancona, Italy*
Alex H. Mirnezami • *Cancer Research UK Centre, University of Southampton Cancer Sciences Division, Southampton University Hospital NHS Trust, Southampton, UK; University Department of Surgery, Southampton University Hospital NHS Trust, Southampton, UK*
David J. Monsma • *Center for Cancer Cell Biology, Van Andel Research Institute, Grand Rapids, MI, USA; College of Human Medicine, Michigan State University, Grand Rapids, MI, USA*
Jennifer K. Mooi • *Olivia Newton-John Cancer Research Institute, Melbourne, VIC, Australia*
Christina Oatway • *Lunenfeld-Tanenbaum Research Institute, Sinai Health System, Toronto, Canada*
Laurence Panicot-Dubois • *Aix Marseille University, INSERM UMR-S1076, C2VN, Marseille, France*
Daniel Raftery • *Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, WA, USA*
Claudia Rengucci • *Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Forlì-Cesena, Italy*
Caroline Saucier • *Department of Anatomy and Cell Biology, Faculty of Medicine and Health Science, Université de Sherbrooke, Sherbrooke, QC, Canada*
A. Emre Sayan • *Cancer Research UK Centre, University of Southampton Cancer Sciences Division, Southampton University Hospital NHS Trust, Southampton, UK*
Thomas J. Semrad • *Division of Hematology/Oncology, Department of Internal Medicine, University of California Davis Comprehensive Cancer Center, Sacramento, CA, USA; Gene Upshaw Memorial Tahoe Forest Cancer Center, Truckee, CA, USA*
Gareth J. Thomas • *Cancer Research UK Centre, University of Southampton Cancer Sciences Division, Southampton University Hospital NHS Trust, Southampton, UK*

Eric Tremblay • *Laboratory of Intestinal Physiopathology, Department of Anatomy and Cell Biology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, QC, Canada*

Anne Trinh • *Li Ka Shing Centre, Cancer Research UK Cambridge Institute, University of Cambridge, Cambridge, UK; Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA*

Jesper Thorvald Troelsen • *Department of Science and Environment, Roskilde University, Roskilde, Denmark*

Pierre H. Vachon • *Département d'anatomie et de biologie cellulaire, Faculté de médecine et des sciences de la santé, Université de Sherbrooke, Sherbrooke, QC, Canada*

Louis Vermeulen • *Laboratory for Experimental Oncology and Radiobiology (LEXOR), Center for Experimental and Molecular Medicine (CEMM), Academic Medical Center and Cancer Center Amsterdam, Amsterdam, The Netherlands*

Paul W. Vos • *Department of Biostatistics, East Carolina University, Greenville, NC, USA*

Yeo Dae Yoon • *Korea Research Institute of Bioscience and Biotechnology, Cheongju, Chungbuk, Republic of Korea*

Jiangjiang Zhang • *Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, WA, USA*

Part I

Molecular Basis of Colorectal Cancer

Chapter 1

Cell Line Models of Molecular Subtypes of Colorectal Cancer

Jennifer K. Mooi, Ian Y. Luk, and John M. Mariadason

Abstract

Colorectal cancer (CRC) is a genetically diverse disease necessitating the need for well-characterized and reproducible models to enable its accurate investigation. Recent genomic analyses have confirmed that CRC cell lines accurately retain the key genetic alterations and represent the major molecular subtypes of primary CRC, underscoring their value as powerful preclinical models. In this chapter we detail the important issues to consider when using CRC cell lines, the techniques used for their appropriate molecular classification, and the methods by which they are cultured in vitro and as subcutaneous xenografts in immune-compromised mice. A panel of commonly available CRC cell lines that have been characterized for key molecular subtypes is also provided as a resource for investigators to select appropriate models to address specific research questions.

Key words Colorectal cancer cells, Molecular subtype, DNA profiling, Chromosomal instability, Microsatellite instability, CpG island methylator phenotype, Signaling pathways

1 Introduction

Colorectal cancers (CRC) are broadly classified according to the global genomic or epigenomic aberrations they harbor. Approximately 80% of sporadic CRCs have chromosomal instability (CIN) characterized by whole or partial chromosomal duplication or deletion, while the remainder have microsatellite instability (MSI), underpinned by inactivation of DNA mismatch repair genes [1, 2]. In addition, approximately 20% of CRCs harbor the CpG island methylator phenotype (CIMP), characterized by increased rates of DNA promoter methylation [3]. CIMP is also highly associated with MSI CRC. In both CIN and MSI CRCs, the underlying genetic instability drives disease progression through the sequential deregulation of five key signaling pathways: Wnt, MAPK, p53, TGFβ, and PI3K [4–6].

Jennifer K. Mooi and Ian Y. Luk contributed equally to this work.

Jean-François Beaulieu (ed.), *Colorectal Cancer: Methods and Protocols*, Methods in Molecular Biology, vol. 1765,
https://doi.org/10.1007/978-1-4939-7765-9_1,

The molecular subtype and mutation spectrum of CRCs impact significantly on patient prognosis and response to therapy. For example, MSI tumors have significantly lower rates of relapse after early stage resection, while in the metastatic setting, tumors harboring *KRAS* mutations are largely refractory to EGFR-targeted therapies [7, 8].

More recently, global gene expression profiling has been used to classify CRCs into one of four consensus molecular subtypes (CMS 1–4) [9]. Prognostic associations of CMS have been described, and associations with treatment are still being investigated. The established molecular classifiers (CIN, MSI, and CIMP) are enriched within particular CMS groups, such as CMS1, which is enriched for MSI and CIMP CRCs. Comparatively, the CMS2 subgroup is enriched for CRCs with high expression of Wnt target genes, CMS3 is enriched for *KRAS* mutant tumors, and CMS4 is enriched for tumors with high stromal content and TGFβ signaling.

Comprehensive characterization of the genetic aberrations, or determining the CMS of a CRC cell line, requires high-throughput genomic approaches and gene expression profiling respectively. These specialized methods are beyond the scope of this chapter. Instead, this chapter will describe the key quality control measures to consider prior to using CRC cell lines, specifically cell line authentication and mycoplasma screening. The chapter then details the methods for MSI and CIMP classification which can be used by investigators needing to characterize new CRC cell lines, patient-derived xenografts, or to confirm the molecular subtypes of existing cell lines. Finally we detail methods for culturing CRC cell lines in vitro and as subcutaneous xenografts in immune-compromised mice.

As a resource, we provide a list of commonly used and well characterized CRC cell lines to aid investigators in selecting appropriate models to address specific research questions (Appendix 1).

2 Materials

2.1 Short Tandem Repeat (STR) Profiling

1. Reagents for isolation of genomic DNA from cells (including various commercially available kits) (*see* **Note 1**).
2. STR profiling Kit. Various commercial kits are available, such as the GenePrint™10 system (Promega, USA).

2.2 Mycoplasma Testing

For PCR and agarose gel electrophoresis

1. *Taq* DNA Polymerase PCR kit (*Taq* Polymerase, dNTP mixture, $MgCl_2$ and PCR buffer).
2. Sense primer GPO-3 (10 μM) (5′-GGGAGCAAACAGGATTAGATACCCT-3′) [10].
3. Antisense primer MGSO (10 μM) (5′-TGCACCATCTGTCACTCTGTTAACCTC-3′) [10].

4. Agarose.
5. 50× Tris-Acetate-EDTA (TAE) Buffer: Prepare by adding 242 g Tris base to 57.1 mL glacial acetic acid, and 100 mL of 500 mM EDTA (pH 8.0) solution, and bring the volume up to 1 L.
6. SYBR Safe DNA Gel Stain (Life Technologies).
7. 1 kb Plus DNA Ladder.

2.3 Cell Culture

All materials need to be sterile and used only in a tissue culture flow hood in order to prevent biological contamination.

1. Base Culture Medium: A number of different culture media are available. Almost all CRC cell lines grow well in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM F-12), RPMI-1640, DMEM (1×) or MEM (Gibco, USA). Occasionally, a cell line may require a specific culture medium. It is therefore best to check with the cell line supplier regarding the most appropriate culture medium for each cell line. When comparing effects across multiple cell lines it is best to use a single culture medium. We have used DMEM-F12 when performing experiments in which drug effects on multiple CRC cell lines are compared [11–13].
2. To prepare complete culture medium, supplement 500 mL of base cell culture medium with the following: 50 mL of fetal bovine serum (FBS) (various suppliers), 5 mL of GlutaMAX 100× supplement (Gibco, USA), 5 mL of Penicillin–Streptomycin antibiotic (10,000 units/mL), and 5 mL of HEPES buffer 1M. (When confident with aseptic technique, the use of antibiotics may be omitted.)
3. Trypsin solution (various suppliers, e.g., TrypLE Express Enzyme, Gibco, USA).
4. Dulbecco's phosphate-buffered saline (DPBS).

2.4 Formalin-Fixed Paraffin-Embedded (FFPE) Cell Blocks

1. Plasma, undiluted (bovine, goat, rabbit, or human) (sources—*see* **Note 2**).
2. Thrombin, topical (bovine), 1000 IU/mL concentration.
3. Long thin wooden stick (e.g., single ended cotton bud on a 15 cm wooden stick).
4. 10% neutral buffered formalin.
5. 80% ethanol.
6. Dulbecco's phosphate-buffered saline (DPBS).

2.5 Xenografts

1. Corning Matrigel matrix.
2. Complete culture medium (*see* Subheading point 2.3.2), without antibiotics.
3. Trypan Blue.

2.6 MSI Analysis

1. For Immunohistochemistry (IHC), primary antibodies for human MLH1, PMS2, MSH2, MSH6 and the relevant secondary antibodies and detection system reagents (*see* **Note 3**).
2. Reagents for isolation of genomic DNA from cells (including various commercially available kits) (*see* **Note 1**).
3. Reagents for PCR and fragment analysis of MSI markers, available as MSI Analysis System Version 1.2 (Promega, USA).

2.7 CIMP Analysis

1. Reagents for isolation of genomic DNA from cells (including various commercially available kits) (*see* **Note 1**).
2. Reagents for bisulfite treatment of gDNA (available in various commercially available kits such as the EpiTect Bisulfite kit, Qiagen).
3. Probes and primers as per Table 1.
4. Methylated DNA reference.
5. Nuclease-free water.
6. Reagents for methylation-specific PCR: Taq DNA polymerase, dNTP mix (dATP, dCTP, dGTP, dUTP), $MgCl_2$, Tween 20, gelatin. These reagents are also available as components of the Epitech MethyLight PCR kit, Qiagen).

3 Methods

3.1 Short Tandem Repeat (STR) Profiling of CRC Cell Lines

Prior to commencing work with cell lines it is important to authenticate that the cell line being used is indeed the correct line. Misidentification of cell lines can occur through human error, by simple mislabeling, or cross-contamination of two or more independent lines.

The authenticity of a cell line can be determined by STR profiling, and is now a requirement of many journals prior to publication. The importance of cell line authentication is underscored by the revelation that some CRC cell lines (such as HT-29 and WiDR) that were previously considered independent are in fact the same cell line.

STR's are short sequences of DNA, typically 2–5 base pairs in length, that are repeated multiple times in a row on a DNA strand [14]. STR profiling exploits the polymorphic (variable) nature of specific STRs located throughout the human genome to generate a unique genetic profile for a given sample with a very low probability of producing a random match.

3.1.1 Genomic DNA Isolation

1. Various methods can be used to isolate gDNA from colon cancer cell lines including well-optimized and validated protocols in the form of commercially available kits. We recommend isolating gDNA using a commercially available kit, as per the manufacturer's instructions (*see* **Note 1**).

Table 1
Probes and primers for MethyLight CIMP classification assay

Gene	Forward primer	Reverse primer	Probe
CACNA1G	TTTTTTCGTTTCGCGTTTAGGT	CTCGAAACGACTTCGCCG	6FAM-AAATAACGCCGAATCCGACAACCGA-BHQ
IGF2	GAGCGGTTTCGGTGTCGTTA	CCAACTCGATTTAAACCGACG	6FAM-CCCTCTACCGTCGCGAACCCGA-BHQ
NEUROG1	CGTGTAGCGTTCGGGTATTTGTA	CGATAATTACGAACACACTCCGAAT	6FAM-CGATAACGACCTCCCGCGAACATAAA-BHQ-1
RUNX3	CGTTCGATGGTGGACGTGT	GACGAACAACGTCTTATTACAACGC	6FAM-CGCACGAACTCGCCTACGTAATCCG-BHQ-1
SOCS1	GCGTCGAGTTCGTGGGTATTT	CCGAAACCATCTTCACGCTAA	6FAM-ACAATTCCGCTAACGACTATCGCGCA-BHQ-1
ALU[a]	GGTTAGGTATAGTGGTTTATAT TTGTAATTTTAGTA	ATTAACTAAACTAATCTTAAACTCCT AACCTCA	6FAM-CCTACCTTAACCTCCC-MGBNFQ

[a]The *ALU* reaction is a non-methylation-dependent reaction used as a control for normalizing concentrations of input bisulfite-converted DNA

2. From cultured cell lines, collect up to 10^6 cells in a microcentrifuge tube.
3. Centrifuge the cell suspension at 300 × *g* for 5 min at 4 °C to form a cell pellet and aspirate the supernatant.
4. Proceed as per manufacturer's instructions.

3.1.2 STR Profiling

1. Perform STR profiling as per the manufacturer's instructions using the reagents provided in the STR profiling kit. We have used the GenePrint™10 System (Promega, USA) which provides reagents for the coamplification of ten human loci (*TH01, TPOX, vWA, Amelogenin, CSF1PO, D16S539, D7S820, D13S317, D5S818, and D21S11*). Collective assessment of these loci prov ides a genetic profile with a very low random match probability (estimated 1 in 10^9).
2. Detection of the amplified fragments can be performed using a number of genetic analyzers, including the Applied Biosystems 3500 or 3500 × L Genetic Analyzer with POP-4™ Polymer (*see* **Note 4**).

3.1.3 Interpreting STR Results

1. An example electropherogram of STR profiling performed on the SW480 CRC cell line is provided in Fig. 1. Examination of the *TH01* locus in the top panel reveals a single peak and the number eight. This means that SW480 is homozygous at both alleles of *TH01* and has eight repeats on each chromosome. In comparison, the *CSF1PO* locus in the middle panel has two peaks, one labeled 13 and one labeled 14. This corresponds to a heterozygous locus in which one allele has 13 repeats and the other has 14.
2. To confirm authenticity, a minimum 80% match across the panel of allele markers is recommended. Reference STR profiles of CRC cell lines can be obtained from databases such as ExPASy Bioinformatics Resource Portal Cellosaurus Database (http://web.expasy.org/cellosaurus) or the relevant source institutions. Reference STR profiles of some commonly used CRC cell lines are provided in Appendix 1.

3.2 Mycoplasma Testing of Cell Lines by PCR

A further important consideration when using cell lines is to ensure they are free of biological contamination. This includes contamination with bacteria, molds, yeasts, and mycoplasma. While bacterial, mold, and yeast contaminations can be detected by visual inspection of the culture medium, the smaller size of mycoplasmas make them much harder to detect. Mycoplasmas are small bacterial organisms (0.15–0.3 μm) that lack a cell wall and have a flexible membrane that allows them to adhere to the host cell surface [15]. They are able to bind and invade into a host eukaryotic cell using specialised tip organelles. Once inside the cell, they multiply

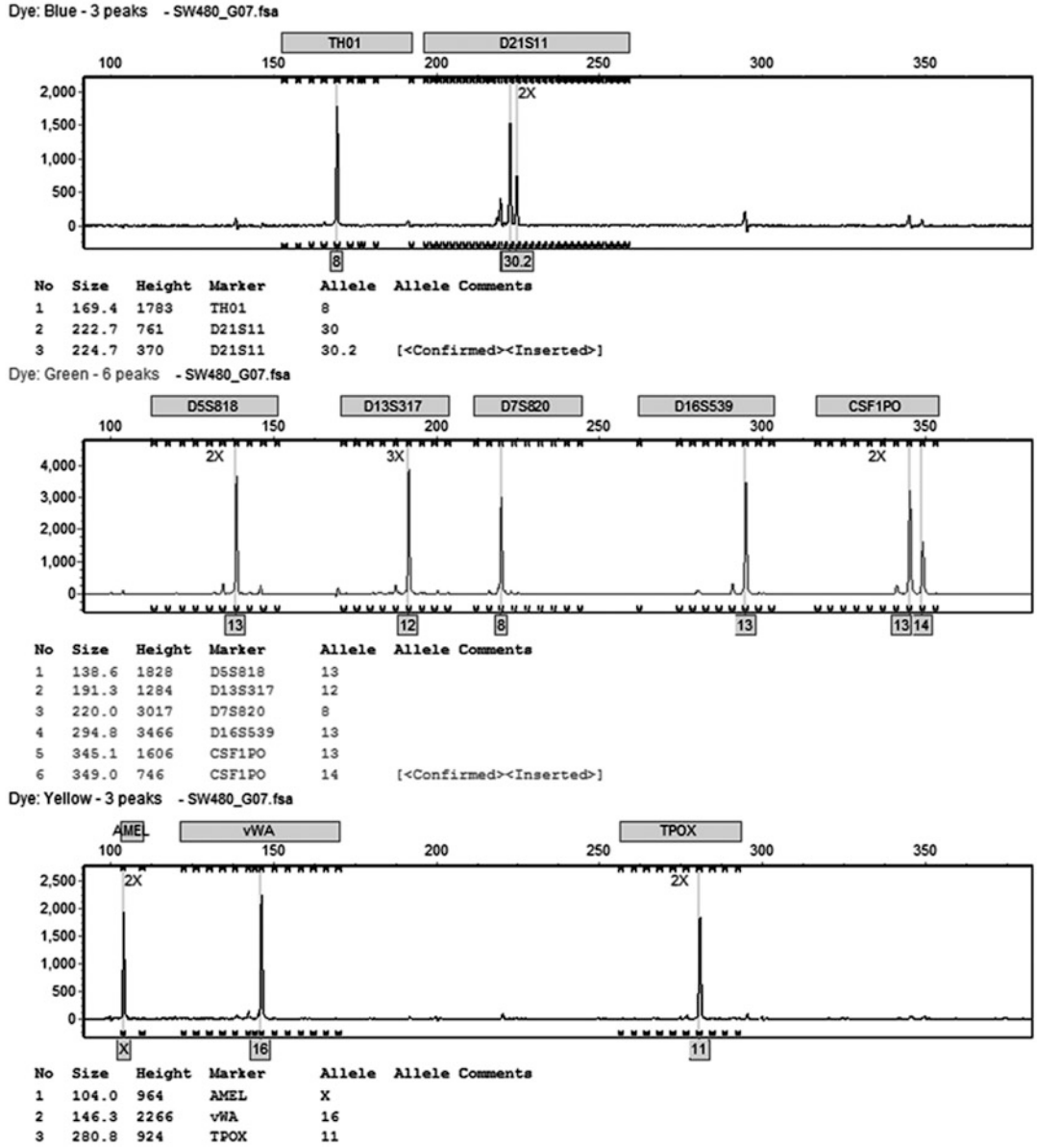


Fig. 1 STR profiling electropherogram of the SW480 colorectal cancer cell line

and compete with the host for biosynthetic precursors and nutrients and can alter cell physiology. Mycoplasma contamination, which may occur during routine cell culture, can be detected using a number of methods including fluorescent staining (e.g., Hoechst staining), ELISA, immunostaining, autoradiography, microbiological assays, or PCR. It is strongly recommended that cell cultures are periodically tested for this contamination. Here we describe a method for detecting mycoplasma contamination by PCR.

Table 2
Reaction mastermix for mycoplasma testing

Component	Volume (μL)
Supernatant (from cell line)	1
10× PCR buffer	2.5
dNTPs (10 mM)	0.5
$MgCl_2$ (50 mM)	0.75
GPO-3 primer (10 μM)	0.5
MGSO primer (10 μM)	0.5
Taq DNA polymerase	0.1
Nuclease free H_2O	19.2
Total reaction volume	*25*

1. Culture colorectal cancer cell line for 48–72 h in regular culture medium without antibiotics. Do not perform any media changes during this period (*see* **Note 5**).
2. At the end of the culture period, collect 1 mL of the culture medium in a microcentrifuge tube.
3. Centrifuge the culture medium at 300 × *g* for 5 min at 4 °C to pellet cell debris. Transfer the supernatant into a fresh microcentrifuge tube.
4. Prepare PCR master mix using the components listed in Table 2.
5. Add 24 μL of PCR mastermix and 1 μL of cell culture supernatant to each PCR tube for a 25 μL reaction (*see* **Note 6**).
6. Mix the reaction in the tubes and spin briefly.
7. Perform PCR using the cycling conditions listed in Table 3.
8. Prepare 2 L of 1× TAE buffer by adding 40 mL of 50× TAE to 1.6 L of MilliQ water.
9. Weigh out 2 g of agarose and add to a conical flask with 100 mL of 1× TAE to create a 2% agarose gel.
10. To dissolve the agarose, heat the mixture in a microwave until completely dissolved (*see* **Note 7**).
11. Add 10 μL of SYBR Safe DNA gel stain to the agarose mix and pour the gel mixture into a sealed gel moulding tank.
12. Place a well-moulding comb into the gel mix appropriate for the number of samples, and allow the gel to set for approximately 30 min.

Table 3
Cycling conditions for mycoplasma testing

Step	Temperature (°C)	Time
Denaturing, 1 cycle	95	5 min
Cycling, 36 cycles		
Denature	94	30 s
Anneal	55	30 s
Elongation	72	1 min
Elongation, 1 cycle	72	10 min
Hold, 1 cycle	4	∞

13. Once set, carefully remove the well-comb from the gel and place the gel into the electrophoresis tank.
14. Pour 1× TAE buffer to completely submerge the gel.
15. Run 10 μL of the PCR-amplified product on the 2% agarose gel at 100 V for 1.5 h. Run an appropriate volume of 1 kb DNA ladder in one well.
16. Visualize and image the gel under UV light. Detection of a 270 bp PCR product indicates a cell line is positive for mycoplasma contamination.

3.3 Growth and Subculture of CRC cell Lines In Vitro

CRC cell lines are maintained and passaged using standard cell culture techniques. The majority of CRC cell lines grow as adherent monolayers (e.g., HCT116, SW948, and SW1116), while some grow as semiadherent cultures (e.g., Colo-201, Colo-205, and Colo-320) or in suspension (e.g., SNU175 and SNUC1). It is important to be aware of the in vitro growth characteristics of the cell line you are working with to carry out the appropriate cell culture technique. The following is a general protocol for the subculture of CRC cell lines.

Carry out all procedures using proper aseptic technique in a laminar flow cabinet. Pre-warm the culture medium, trypsin, and DPBS to 37 °C prior to commencing.

1. For adherent cell lines, aspirate the old culture medium when cells reach approximately 80% confluence. For semiadherent cell lines, collect the medium containing the nonadherent population with a pipet and transfer to a fresh falcon tube.
2. Add 5 mL of pre-warmed sterile PBS per 25 cm^2 of culture flask to wash the adherent cells on the flask and to remove any residual culture medium.
3. Aspirate the PBS.

4. Add 1 mL of trypsin per 25 cm^2 of culture flask and incubate at 37 °C for approximately 5 min with intermittent tapping of the side of the flask to induce cell detachment (*see* **Note 8**).
5. Once the cells have detached, quickly neutralize the trypsin by adding an equal volume of fresh FBS-containing culture medium to the cell suspension, gently resuspend the cells by pipeting and transfer to a fresh tube. For semiadherent cell lines, add the detached adherent cell population to the non-adherent population collected previously.
6. Centrifuge at 300 × *g* at 4 °C for 5 min to pellet the cells.
7. Remove the supernatant and resuspend the cell pellet in an appropriate volume of fresh medium.
8. At this point cell number can be quantified using either a haemocytometer or an automated cell counter (e.g., Bio-Rad TC10), and an appropriate number of cells seeded into flasks, plates, or dishes as required for experimentation. Trypan Blue staining can be used to enumerate dead versus viable cells (*see* **Note 9**).
9. For routine maintenance of the cell line, cells should be diluted 1:5 to 1:10 in fresh medium (depending on the growth characteristics of the cell line) and seeded into a new flask.
10. For suspension cell lines no detachment procedure is necessary. Simply carry out steps 6–9 above to passage cells or change culture medium.

3.4 Creation of Formalin-Fixed Paraffin-Embedded Cell Blocks from CRC Cell Lines Cultured In Vitro

Formalin-fixed paraffin-embedded (FFPE) blocks of CRC cell lines grown in vitro can be created for subsequent histopathological, immunohistochemical and fluorescence-based analyses.

1. Culture the CRC cell line using the in vitro culture technique described in Subheading 3.3 to approximately 80% confluence in a 175 cm^2 tissue culture flask, to obtain approximately 10^7cells (*see* **Note 10**).
2. Remove the culture medium, and wash the cells in cold DPBS. Aspirate the DPBS.
3. Add 10 mL of cold DPBS and remove the monolayer of cells from the culture flask by scraping gently with small angled rubber scraper.
4. Transfer the cell suspension into a 15 mL tube.
5. Centrifuge the cell suspension at 4 °C, at 300 × *g*, for 5 min.
6. Remove the supernatant, and resuspend the cell pellet in 10 mL of fresh DPBS and centrifuge again as above.
7. Remove the supernatant to leave the cell pellet at the bottom of the tube. Add 150 μL of plasma and 150 μL of thrombin (*see* **Note 11**) onto the pellet and use a thin wooden stick to gently

resuspend. Leave the end of the wooden stick in the centre of pellet and allow the clot to form around it by incubating for 5–10 min at room temperature.

8. Once the clot has formed, gently lift the clot out of the tube using the wooden stick. The cell pellet should have clotted and be attached to the end of the stick.
9. Gently dab the cell clot on a piece of filter paper to remove any excess liquid.
10. Use a scalpel blade to transfer the cell clot off the end of the stick and into a histology cartridge, lined with porous membranes to protect the cell clot (*see* **Note 12**). Fix in 10% formalin for 24–48 h (*see* **Note 13**).
11. Process and embed the sample into a formalin block using standard histology procedures.

3.5 Growth of CRC Cell Lines as Xenografts

1. Grow CRC cells to approximately 80% confluence in a 175 cm^2 tissue culture flask and detach by trypsinization as described in Subheading 3.3.
2. Determine cell number as described in Subheading 3.3 and calculate the volume of cell suspension required for 2×10^6 viable cells (*see* **Note 9**).
3. Transfer the volume of cell suspension containing 2×10^6 cells into a sterile microcentrifuge tube.
4. Centrifuge cells at 300 × *g* for 5 min at 4 °C to form a cell pellet.
5. Resuspend the cell pellet in 75 μL of antibiotic-free complete culture medium.
6. To the resuspended cells, add 75 μL of matrigel matrix making sure the mixture is homogenous to create a cell-matrigel mixture (*see* **Note 14**).
7. Inject the cell-matrigel mixture subcutaneously into the flanks of Balb/*c nu/nu* immunodeficient mice (*see* **Notes 15** and **16**).
8. Monitor the animals for any visible signs of distress as per Institute Review Board/Animal Ethics Committee guidelines.
9. Monitor tumor growth at regular intervals (every day or every second day) by measurement of the length and the width of the tumor and computation of tumor volume according to the formula $(4/3 \times \text{PI} ((\text{tumor width} + \text{tumor length})/4)^3)$. Once tumors have reached the maximum size approved by the Institute Review Board/Animal Ethics Committee, animals should be humanely euthanized. If required, tumors can be extracted and either snap frozen or fixed in 10% buffered formalin for further analysis.

3.6 Microsatellite Instability (MSI) Analysis in CRC Cell Lines

The MSI status of a cell line can be determined in multiple ways. In this chapter we will describe two commonly used methods:

1. Detecting loss of expression of the mismatch repair proteins (MLH1, PMS2, MSH2 and MSH6) by immunohistochemistry (IHC).
2. Assessment of alteration in length of specific microsatellite sequences in the genome by PCR and fragment analysis (commonly referred to as Microsatellite Testing) (*see* **Note 17**).

3.6.1 Loss of Expression of Mismatch Repair Proteins by Immunohistochemistry

Microsatellite instability is caused by loss of function of one or more of the mismatch repair proteins MLH1, PMS2, MSH2 and MSH6. The MSI status of a CRC cell line can be determined by immunohistochemical assessment of expression of all four MMR proteins, or if resources are limited, just MLH1 and MSH2 (*see* **Note 18**). This can be performed on FFPE sections of colon cancer cell lines prepared as cell blocks (per Subheading 3.4) or grown as xenografts.

1. Cut the required number of 4 μm-thick sections of a CRC cell line from FFPE blocks, and mount on electrostatic glass slides.
2. Carry out standard IHC staining for each MMR protein (*see* **Note 18**).
3. Optimal IHC staining protocols including antigen retrieval method, primary antibody concentration, incubation times and secondary antibody-detection system will need to be optimized by the end-user for each primary antibody used.
4. Interpretation of IHC staining results: staining is interpreted as either the presence or absence of nuclear staining of each MMR protein in tumor cells. Loss of any one of the four MMR proteins is considered evidence for microsatellite instability (MMR-deficient), while positive staining in all four proteins (or two if only MLH1 and MSH2 are tested) is considered evidence of a microsatellite stable (MMR-proficient) cell line (*see* **Note 19**) (Fig. 2).

3.6.2 Microsatellite Testing

Loss of MMR proteins result in failure to correct DNA replication errors. These errors occur at high frequency in repetitive DNA sequences (microsatellites) due to "slippage" of the polymerase in these regions, hence the term microsatellite instability. A number of nonpolymorphic microsatellites have been identified in the human genome and detection of alterations in the length of these microsatellites by their PCR amplification followed by fragment analysis provides a reliable means of identifying microsatellite instability in CRC cell lines or tumors.

1. Isolate genomic DNA from CRC cell lines. Various methods can be used to isolate gDNA from CRC cell lines including

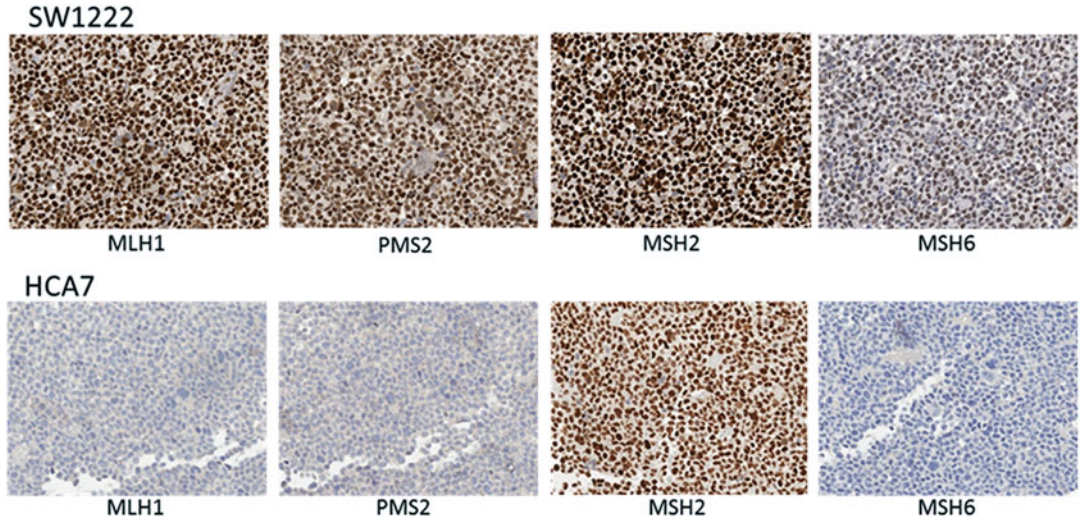


Fig. 2 IHC for MMR proteins on SW1222 and HCA7 cells (in cell block sections). In SW1222, all four MMR proteins are expressed indicating microsatellite stable status. In HCA7, expression of MLH1, PMS2, and MSH6 is lost, indicating microsatellite instability (*see* **Note 19**)

well-optimized and validated protocols in the form of commercially available kits. We recommend isolating gDNA using a commercially available kit, as per the manufacturer's instructions (*see* **Note 1**).

2. PCR amplification of a panel of microsatellite markers. The commercially available kit, MSI Analysis System Version 1.2 (Promega, USA) [16] provides all of the reagents required for this analysis. The kit contains reagents to perform a multiplex PCR assay of five validated mononucleotide markers (*BAT-25*, *BAT-26*, *NR-21*, *NR-24*, and *MONO-27*) (*see* **Note 20**). The MSI Analysis System allows for multiplexing of PCR products of the panel of markers in a single sample using probes conjugated to individual fluorophores. Perform PCR amplification of cell line gDNA using the reagents provided according to the manufacturer's instructions. We have provided additional guidance from our experience of using this protocol in **Notes 21–23**. The user manual for the MSI Analysis System Version 1.2 can be found at www.promega.com.

3. Detection of amplified PCR products (fragment analysis). This step requires resolution of the fluorophore-labeled PCR products by capillary electrophoresis and detection of amplified fragments of various lengths using an appropriate instrument. The detailed instrument-specific protocol for spectral calibration required for recognition of the multiplexed fluorescent fragments is available in the User Manual for the MSI Analysis System Version 1.2 (www.promega.com). We have used the

Applied Biosystems 3730 DNA Analyzer, with POP-7™ polymer and a 36 cm capillary array for resolution and detection of PCR products generated using the Promega MSI Analysis System. *See* **Notes 24** and **25** for additional guidance regarding this step.

4. Interpretation of microsatellite instability. Inspection of electropherogram peaks generated from the fragment analysis is required to interpret microsatellite instability. For each locus, any shift in position of the peak allele compared to the position in the reference electropherograms provided in the manual is regarded as instability. *Instability at two or more of the five markers is diagnostic of MS*I (*see* **Note 26**). Analysis of electropherograms from samples of known MSI status can be helpful in the interpretation of results (*see* **Note 23**).

3.7 Assessment of CpG Island Methylator Phenotype (CIMP) in CRC Cell Lines

There are a number of methods available for assessing the CIMP status of CRC tumors and cell lines [17–21]. All of these methods involve bisulfite treatment of gDNA which converts unmethylated cytosine residues to uracil, while methylated cytosine remains unaffected. Thus bisulfite-treated gDNA reveals methylation-specific information on DNA sequences which can be analyzed by a number of downstream assays following PCR amplification. In addition, the methylation status of different panels of marker genes can be used to assess CIMP status [3, 22–24] (*see* **Note 27**). Here, we describe a method that can be used for assessing CIMP status of CRC cell line samples using the MethyLight assay, a multiplex PCR assay which simultaneously detects the methylation status of five CIMP marker genes (*CACNA1G, IGF2, NEUROG1, RUNX3,* and *SOCS1*) as described by Weisenberger *et al.* [17, 18].

3.7.1 Isolation of Genomic DNA from CRC Cell Lines

Various methods can be used to isolate gDNA from CRC cell lines including well-optimized and validated protocols in the form of commercially available kits. We recommend isolating gDNA using a commercially available kit, as per the manufacturer's instructions (*see* **Note 1**).

3.7.2 Bisulfite Conversion of Extracted gDNA

Various commercially available kits can be used for this step, such as the EpiTect Bisulfite kit (Qiagen). We recommend performing bisulfite conversion using the reagents provided in one of these commercially available kits by following the manufacturer's instructions.

3.7.3 MethyLight PCR Assay

The MethyLight assay uses "methylation-specific" primers to only amplify loci that are methylated. Primers are specifically designed to be complementary only to unconverted (methylated) cytosines on bisulfite-treated gDNA. A methylation-prone CpG dinucleotide is also usually placed at the 3′-end of the primer to improve sensitivity.

Table 4
Reaction mastermix for methylation-specific PCR

Component	Final concentration
$MgCl_2$	3.5 mM
Tween-20	0.01%
Gelatin	0.05%
Taq polymerase	0.1 U
Primers (forward and reverse)	300 nM each
Probes	100 nM each
dNTPs	200 μM
Template bisulfite-converted DNA[a]	≤100 ng/reaction
Nuclease-free H_2O	[b]

Include a reaction for methylated DNA reference (positive control)
[a]Template DNA concentrations are adjusted and optimized by end-user
[b]Make up to final reaction volume

This method is highly sensitive and specific for interrogating CpG-rich loci with high methylation density such as the CIMP-high markers listed above.

1. Add the components for the PCR reaction mix to the desired final reaction volume (25–50 μL) as per Table 4.
2. Probes and primer sequences for the CIMP MethyLight assay have been previously validated and are listed in Table 1 (Materials) [22]. The first five markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*) form the panel for CIMP classification. The *ALU* marker is a non-methylation-dependent reaction used as a control for normalizing the concentrations of input bisulfite-converted DNA.
3. Include a fully methylated DNA reference sample in the PCR reactions to serve as a positive control and for computing the percentage methylation of your sample (*see* **Note 28**). A negative control reaction (nuclease-free water) should also be included.
4. Perform PCR using the cycling conditions provided in Table 5.

3.7.4 Data Analysis and Interpretation

1. The percent methylation rate (PMR) of each locus is calculated as follows:

$$\mathrm{PMR} = 100 \times (\mathrm{Ct\ CIMP\ marker}/\mathrm{Ct\ ALU})_{\mathrm{sample}}/(\mathrm{Ct\ CIMP\ marker}/\mathrm{Ct\ ALU})_{\mathrm{methylated\ DNA\ reference}}.$$

Table 5
Cycling conditions for methylation PCR analysis

Step	Temperature	Time
Initial PCR activation	95 °C	5 min
2-step cycling:		
Denaturation	95 °C	15 s
Annealing/extension	60 °C	60 s
Number of cycles	40–50[a]	

[a]No. of cycles depends on amount of template DNA, user to optimize

Ct: cycle threshold.

Sample: test sample.

Methylated DNA reference (*see* **Note 28**).

CIMP marker: *CACNA1G, IGF2, NEUROG1, RUNX3*, or *SOCS1*.

2. A CIMP marker is considered methylated if the PMR is $\geq$10. Cell line samples with methylation in $\geq$3 of these five markers are classified as CIMP-high, those with two or fewer methylated markers are considered CIMP-low, and those unmethylated at all five markers are considered CIMP-negative.

4 Notes

1. A number of kits suitable for extraction of genomic DNA (gDNA) from cell lines can be used. It is also possible to isolate gDNA from cell line xenografts that have been freshly frozen or formalin-fixed and paraffin-embedded (FFPE). In the case of extracting gDNA from FFPE material, commercially available kits specific for this purpose need to be used. We recommend isolating gDNA from in vitro cultured cells whenever possible as this produces the highest quality of gDNA.
2. We use human plasma obtained via a hospital blood bank. However, plasma from any animal species would be suitable and can be purchased from a number of biomedical research suppliers. Make sure plasma, not serum, is used. The coagulation factors in plasma are required to interact with thrombin to form the cell clot.
3. Primary antibody targeting *human* MMR proteins should be used.
4. POP-4 polymer is a separation matrix for performing DNA sequencing and fragment analysis applications.

5. Cells need to be cultured for this time period in order for any mycoplasma contamination that may be present to reach detectable levels in the culture medium. During this period, we recommend culturing cells in antibiotic-free medium to avoid masking of any low level contamination.
6. If possible include 1 μL of supernatant from a known mycoplasma-positive sample as a positive control and 1 μL of H_2O as a negative control.
7. Usually 1 min of heating is required per 100 mL of agarose/TAE mixture. Microwave in intervals to prevent overflow. If the agarose has not dissolved, continue microwaving until the agarose is completely dissolved.
8. Incubation time for each cell line will vary as some CRC cell lines are more adherent than others.
9. Trypan Blue selectively stains dead cells and can be used to determine the percentage of viable cells in the cell suspension. When using Trypan Blue, add 10 μL of Trypan Blue to 10 μL of cell suspension (1:1 ratio), mix well by resuspending with a micropipette. Use this mixture on the counting slide.
10. User can modify the number of cells used to generate the cell pellet for cell block creation. Larger numbers of cells can be used if a larger-sized cell pellet is desired for the paraffin block. We find that a minimum of 2×10^7 cells is required to produce an average-sized pellet that is easy for handling and embedding into a standard paraffin block. For accurate comparison between cell lines, we recommend adding fresh medium to the culture approximately 8 h prior to collection.
11. When working with a large cell pellet, a longer incubation period or more thrombin and plasma may be required to induce clotting. If a clot has not formed after 15 min, add a further 150 μL of thrombin and 150 μL of plasma to the cell pellet.
12. We use two porous sponge sheets cut to size, to line the histology cartridge. The cell clot is then placed in between the sponge sheets which protects the clot in the cartridge. An alternative to the sponge sheets is porous cigarette rolling paper (purchased from any tobacconist). Place the cell clot in the centre of the cigarette paper and fold the paper around the clot, then place in histology cartridge.
13. Do not leave cell clot in formalin for longer than 48 h. If necessary, after 24–48 h remove the sample from formalin and store in 80% ethanol before processing and paraffin embedding. If working with a small cell pellet, staining the fixed pellet with eosin prior to embedding may help to improve

the visibility of the cell pellet when embedded into a paraffin block.

14. Ensure all procedures or samples containing matrigel are performed on ice as matrigel will solidify when it begins to warm. It also helps to use pipette tips which have been cooled.
15. We use a 29G × ½" needle with a 0.5 ml syringe. Ensure that needle and syringes are cooled (or on ice) prior to use to prevent the matrigel from solidifying.
16. Be sure to rid the needle and syringe of all air bubbles prior to injecting.
17. Fragment analysis of microsatellite markers will not reveal which MMR gene/proteins are deficient, whereas IHC analysis for the individual MMR proteins (MLH1, PMS2, MSH2 and MSH6) will identify which of these are lost resulting in microsatellite instability.
18. The MMR proteins function as heterodimers, with MLH1 partnering with PMS2, and MSH2 partnering with MSH6 for stability. If only two proteins are to be tested, then assessment of MLH1 and MSH2 expression provides the best sensitivity and specificity, as these proteins are required for stabilizing their corresponding partner protein. Comparatively, loss of PMS2 or MSH6 can be compensated for by other minor proteins, such as MSH3, MLH3 or PMS1. IHC testing for loss of MLH1 and MSH2 provides 92% sensitivity and 100% specificity for detecting MSI, compared to assessment of MSI status by fragment analysis [25]. To increase sensitivity, IHC for all four MMR proteins should be performed.
19. Due to the dimerization of MMR proteins, loss of staining of both major and minor proteins in a dimer pair commonly occurs, e.g., MLH1 and PMS2 or MSH2 and MSH6. Thus the concurrent loss of expression of these protein pairs provides added confidence in calling a cell line as MSI. However in the case of isolated loss of a minor protein such as PMS2, the major protein MLH1 may remain intact due to redundancy in binding to other minor proteins, although in some cases this can still result in microsatellite instability [26]. Confirmatory microsatellite testing by fragment analysis is recommended in such cases. In our experience, CRC cell lines with microsatellite instability typically demonstrate loss of more than two MMR proteins on IHC testing. The loss of additional MMR proteins may reflect secondary mutations subsequent to development of MSI.

20. An alternative to assessment of the microsatellite markers in the Promega MSI Analysis system is a different panel of microsatellite markers, the "Bethesda panel," which were recommended by the National Cancer Institute in 1997. The Bethesda panel consists of two mononucleotide markers *BAT-25, BAT-26* (which are also part of the Promega panel) and three dinucleotide markers, *D2S123, D5S346*, and *D17S250* [27]. The exclusive use of mononucleotide markers in the Promega panel has been reported to provide high sensitivity and specificity of MSI detection (almost 100% for both). Furthermore, the monomorphic nature of these markers (i.e., almost all individuals are homozygous for the common allele of the given marker) simplifies data interpretation [16, 28].
21. Accurate quantification of template DNA is important when using the MSI Analysis System. DNA isolated from cell lines cultured in vitro or from fresh frozen xenograft material can be quantified by NanoDrop spectrophotometry. For quantification of DNA isolated from FFPE material we recommend using the Qubit fluorescence-based assay, as NanoDrop is less accurate in this setting [29, 30].
22. Promega recommends using 1–2 ng of genomic DNA as template in the PCR reactions. We find that the user may need to adjust the amount of template DNA due to variations in DNA quality related to individual DNA isolation methods and sample properties, especially when dealing with low concentrations. If working with a large number of samples, we suggest optimization of the assays using various amounts of DNA template (1, 2, 5, 10 ng) from a small number of samples prior to commencing.
23. Assay controls: For the amplification step, the Promega MSI Analysis System kit provides a positive amplification control. Nuclease-free water can be used as a negative amplification control. To facilitate data analysis, we suggest including cell line samples of known microsatellite status (stable and unstable) to serve as analysis controls (*see* Appendix 1).
24. Matrix standards: Spectral calibration for the fluorescent dyes used in the MSI Analysis System is required for each genetic analyzer machine in order to evaluate the multiplexed fluorescent fragments. Matrix standards used for spectral calibration are specific to the genetic analyzer machine and can be purchased from Promega (PowerPlex Matrix Standards, Promega). It is important to consult Promega if unsure about which matrix standards to use.

25. The Promega MSI Analysis System was developed and optimized for use with Applied Biosystem's Genetic Analyzers using POP-4™ polymer, however amplification products can also be detected using instruments with POP-7™ polymer. Promega has a published protocol for this which can be found online (http://manualzz.com/doc/6951420/use-of-the-msi-analysis-system-with-the-applied).
26. The *Penta C/ Penta D* loci which are included in the Promega assay are not required for interpretation of MSI status of CRC cell line samples, as there is typically no paired normal DNA sample. These markers are useful only when using this assay for analysis of samples with paired tumor and normal DNA to confirm correct pairing of tumor and normal samples.
27. Various panels of CpG gene loci can be used to define CIMP-high status. These include the original "Issa panel" of five markers: *MINT1, MINT2, MINT31, CDKN2A (p16)*, and *MLH1* [3], and another panel of five markers (*CACNA1G, IGF2, NEUROG1, RUNX3*, and *SOCS1*) described by Weisenberger et al. (also referred to as the "Laird Panel") [22]. Subsequent studies have further extended the latter panel to eight markers (*CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1, CDKN2A, CRABP1*, and *MLH1*) or 16 markers: (*CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1, CDKN2A, CRABP1, MLH1, CHFR, HIC1, IGFBP3, MGMT, MINT1, MINT31, MLH1, CDKN2A/ARF (p14)*, and *WRN* [23, 31].
28. A methylated DNA reference sample is human DNA that has been completely methylated by in vitro treatment using *Sssl methylase*, and bisulfite-converted. Amplification of this reference template in the MethyLight assay allows the extent of methylation of each locus in your test samples to be expressed as a percentage of this fully methylated reference (percent methylation rate, PMR). This methylated DNA reference sample can be purchased separately from Qiagen (EpiTech control DNA, methylated #59655, Qiagen).

Appendix 1

Reference STR profiles and properties of 30 commonly used CRC cell lines

CRC cell line name	In vitro growth	STR (short tandem repeats) profiles of cell lines at 10 loci										MSI/MSS status	CIMP status
		AMEL	CSF1PO	D5S818	D7S820	D13S317	D16S539	D21S11	TH01	TPOX	vWA		
CACO2	Adherent	X	11	12, 13	11, 12	11, 13, 14	12, 13	30	6	9, 11	16, 18	MSS	Neg/low
COLO201[a]	Semiadherent	X	11, 12	10, 13	9, 10	10	12, 13	30.2, 33.2	8, 9	11	15	MSS	High
COLO205[a]	Semiadherent	X	11, 12	10, 13	9, 10	10, 12	12, 13	30.2, 33.2	8, 9	11	15	MSS	High
COLO320	Semiadherent	X	11	12	9, 12	11	11, 12	33.2	9	8, 9	15, 18	MSS	Neg/low
DLD1	Adherent	X, Y	11, 12	13	10, 12	8, 11	12, 13	29, 32.2	7, 9.3	8, 11	18, 19	MSI	High
HCA7	Adherent	X	11, 14	8, 12	11, 12	9	10, 11	26, 28.2	6, 7	8, 10	14, 16	MSI	High
HCT116	Adherent	X, Y	7, 10	10, 11	11, 12	10, 12	11, 13	29, 30	8, 9	8, 9	17, 22	MSI	High
HCT15[b]	Adherent	X, Y	12	13	10, 12	8, 11	12, 13	29, 32.2	7, 9.3	8, 11	18, 19	MSI	High
HCT8[b]	Adherent	X, Y	12	13	10, 12	8, 11	12, 13	29, 32.2	7, 9.3	8, 11	18, 19	MSI	High
HT115	Adherent	X	7, 12	12	11, 12	13	10	30, 32.2	6, 9	8	18	MSS	Neg/low
HT29[c]	Adherent	X	11, 12	11, 12	10	11, 12	11, 12	29, 30	6, 9	8, 9	17, 19	MSS	High
KM12	Adherent	X	10, 12	10, 17	8, 9	12, 15	11	27	9.3	11, 12	17	MSI	High
LIM1215	Adherent	X, Y	10	9, 12	9	8, 12	8, 12	29, 31	9.3, 10	8	16	MSI	Neg/low
LIM1899	Adherent	X, Y	10, 11	11, 14	8, 9	9, 12	10, 11, 12	29	6, 9.3	9, 11	18, 19	MSI	Neg/low
LIM2405	Adherent	X, Y	14, 15	12, 13	7, 8	8, 11	14, 15	29, 30	9.3	8	14, 18	MSI	High
LIM2537	Adherent	X	9	10	8, 11	8, 14	12	28, 29, 30	6	8, 9	14, 17	MSI	High
LIM2551	Semiadherent	X, Y	9, 10, 11	12, 16	9, 11	8, 11	11	27, 28, 33.2	6, 9.3	8, 11	17, 19	MSI	Neg/low
LS174T[d]	Adherent	X	10, 13, 14	11, 15, 16	10.3, 11	10	11, 13	29, 31	6, 7	8, 9	15, 17, 18	MSI	Neg/low
LS180[d]	Adherent	X	10, 13	10, 15	9.3, 11	10	11, 13	29, 31	6, 7	8, 9	15, 18	MSI	Neg/low
RKO	Adherent	X	8, 10	11, 13, 15	8, 10	8, 11	12, 13	27, 30	6, 10	11	15, 16, 17, 22	MSI	High
SNU175	Suspension	X	10, 12	11	11	9, 12	9, 12	30, 32.2	7, 9	8, 11	14, 17	MSI	Neg/low

(continued)

CRC cell line name	In vitro growth	STR (short tandem repeats) profiles of cell lines at 10 loci										MSI/MSS status	CIMP status
		AMEL	CSF1PO	D5S818	D7S820	D13S317	D16S539	D21S11	TH01	TPOX	vWA		
SNUC1	Suspension	X	12	10	10, 11	9, 11	9, 11	30	7, 9	11, 12	16, 18	MSS	Neg/low
SW1116	Adherent	X, Y	10, 11	11, 12	12	11, 14	9, 12	28, 29	6	8, 11	14, 19	MSS	Neg/low
SW1222	Adherent	X, Y	11	9	10, 12	9, 13	9, 10	29	7	10	18	MSS	Neg/low
SW403	Adherent	X	10, 13	11	8, 9	13	10, 12	28, 29	6	8, 9	14, 18	MSS	Neg/low
SW480[e]	Adherent	X	13, 14	13	8	12	13	30, 30.2	8	11	16	MSS	Neg/low
SW620[e]	Adherent	X	13, 14	13	8, 9	12	9, 13	30, 30.2	8	11	16	MSS	Neg/low
SW948	Adherent	X	12	11	9, 11	10, 11	11, 12	25.2, 29	6, 9.3	8, 11	16, 18	MSS	Neg/low
T84	Adherent	X	10	12	8, 10	9	10, 11	31	6, 9	8, 11	17, 18	MSS	Neg/low
WIDR[c]	Adherent	X	11, 12	11, 12	10	11, 12	11, 12	29, 30	6, 9	8, 9	17, 19	MSS	High

Reference STR profiles were obtained from ExPASy Bioinformatics Resource Portal Cellosaurus Database, and data on LIM cell lines were obtained from the source institute (Olivia Newton-John Cancer Research Institute)

[a,b,c,d,e]Cell lines derived from the same individual are marked by same symbols

References

1. Shibata D, Peinado MA, lonov Y et al (1994) Genomic instability in repeated sequences is an early somatic event in colorectal tumorigenesis that persists after transformation. Nat Genet 6 (3):273–281
2. Lengauer C, Kinzler KW, Vogelstein B (1997) Genetic instability in colorectal cancers. Nature 386(6625):623–627
3. Issa J-P (2004) CpG island methylator phenotype in cancer. Nat Rev Cancer 4(12):988–993
4. Bellam N, Pasche B (2010) TGF-β signaling alterations and colon cancer. In: Pasche B (ed) Cancer genetics. Springer, Boston, MA, pp 85–103. https://doi.org/10.1007/978-1-4419-6033-7_5
5. Cathomas G (2014) PIK3CA in colorectal cancer. Front Oncol 4:35. https://doi.org/10.3389/fonc.2014.00035
6. Colussi D, Brandi G, Bazzoli F et al (2013) Molecular pathways involved in colorectal cancer: implications for disease behavior and prevention. Int J Mol Sci 14(8):16365–16385. https://doi.org/10.3390/ijms140816365
7. Barras D (2015) BRAF mutation in colorectal cancer: an update. Biomark Cancer 7(Suppl 1):9–12. https://doi.org/10.4137/bic.s25248
8. Morkel M, Riemer P, Bläker H et al (2015) Similar but different: distinct roles for KRAS and BRAF oncogenes in colorectal cancer development and therapy resistance. Oncotarget 6(25):20785–20800
9. Guinney J, Dienstmann R, Wang X et al (2015) The consensus molecular subtypes of colorectal cancer. Nat Med 21(11):1350–1356. https://doi.org/10.1038/nm.3967
10. Young L, Sung J, Stacey G et al (2010) Detection of mycoplasma in cell cultures. Nat Protoc 5(5):929–934
11. Tögel L, Nightingale R, Chueh AC et al (2016) Dual targeting of bromodomain and extra-terminal domain proteins, and WNT or MAPK signaling, inhibits c-MYC expression and proliferation of colorectal cancer cells. Mol Cancer Ther 15(6):1217
12. Shin J, Carr A, Corner GA et al (2014) The intestinal epithelial cell differentiation marker intestinal alkaline phosphatase (ALPi) is selectively induced by histone deacetylase inhibitors (HDACi) in colon cancer cells in a kruppel-like factor 5 (KLF5)-dependent manner. J Biol Chem 289(36):25306–25316. https://doi.org/10.1074/jbc.M114.557546
13. Wilson AJ, Chueh AC, Tögel L et al (2010) A coordinated Sp1/Sp3-mediated transcriptional response involving immediate-early gene induction is linked to HDAC inhibitor-induced apoptosis in colon cancer cells. Cancer Res 70(2):609–620. https://doi.org/10.1158/0008-5472.CAN-09-2327
14. Butler JM (2007) Short tandem repeat typing technologies used in human identity testing. BioTechniques 43(4):ii–iv
15. Nikfarjam L, Farzaneh P (2012) Prevention and detection of mycoplasma contamination in cell culture. Cell J 13(4):203–212
16. Bacher JW, Flanagan LA, Smalley RL et al (2004) Development of a fluorescent multiplex assay for detection of MSI-high tumors. Dis Markers 20(4–5):237–250
17. Eads CA, Danenberg KD, Kawakami K et al (2000) MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 28(8):E32
18. Weisenberger DJ, Campan M, Long TI et al (2005) Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 33(21):6823–6836. https://doi.org/10.1093/nar/gki987
19. Gu H, Bock C, Mikkelsen TS et al (2010) Genome-scale DNA methylation mapping of clinical samples at single-nucleotide resolution. Nat Methods 7(2):133–136. https://doi.org/10.1038/nmeth.1414
20. Wojdacz TK, Dobrovic A (2007) Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. Nucleic Acids Res 35(6):e41. https://doi.org/10.1093/nar/gkm013
21. Jasmine F, Rahaman R, Roy S et al (2012) Interpretation of genome-wide infinium methylation data from ligated DNA in formalin-fixed, paraffin-embedded paired tumor and normal tissue. BMC Res Notes 5:117. https://doi.org/10.1186/1756-0500-5-117
22. Weisenberger DJ, Siegmund KD, Campan M et al (2006) CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 38(7):787–793. https://doi.org/10.1038/ng1834
23. Nosho K, Irahara N, Shima K et al (2008) Comprehensive biostatistical analysis of CpG island methylator phenotype in colorectal cancer using a large population-based sample. PLoS One 3(11):e3698. https://doi.org/10.1371/journal.pone.0003698
24. Ogino S, Kawasaki T, Brahmandam M et al (2006) Precision and performance

characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol Diagn 8 (2):209–217. https://doi.org/10.2353/jmoldx.2006.050135

25. Lindor NM, Burgart LJ, Leontovich O et al (2002) Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. J Clin Oncol 20(4):1043–1048. https://doi.org/10.1200/JCO.2002.20.4.1043
26. Shi C, Washington K (2012) Molecular testing in colorectal cancer: diagnosis of lynch syndrome and personalized cancer medicine. Am J Clin Pathol 137(6):847–859. https://doi.org/10.1309/AJCPI83DINULUJNI
27. Berg KD, Glaser CL, Thompson RE et al (2000) Detection of microsatellite instability by fluorescence multiplex polymerase chain reaction. J Mol Diagn 2(1):20–28
28. Murphy KM, Zhang S, Geiger T et al (2006) Comparison of the microsatellite instability analysis system and the Bethesda panel for the determination of microsatellite instability in colorectal cancers. J Mol Diagn 8 (3):305–311. https://doi.org/10.2353/jmoldx.2006.050092
29. Sah S, Chen L, Houghton J et al (2013) Functional DNA quantification guides accurate next-generation sequencing mutation detection in formalin-fixed, paraffin-embedded tumor biopsies. Genome Med 5(8):77. https://doi.org/10.1186/gm481
30. Simbolo M, Gottardi M, Corbo V et al (2013) DNA qualification workflow for next generation sequencing of histopathological samples. PLoS One 8(6):e62692. https://doi.org/10.1371/journal.pone.0062692
31. Ogino S, Kawasaki T, Kirkner GJ et al (2007) Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn 9 (3):305–314. https://doi.org/10.2353/jmoldx.2007.060170