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ACMG technical standards and guidelines for genetic testing for inherited colorectal cancer (Lynch syndrome, familial adenomatous polyposis, and MYH-associated polyposis)

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Lynch syndrome, familial adenomatous polyposis, and Mut Y homolog (MYH)-associated polyposis are three major known types of inherited colorectal cancer, which accounts for up to 5% of all colon cancer cases. Lynch syndrome is most frequently caused by mutations in the mismatch repair genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* and is inherited in an autosomal dominant manner. Familial adenomatous polyposis is manifested as colonic polyposis caused by mutations in the *APC* gene and is also inherited in an autosomal dominant manner. Finally, MYH-associated polyposis is caused by mutations in the *MUTYH* gene and is inherited in an autosomal recessive manner but may or may not be associated with polyps. There are variants of both familial adenomatous polyposis (Gardner syndrome—with extracolonic features—and Turcot syndrome, which features medulloblastoma) and Lynch syndrome (Muir–Torre syndrome features sebaceous skin carcinomas, and Turcot syndrome features glioblastomas). Although a clinical diagnosis of familial adenomatous polyposis can be made using colonoscopy, genetic testing is needed to inform at-risk relatives. Because of the overlapping phenotypes between attenuated familial adenomatous polyposis, MYH-associated polyposis, and Lynch syndrome, genetic testing is needed to distinguish among these conditions. This distinction is important, especially for women with Lynch syndrome, who are at increased risk for gynecological cancers. Clinical testing for these genes has progressed rapidly in the past few years with advances in technologies and the lower cost of reagents, especially for sequencing. To assist clinical laboratories in developing and validating testing for this group of inherited colorectal cancers, the American College of Medical Genetics and Genomics has developed the following technical standards and guidelines. An algorithm for testing is also proposed.

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1 INTRODUCTION

Colorectal cancer (CRC) can be characterized as sporadic, familial, or hereditary. The American Cancer Society expects ~142,000 new cases of CRC in 2013. It is estimated that 20–30% of all CRCs are familial, which includes CRCs of multifactorial inheritance. CRCs due to inherited highly penetrant single-gene mutations may account for an additional 5% of all colon cancer cases.[1,2]

Of the three major types of inherited CRC, Lynch syndrome—which was previously known as hereditary nonpolyposis CRC—is typically recognized as an assemblage of associated cancers characterized by defective mismatch repair (MMR) leading to microsatellite instability (MSI). Lynch syndrome is most frequently caused by mutations in the MMR genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* and is inherited in an autosomal dominant manner. Familial cases that meet the Amsterdam criteria but wherein tumors show MSI-low or MSI-stable profiles and have no mutation in the MMR genes are now classified as familial CRC type X, which is likely to be a heterogeneous collection of disorders.[1] Familial adenomatous polyposis (FAP) is manifested as extensive colonic polyposis caused by mutations in the *APC* gene and inherited in an autosomal dominant manner. Lastly, in 2002, an autosomal recessive condition, MYH-associated polyposis (MAP), caused by mutations in the *MUTYH* gene,

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was described.[3] Lynch syndrome and FAP have sometimes also been referred to as Gardner syndrome (FAP with extracolonic features), Muir–Torre syndrome (Lynch syndrome with sebaceous skin lesions), and Turcot syndrome (FAP with medulloblastomas or Lynch syndrome with glioblastomas). Clinical testing for these genes has progressed rapidly in the past few years with advances in technologies and the lower cost of reagents, especially for sequencing.[4,5]

The guidelines in this document are limited to inherited CRCs only. The Evaluation of Genomics Applications in Practice and Prevention Working Group has published recommendations for genetic testing strategies in newly diagnosed individuals with CRC. The Evaluation of Genomics Applications in Practice and Prevention Working Group found sufficient evidence to recommend screening all newly diagnosed patients with CRC for Lynch syndrome to prevent morbidity and mortality in their at-risk relatives. The Working Group found that measurements of clinical validity varied with each test and strategy. Although several testing strategies were potentially effective, none was clearly superior. A follow-up study found that immunohistochemistry (IHC) with reflex to serine/threonine-protein kinase B-Raf (*BRAF*) testing was the most cost-effective method of universal screening for Lynch syndrome.[6,7] This strategy can identify a significant number of Lynch syndrome patients.[8–10]

A description of genomic sequence and a listing of mutations and single-nucleotide polymorphisms (SNPs) for genes is provided in this document. The entire genomic sequence along with the structural motifs of the genes described in this document can be found at http://www.genome.ucsc.edu. A listing of mutations can be found in the Human Gene Mutation Database at http://www.hgmd.cf.ac.uk/ac/search.php and the InSight database at http://www.insight-group.org. SNPs are listed in http://www.ncbi.nih.gov/dbSNP (build 135) and the University of California Santa Cruz Genome Browser (http://www.genome.ucsc.edu).

2 LYNCH SYNDROME, PREVIOUSLY TERMED HEREDITARY NONPOLYPOSIS CRC

Lynch syndrome is the most common form of inherited CRC. This syndrome is an autosomal dominant disease with a population incidence of ~1 in 4–500 and is responsible for ~3% of all colon cancer cases. Lynch syndrome is characterized by genetic heterogeneity and is known to be associated with mutations in at least four MMR genes: *MLH1*, *MSH2*, *MSH6*, and *PMS2*.

2.1 Gene symbol/chromosome locus, OMIM, and transcript number

Lynch syndrome (OMIM 120435) is a genetically heterogeneous disease caused by mutations in the following MMR genes:

MLH1: MutL, *Escherichia coli*, homolog of, 1; located on chromosome 3p21.3 (OMIM:120436), NM_000249.5
MSH2: MutS, *E. coli*, homolog of, 2; located on chromosome 2p22-p21 (OMIM:609309), NM_000251.1
MSH6: MutS, *E. coli*, homolog of, 6; located on chromosome 2p16 (OMIM:600678), NM_000179.2
PMS2: Postmeiotic segregation increased *Saccharomyces cerevisiae*, 2; located on chromosome 7p22 (OMIM:600259), NM_000535.5

2.2 Other loci

PMS1: Postmeiotic segregation increased *S. cerevisiae*, 1; located on chromosome 2q31-q33 (OMIM:600258)
TGFBR2: Transforming growth factor-β receptor, type II; located on chromosome 3p22 (OMIM:190182)
MLH3: MutL, *E. coli*, homolog of, 3; located on chromosome 14q24.3 (OMIM:604395)
EpCAM: Also known as *TACSTD1*, located on chromosome 2p21 (OMIM:185535)
BRAF: Located on chromosome 7q34 (OMIM:164757)

2.3 Brief clinical description

Patients with Lynch syndrome have up to an 80% lifetime risk of developing colon cancer and, in women, up to a 60% lifetime risk of developing endometrial carcinoma. Affected individuals are also at greater risk for other cancers, such as stomach, ovarian, small-bowel, biliary, renal pelvis, and ureteral cancers. In contrast to patients with FAP, patients with Lynch syndrome develop adenomas at a normal rate, but these precursor lesions progress more rapidly through the stages of carcinogenesis. Relative to sporadic CRC, adenomas and carcinomas in Lynch syndrome occur more often in the proximal colon.[11–14]

The average age of onset for CRC in Lynch syndrome is 61 years. The age of diagnosis of Lynch syndrome–associated endometrial cancer is 46–62 years. Lynch syndrome–associated ovarian cancers have an age of onset of ~42 years, and 30% of individuals with a diagnosis of ovarian cancer are diagnosed by the age of 40. Among women with Lynch syndrome who develop both colon and endometrial cancers, 50% present first with endometrial cancer.[11,13,15,16]

The clinical diagnosis of Lynch syndrome has historically been based on the Amsterdam criteria, although the revised Bethesda criteria are commonly used.[17–19] In 1990, the Amsterdam criteria were initially developed as a research tool to enrich for a more homogeneous population, but when used clinically, these criteria identify only ~60% of patients with Lynch syndrome.[20,21] This lack of sensitivity led to the development of the revised criteria (Amsterdam II Criteria), which take into account the presence of extracolonic cancers and have a detection sensitivity of ~80%.[22] The Bethesda Guidelines were developed by the National Cancer Institute to advise on the testing of tumors for MSI when CRC occurred before the age of 50, when a synchronous or metachronous colon cancer or other related cancer was present, and when there was a significant family history.[23,24]

2.4 Mode of inheritance

Lynch syndrome is inherited in an autosomal dominant manner. The majority of Lynch syndrome patients inherit a mutation in

one of the MMR genes from a parent; however, cancer development is variable with regard to age, and the mutation may show incomplete penetrance. De novo mutations have been recently reported with a mutation rate of ~1.6%.[25] Rare biallelic MMR mutations have been reported in the *MLH1, MSH2, MSH6,* and *PMS2* genes, and this leads to a more severe phenotype known as constitutional MMR deficiency.[26,27]

2.5 Gene description/normal gene product

2.5.1 *MLH1* gene

The *MLH1* gene is 72,558 bases in length and consists of 19 coding exons; the translated protein contains 756 amino acids. The protein MLH1 dimerizes with the protein product of the *PMS2* gene to coordinate the binding of other proteins involved in MMR, including the helicases, the protein encoded by *EXO1*, proliferating cell nuclear antigen, single-stranded DNA-binding protein (Replication protein A, RPA), and DNA polymerases.

2.5.2 *MSH2* gene

The *MSH2* gene is 159,343 bases in length and consists of 16 coding exons; the translated protein contains 934 amino acids. The MSH2 protein forms a heterodimer with either MMR protein MSH6 or MSH3 and functions to identify mismatches. A sliding clamp model has been put forward to describe the structure of the heterodimer. Mismatches in the DNA are thought to be detected as the clamp slides along the DNA.

2.5.3 *MSH6* gene

The *MSH6* gene is 23,871 bases in length and consists of 10 coding exons; the translated protein contains 1,360 amino acids. The MSH6 protein forms a heterodimer with the DNA MMR protein MSH2 and functions in the identification of mismatches by a sliding clamp model.

2.5.4 *PMS2* gene

The *PMS2* gene is 35,887 bases in length and consists of 15 coding exons; the translated protein contains 862 amino acids. The PMS2 protein dimerizes with the MLH1 protein (see MLH1 for details on the function of this protein dimer). The *PMS2* gene has at least 15 pseudogenes, which have a significantly high homology with the active gene.[28]

2.6 Function of MMR genes

MMR genes are involved in numerous cellular functions, including the following:

1. Repairing DNA synthesis errors;
2. Repairing double-stranded DNA breaks;
3. Apoptosis;
4. Antirecombination;
5. Destabilization of DNA.

These responsibilities make MMR proteins extremely important in the basic maintenance of the genetic material and regulation of the cellular cycle. When MMR is lost or is defective, there is a decrease in apoptosis and an increase in cell survival. This can provide a selective growth advantage to the cell, thereby causing a greater susceptibility to tissue-specific cancers.

2.7 Mutational mechanism/abnormal gene product

Approximately 50% of the mutations are in the *MLH1* gene and 40% are in the *MSH2* gene. Mutations in *MSH6* account for ~7–10% of families with Lynch syndrome, and mutations in *PMS2* are responsible for <5% of Lynch families. About 1–3% of mutations are in the *EpCAM* gene. The contribution of mutations from *MSH6*, *PMS2,* and *EpCAM* genes is an estimate, and different studies have reported varying numbers. These genes cooperatively participate to repair nucleotide mismatch errors arising in DNA replication, and deficiencies in any one of the repair genes can lead to Lynch syndrome. In the somatic tumor tissue, mutations in the MMRs genes result in high levels of MSI.[29–34]

2.8 Mutation spectrum, prevalence, and ethnic association of common mutations

Mutations in the MMR genes have been observed in all ethnic groups. More than 2,000 different mutations (Human Gene Mutation Database, http://www.hgmd.org) have been identified in the four MMR genes: 875 in the *MLH1* gene, 860 in the *MSH2* gene, 290 in the *MSH6* gene, and 111 in the *PMS2* gene. Mutations can be of the missense, nonsense, splice site, or regulatory types. Large deletions are also responsible for 5–10% of *MLH1* gene mutations. Large deletions appear to be especially common in the *MSH2* gene, where they reportedly account for 17–50% of mutations. Large deletions and duplications are rarely seen in *MSH6* or *PMS2* (MMR sequence variant database, http://www.med.mun.ca/mmvariants/). There are few mutation hotspots. The splice site mutation in intron 5 of the *MSH2* gene, c. 942+3A>T, has been repeatedly seen in different ethnicities, including Caucasians, African Americans, and Asians. A deletion of exon 16 in *MLH1* is a founder mutation detected in 29 families in Finland, and a deletion of exons 1–6 in *MSH2* is a founder mutation in 18,981 individuals in the United States. The p.A636P mutation in the *MSH2* gene has been found in 0.59% of the Ashkenazi Jewish population with CRC.[35]

2.9 Testing criteria

Individuals meeting any of the following revised Bethesda criteria[23] are recommended for MSI testing:

1. CRC diagnosed before age 50;
2. Presence of synchronous or metachronous CRC or other hereditary nonpolyposis CRC-related tumor, regardless of age;
3. CRC in an individual younger than 60 years of age exhibiting tumor-infiltrating lymphocytes;
4. CRC at any age, plus CRC or hereditary nonpolyposis CRC-related tumor diagnosed before the age of 50 years in at least one first-degree relative;

5. CRC at any age, plus CRC or hereditary nonpolyposis CRC-related tumor diagnosed before the age of 50 years in two or more first- or second-degree relatives

2.10 Algorithm for testing

A suggested algorithm for Lynch syndrome testing is shown in **Figure 1**. This algorithm takes advantage of certain molecular features of a tumor to determine whether it is likely to be associated with Lynch syndrome and then how best to proceed with the germ line analysis.

2.10.1 MSI analysis

Because nearly all CRCs associated with Lynch syndrome exhibit MSI, the first step is to test the tumor (usually a CRC) for MSI either directly by PCR of microsatellite repeats and/or by IHC.[36–38] Although usually concordant, discordant results from these two techniques are possible. For example, a missense mutation may destroy the function of an MMR protein without affecting its antigenicity, thus leading to an abnormal MSI result and a normal IHC result. Conversely, it is possible that some MMR-deficient tumors, such as endometrial cancers or colonic adenomas, may not have accumulated sufficient replication errors to be detected by PCR of microsatellite repeats; in those cases, IHC may show an abnormal result, whereas PCR may not. In addition, some tumors associated with the *MSH6* germ line mutation have been reported to be microsatellite stable by PCR.[39,40] Approximately 5% of cases that demonstrate MSI have normal IHC for all proteins. Therefore, an abnormal result with either test should probably trigger the next set of tests in the algorithm, and consideration should be given to performing both tests, especially if the index of suspicion for Lynch syndrome is high. In addition, the IHC results can be used to guide the germ line analysis (see below) and determine whether or not *BRAF* or *MLH1* promoter methylation testing should be performed.

2.10.2 Methylation of the MLH1 promoter "region C"

Approximately 10–15% of sporadic CRCs also exhibit MSI. The molecular basis for instability in these tumors is most often methylation of the MLH1 promoter, leading to loss of both mRNA and protein expression.[41–49] MLH1 promoter

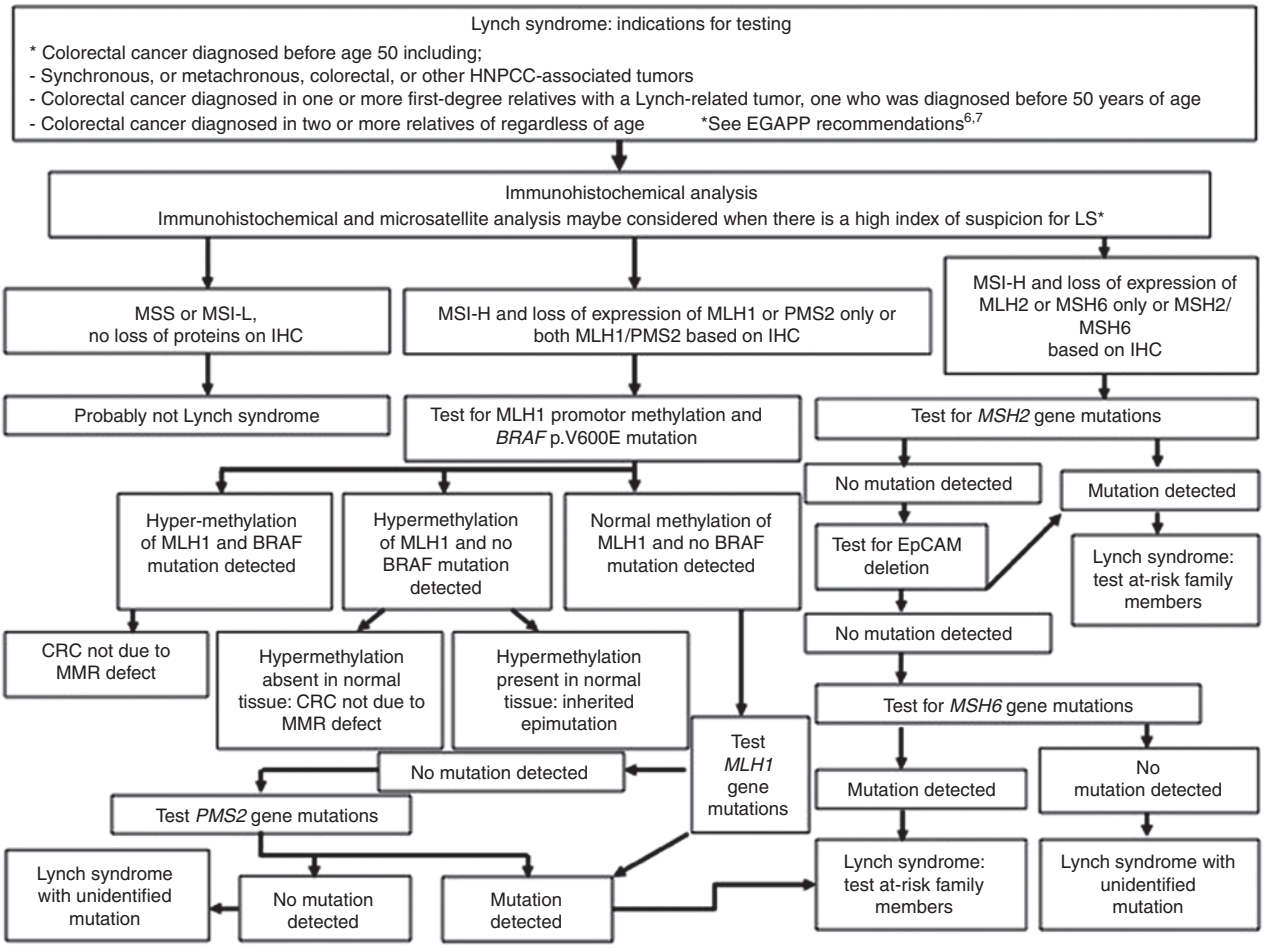

Figure 1 Lynch syndrome: indications for testing. CRC, colorectal cancer; EGAPP, Evaluation of Genomics Applications in Practice and Prevention Working Group; HNPCC, hereditary non-polyposis colorectal cancer; IHC, immunohistochemistry; LS, Lynch syndrome, MMR, mismatch repair; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; MSS, microsatellite-stable.

region "C" is a small proximal region (−248 to −178 relative to the transcription start site) in which the methylation status invariably correlates with the lack of MLH1 expression. The typical IHC profile of one of these sporadic unstable tumors is loss of expression of both MLH1 and PMS2. This is because the stability of PMS2 depends on its ability to form a complex with MLH1 (a similar situation exists with MSH2 and MSH6). The converse, however, is usually not true because tumors with defects in PMS2 or MSH6 may maintain expression of MLH1 or MSH2, respectively. A tumor with loss of MLH1 and PMS2 may be either sporadic or associated with Lynch syndrome because either promoter methylation or a germ line mutation in MLH1 will lead to this same IHC profile. A way to distinguish between these two possibilities is desirable, so as to avoid unnecessary germ line analysis of patients with[50,51] sporadic tumors. It must be noted that germ line MLH1 hypermethylation, although rare, has been documented.[52,53]

2.10.3 *BRAF* gene mutation p.V600E

More than half of sporadic MSI tumors (50–68%) have a specific mutation in the *BRAF* gene, p.V600E, which is rarely detected in Lynch syndrome–associated cancers.[41,43,54] Therefore, if an unstable tumor harbors the *BRAF* gene p.V600E mutation, it is most likely sporadic and germ line testing is not necessary. If the *BRAF* mutation is not present, then the tumor may be either sporadic or associated with Lynch syndrome.

2.10.4 MMR protein expression

Lack of expression of MSH2, MSH6, or PMS2 (with maintenance of MLH1 expression) is usually not seen in sporadic unstable tumors, and proceeding to germ line testing should be based on these IHC profiles. The IHC profile can also be used to choose the gene(s) of interest for germ line evaluation. In cases in which only one gene product is not expressed (typically MSH6 or PMS2), then that gene can be evaluated. If two gene products are not expressed (usually MLH1/PMS2 or MSH2/MSH6), then either *MLH1* or *MSH2* is the likely culprit, and molecular analysis can begin for one of those genes. Simultaneous evaluation of both genes that form a complex can also be considered because the interplay of gene inactivation, complex stability, and IHC expression may not always be predictable.

A germ line evaluation without MSI or IHC tumor testing can be justified in cases with a high enough index of suspicion for Lynch syndrome (e.g., a very strong family history of Lynch syndrome–associated tumors occurring at young ages), when tumor is not available for MSI or IHC, or when the family history is suspicious for Lynch syndrome and the consultand is cancer free. Because the screening tests described above may not be perfect, with a high enough index of suspicion a germ line evaluation should be performed regardless of the results of the screening tests. IHC results may still be helpful in guiding the germ line evaluation and, perhaps, help in determining the relevance of amino acid changes of uncertain significance.

2.11 Sensitivity and specificity

A combination of Sanger sequencing and deletion detection can detect >80% of mutations in *MLH1* and *MSH2* genes. The detection rate is unknown for *MSH6* and *PMS2* genes.

2.12 Diagnostic versus predictive testing

Molecular testing for mutations in MMR genes is used for confirmative diagnosis.[55] Positive results are considered diagnostic of Lynch syndrome. Penetrance of colon cancer associated with mutations in MMR genes is less than 100%. Therefore, some individuals with a cancer-predisposing mutation in one of the MMR genes may never develop colon cancer.[5,56,57]

2.13 Guidelines for detection of mutations in the MMR genes: methods

All general guidelines for PCR and DNA sequencing in the American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines for Clinical Genetics Laboratories apply (http://www.acmg.net). The following additional details are specific for MMR gene testing. For this test, there are two valid methods with different strengths and weaknesses.

2.13.1 Sample requirements and processing

Normal and tumor tissues are required to perform MSI and IHC testing. Either fresh-frozen tissue or formalin-fixed paraffin-embedded (FFPE) tissue block can be used. The fresh-frozen or the FFPE tissue block should be carefully chosen. It is preferable that it contain adequate normal and tumor tissues. The tissue is sectioned and used for hematoxylin and eosin staining, which is used to identify the boundaries of the normal and tumor tissues. Once the sections are obtained (~3–5 μm), IHC can be performed, and DNA can be extracted for MSI. DNA extracted from a blood sample can also be used as a representative normal tissue from the patient.

For mutation detection in the MMR genes via Sanger sequencing, DNA extracted from FFPE tissue can be used, except for the *PMS2* gene, because the quality of DNA obtained from FFPE tissue is compromised. DNA extracted from a blood sample is required for MMR gene sequencing. Refer to ACMG Standards and Guidelines for Clinical Genetics Laboratories, Section G3, for technical details.

2.13.2 IHC

IHC is performed on all four MMR proteins: MLH1, MSH2, MSH6, and PMS2. It is necessary to analyze the expression of all four relevant MMR proteins because loss of either MSH6 or PMS2 may occur and because assessment of only MLH1 and MSH2—or only MLH1, MSH2, and MSH6 (as is performed in some laboratories)—may miss abnormalities in expression.

The tissue sections are incubated with monoclonal antibodies against MLH1, MSH2, MSH6, and PMS2. All sections are examined for expression of MMR proteins in the nucleus and adjacent nonneoplastic tissue elements and subsequently defined as showing presence or absence of these proteins.

2.13.3 DNA extraction

DNA extractions from fresh tissue or FFPE sections can be performed using routine DNA extraction methods after pretreatment of the tissue sections as described in section 2.13.1. The sections are deparaffinized with xylene and enzymatically digested with proteinase K for tissue dissolution. The digested sections (DNA lysates) can be directly used for MSI testing, or DNA can be extracted using standard DNA extraction methods using glycogen as a carrier to enhance yield.[58]

2.13.4 *BRAF* gene p.V600E missense mutation

The p.V600E mutation can be detected using a targeted mutation detection technique, such as restriction enzyme digestion, allele-specific primer extension, or real-time PCR. Refer to ACMG Standards and Guidelines for Clinical Genetics Laboratories, Section G8.7, for technical details.

2.13.5 MLH1 promoter methylation region "C"

Bisulfite modification with real-time (quantitative) methylation-specific PCR analysis to detect the methylated and unmethylated allele is the commonly used method.[59–64] The first step of the methylation-specific PCR procedure is chemical conversion of DNA by sodium bisulfite. Genomic DNA is treated with sodium bisulfite, which deaminates unmethylated cytosines to uracil. 5-Methyl cytosines are resistant to this deamination and following sodium bisulfite treatment will remain as cytosines. After desulfonation and purification, the DNA is used as a template in a real-time TaqMan PCR reaction with amplification primers that are designed to avoid cytosines in CpG dinucleotides. This allows for the equivalent amplification of both methylated and unmethylated alleles. During PCR amplification, different fluorescently labeled TaqMan probes are used to discriminate between methylated and unmethylated DNA sequences by recognizing either cytosine or uracil (thymine) if the sample DNA was methylated or unmethylated, respectively. By interpolation on standard curves, generated by titration of a sample from an unaffected individual, the amount of methylated and unmethylated DNA can be determined. A methylation index, defined as the amount of methylated DNA divided by methylated plus unmethylated DNA, can be calculated. Deviation from a predetermined normal range established by the laboratory would be indicative of disease. Refer to ACMG Standards and Guidelines for Clinical Genetics Laboratories, Section G8.7, for technical details.

2.13.6 Methods for MSI determination

Methods for MSI determination by PCR have already been described (refer to ACMG Standards and Guidelines for Clinical Genetics Laboratories, Section G9, for technical details). With respect to PCR, the National Cancer Institute panel of microsatellite repeats, BAT25, BAT26, D2S123, D17S250, and D5S346, can be used. Most laboratories are using a commercially available five-marker mononucleotide or quasimonomorphic panel. A quasimonomorphic panel consisting of BAT25, BAT26, NR21, NR22, NR24, and other microsatellite repeats has also been described (**Table 1**).[17,65–68] If 30% or more of the repeats are unstable, a tumor is classified as MSI-high (MSI-H). If fewer than 30% of repeats are unstable, a tumor is classified as MSI-low (MSI-L), and if no repeats are unstable, a tumor is classified as microsatellite stable. A MSI-L profile does not appear to be a good predictor of Lynch syndrome, so this result is grouped with the microsatellite-stable (MSS) type and does not lead to further testing. The most widely used microsatellite panel at this time is probably the Bethesda consensus panel, a combination of two mononucleotide repeats and three dinucleotide repeats (NCI panel). The quasimonomorphic panel consists of five mononucleotide repeats, and it appears that the two panels are highly concordant with respect to the designation of high degrees of MSI.[66–70] Instability in the mononucleotide repeat BAT26 correlates very well with high degrees of tumor instability, especially with respect to sporadic unstable tumors; however, it is recommended that BAT26 not be used by itself in the evaluation of potential Lynch syndrome–affected individuals because BAT26 stability has been reported in some Lynch syndrome–associated microsatellite unstable tumors, especially those with large *MSH2* deletions. It has also been shown that both BAT25 and BAT26 in ~28% of African Americans can be polymorphic at one of the loci and therefore could be incorrectly classified as MSI positive.[38,69]

2.13.7 Detection of point mutations in the MMR genes: methods for PCR

2.13.7.1 PCR method. Several sets of primers, PCR conditions, and methods of separation and detection have been published. Other primers and methods can be used if equivalence is demonstrated.[39,71]

2.13.7.2 Primer design. All PCR reactions, for any locus, can theoretically fail to detect an allele if there is a polymorphism at a primer-binding site. The dbSNP database build 135 (http://www.ncbi.nih.gov/dbSNP) and the 1000 Genomes mutation database update the SNPs in the human genome on a regular basis. It is important that laboratories check for the presence of SNPs under primers minimally on an annual basis.

2.13.7.3 PCR product. Patient amplicon sizes should be determined by running the PCR products on an agarose gel using a 100-bp standard ladder.

2.13.8 Methods for mutation detection by scanning the MMR genes

Historically, mutational evaluation of MMR genes was accomplished using a scanning method such as single-strand conformation polymorphism, conformation strand gel electrophoresis, denaturing gradient gel electrophoresis, or denaturing high-pressure liquid chromatography, followed by Sanger sequencing of aberrant amplicons. In addition, other scanning techniques, such as temperature-gradient capillary electrophoresis and mutation scanning by high-resolution melting analysis, have also been used. Sanger sequencing of all coding exons of the MMR genes is now considered the gold standard for mutation detection. An important aspect of mutational evaluation

is the detection of relatively large deletions of an exon or more. Such deletions are not uncommon, especially in the *MSH2* gene, and will not be detected by sequencing because the non-deleted allele will still be amplified. Refer to ACMG Standards and Guidelines for Clinical Genetics Laboratories, Section G11, for technical details.

2.13.9 Methods for sequencing

2.13.9.1 Sanger sequencing. PCR amplification is performed on all coding exons and intron/exon boundaries of the relevant MMR gene(s) *MLH1*, *MSH2*, *MSH6*, and *PMS2* in patients' genomic DNA. IHC or IHC and MSI results together may help to narrow down the sequencing to one or two genes of the MMR complex. Due to the high homology of the *PMS2* functional gene and pseudogenes, it is difficult to find PCR and/or sequencing primer-binding sites that allow amplifying and sequencing of only the functional gene (see **Supplementary Tables S1–S4** online for PCR conditions).[72] Long-range PCR using functional gene-specific primers can overcome this problem. Refer to ACMG Standards and Guidelines for Clinical Genetics Laboratories, Section G10, for technical details.[73,74]

2.13.9.2 Next-Generation Sequencing. The cost of next-generation sequencing (NGS) has dropped rapidly in the past 2 years, and it is now possible to sequence all genes implicated in CRC in a panel for a lower cost than that for doing Sanger sequencing. NGS can be used to sequence all four genes using

Table 1 Microsatellite markers for microsatellite instability

Marker name	Primer sequence Forward (fluorescently labeled) Reverse	Chromosome	Gene near marker	Repeat size	Allele size range (bp)
NCI panel markers[44]					
BAT-25	VIC 5′-TCG CCT CCA AGA ATG TAA GT 5′-TCT GCA TTT TAA CTA TGG CTC	4q11-12	C-Kit	Mononucleotide	110–130 (122)
BAT-26	NED 5′-TGA CTA CTT TTG ACT TCA GCC 5′-AAC CAT TCA ACA TTT TTA ACC C	2p	MSH2	Mononucleotide	112–120 (117)
D2S123	VIC 5′-AAA CAG GAT GCC TGC CTT TA 5′-GGA CTT TCC ACC TAT GGG AC	2p16	MSH2	Dinucleotide	197–227
D17S250	FAM 5′-GGA AGA ATC AAA TAG ACA AT 5′-GCT GGCCAT ATA TAT ATT TAA ACC	17q11.2-q12	BRCA1	Dinucleotide	130–170
D5S346	FAM 5′-ACT CAC TCT AGT GAT AAA TCG 5′AGC AGA TAA GAC AGT ATT ACT AGT T	5q21	APC	Dinucleotide	96–129
Quasimonomorphic mononucleotide markers[45]					
BAT-25	NED 5′-TCG CCT CCA AGA ATG TAA GT 5′-TCT GCA TTT TAA CTA TGG CTC	4q11-12	C-Kit	Mononucleotide	110–130 (122)
BAT-26	5′-TGA CTA CTT TTG ACT TCA GCC FAM 5′-AAC CAT TCA ACA TTT TTA ACC C	2p	MSH2	Mononucleotide	112–120 (117)
NR-21	5′-TAA ATG TAT GTC TCC CCT GG VIC 5′-ATT CCT ACT CCG CAT TCA CA	14q11.2	SLC7A8	Mononucleotide	103
NR-22	5′-GAG GCT TGT CAA GGA CAT AA FAM 5′-AAT TCG GAT GCC ATC CAG TT	11q24-q25	Transmembrane precursor protein B5	Mononucleotide	142
NR-24	5′-CCA TTG CTG AAT TTT ACC TC VIC 5′-ATT GTG CCA TTG CAT TCC AA	2q11.2	Zinc finger 2 (ZNF-2)	Mononucleotide	132
Alternate panel markers[46–48]					
D18S35	VIC 5′-AGC TAG ATT TTT ACT TCT CTG 5′-CTG GTT GTA CAT GCC TGA C	18q21	DCC	Dinucleotide	93–130
TP53-DI	NED 5′-AGG GAT ACT ATT CAG CCC GAG GTG 5′-ACT GCC ACT CCT TGC CCC ATT C	17p13	P53	Dinucleotide	95–140
TP53-PENTA	FAM 5′-ACT CCA GCC TGG GCA ATA AGA GCT 5′-ACA AAA CAT CCC CTA CCA AAC AGC	17p13	P53	Pentanucleotide	105–150
D1S2883	VIC 5′-AAA TCT GGT CTT CTG TTT TCA CTA T 5′-TTC CAA ATG TTG ACT CTG C	1q24	HPC1	Dinucleotide	170–220
FGA	FAM 5′-GCC CCA TAG GTT TTG AAC TCA 5′-TGA TTT GTC TGT AAT TGC CAG C	4q28	Fibrinogen alpha polypeptide	Tetranucleotide	160–230

The markers recommended by the National Cancer Institute[44] and quasimonomorphic markers[45] are listed.

target-enrichment methodologies such as digital PCR and in-solution hybridization. The *PMS2* gene cannot be sequenced using this technology due to the presence of pseudogenes (http://www.ucsc.edu). Target enrichment involves selection using a PCR-based method, such as highly multiplex PCR and digital PCR, or in-solution hybridization–based methods. Once the genes are selected, NGS can be performed using short- or long-read technologies. Data analysis is complex for NGS and requires significant bioinformatics input. The amount of data generated is large, and a major effort is required for annotation and variant classification.

It is important to note that all regions of interest (exons) may not be amenable to NGS, and some exons will have to be sequenced using Sanger sequencing to complete clinical testing. Furthermore, single-exon and multiexon deletions and duplications may not be detected and may require other methodologies. At this time, methylation analysis has not been adopted as a standard using NGS in clinical testing.

2.13.9.3 Sequence variation with unknown clinical consequences. Sequencing often reveals previously unreported and/or uncharacterized variants. These variants can be missense mutations or a variation located within a splice site consensus sequence in which the contribution to disease cannot be predicted; these are reported as variants of uncertain significance. RNA analyses may be helpful to determine splicing defects. Following the recommendation from ACMG for interpretation of sequence variants, a missense mutation that leads to a nonconservative substitution of an evolutionarily conserved amino acid is more likely to be disease causing than a missense mutation that alters an amino acid that is not evolutionarily conserved.[73] This classification should be approached with caution as more data from human genome sequencing and allele frequencies in different populations become available. Some variants may be rare polymorphisms in a particular population rather than disease-causing mutations. Family segregation studies may clarify the pathologic or benign nature of missense mutations.

2.13.10 Methods for large gene rearrangements

The two main methods to detect large deletions are Southern blot hybridization and multiplex ligation-dependent probe amplification (MLPA), with MLPA becoming more commonly used. Deletion of two or more sequential exons detected by MLPA is a fairly dependable result because each deleted exon can be considered confirmation of the other deleted exon(s). If only one exon is deleted, and no heterozygous polymorphism is detected by sequencing (especially under the probe), then a second confirmatory method is recommended. If MLPA is the original method used, then a Southern blot or quantitative PCR can be used as a confirmatory test. Other methods used for detection of large gene rearrangements include multiplex amplifiable probe hybridization and real-time (quantitative) PCR analysis. Refer to ACMG Standards and Guidelines for Clinical Genetics Laboratories, Section G11, for technical details.

2.13.10.1 Gene-targeted array-based comparative genomic hybridization (aCGH). Gene-targeted array-based comparative genomic hybridization (aCGH) can be used to detect single-exon and multiexon deletions and duplications.[75] This method is not dependent on a single probe and can be extremely sensitive because multiple probes are designed for each exon, thus avoiding allele dropout due to the presence of SNPs under probe, which is a major drawback of MLPA. Refer to ACMG Standards and Guidelines for Clinical Cytogenetics for technical details.

There are fewer concerns for contiguous deletion involving more than one exon; however, a confirmation is needed for a single-exon deletion detected by assays other than aCGH. A false-positive result can be caused by a SNP under the primer, probe, or restriction endonuclease digestion site, which will affect the efficiency of the primer and/or probe annealing to the target or abolish a restriction enzyme digesting site. This is usually not a problem because sequencing will detect the polymorphism and indicate the presence of two alleles. Confirming a true positive typically involves performing another deletion detection assay. Possible deletion assays include Southern blot and an MLPA assay for the same gene, which uses different probes. A real-time (quantitative) PCR assay can also be used for this purpose. Using gene-targeted CGH arrays avoids any dropout because the detection of the deletion is based on multiple probes.

Searching the GenBank dbSNP database and selecting the primer and probe sites without any reported SNPs in the assay development can eliminate false positives. The SNP can be easily detected by sequencing that exon, or the single-exon deletion can be confirmed by secondary tests with the primers and then probing of a different sequence region than that in the primary test, e.g., a quantitative real-time (quantitative) PCR can be used as a confirmatory method.

2.13.11 EpCAM deletion

A common deletion in the 3′ region of the *EpCAM* gene causes somatic hypermethylation of MSH2 because the two genes are adjacent to each other on chromosome 2. Tumors arising from *EpCAM* gene deletion demonstrate an MSI-high profile and loss of MSH2 and/or MSH6 by IHC.[76,77] Deletions in the 3′ region of the *EpCAM* gene can be detected using Southern blot, MLPA, or gene-targeted aCGH and should be analyzed in patients with IHC results showing loss of MSH2 and/or MSH6.

2.14 Interpretation

The following elements must be included in the report.

2.14.1 MSI by PCR

The panel of microsatellites analyzed, as well as the results from each locus, should be reported. An MSI-high profile is reported if 40% or more of the repeats are unstable; an MSI-stable profile is reported if no repeats are unstable, and an MSI-low profile is reported if fewer than 40% of repeats are unstable. Only a high degree of MSI is considered to be indicative of potential Lynch syndrome.

2.14.2 IHC results

The results for antibody staining for all four MMR proteins (MLH1, MSH2, MSH6, and PMS2) should be reported as protein presence (normal staining), protein absence (negative staining), or uninterpretable. "Uninterpretable" refers to a lack of tumor staining without internal control positivity. Quantification of the strength of antibody staining is not recommended.

2.14.3 Mutational testing of MMR genes

The gene; mutation (nucleotide position); amino acid alteration, if present; deletion or insertion, if present; and transcript number should be reported. Changes should be classified as deleterious (pathogenic), benign, or a variant of uncertain significance. Refer to ACMG recommendations for standards for reporting and interpretation of sequence variation, revised in 2007.[73]

Laboratories are strongly encouraged to deposit data from clinical sequencing into public databases such as ClinVar and other Leiden Open (source) Variation Database (LOVDs) (http://www.ncbi.nlm.nih.gov/clinvar, http://www.hgvs.org) in order to update our understanding of genomic variants that will lead to enhanced patient care.

3 FAMILIAL ADENOMATOUS POLYPOSIS

FAP and several related colon cancer phenotypes are caused by mutations in the *APC* gene. The *APC* gene is a tumor suppressor gene, sometimes referred to as a "gatekeeper," that is responsible for regulating the Wnt pathway. In accordance with Knudson's two-hit hypothesis, both alleles of the *APC* gene are inactivated in tumors, resulting in loss of the functional protein.[78] Germ line mutations in the *APC* gene are responsible for FAP and have been well characterized.[79] Identification of a germ line mutation in an affected individual is useful for confirmation of diagnosis and clinical management of presymptomatic family members.

3.1 Gene symbol/chromosome locus, OMIM, and transcript number

APC: Adenomatous polyposis coli; located on chromosome 5p22.2 (OMIM 611731), NM_000038.5

3.2 Other loci

None.

3.3 Brief clinical description

FAP is a heritable colon cancer predisposition disorder. FAP accounts for 1%[1] of all CRC. The disease is characterized by the presence of a large number of colorectal adenomatous polyps (>100) that begin to form at a mean age of 16 years. Variable features include extracolonic polyps, dental abnormalities, congenital hypertrophy of the retinal pigment epithelium, soft tissue tumors, and desmoid tumors. FAP accompanied by non-gastrointestinal maladies was historically referred to as Gardner syndrome before molecular evidence that Gardner syndrome and FAP are both due to mutations in the *APC* gene. Attenuated adenomatous polyposis (AFAP) is characterized by fewer colonic polyps (average of 30) and occurs later in life (average age of onset ~40 years). FAP, AFAP, and Gardner syndrome are caused by germ line mutations in the adenomatous polyposis coli cancer gene (*APC*).

3.4 Mode of inheritance

FAP is an autosomal dominant condition in which every affected individual has a 50% chance of passing on the disease allele to each of his/her offspring. Although *APC* gene mutations are inherited in an autosomal dominant manner, ~20% of FAP cases can have de novo germ line mutations in the *APC* gene.[80,81] In addition, individuals without affected parents, and with or without affected siblings, can have adenomatous polyposis due to recessive inheritance of two mutations in the *MUTYH* gene (see section 4.4).

3.5 Gene description/normal gene product

The *APC* gene is 91 kb in length and encodes a transcript of 10.4 kb. The protein product of this gene is 309 kD long, with 2,843 amino acids, and has 15 exons. The protein belongs to the Wnt signal transduction pathway. Absence of functional APC gene product leads to aberrant transcription of c-myc, cyclin-D, and other target molecules. The normal gene product is also associated with cell adhesion and microtubule assembly.

3.6 Mutational mechanism/abnormal gene product

More than 1,500 different germ line mutations have been characterized in the *APC* gene (Human Gene Mutation Database, http://www.hgmd.org). The majority of mutations are point mutations, including nucleotide substitutions, deletions, or insertions, and most of them result in the introduction of a termination codon. Small subsets of missense mutations have been functionally characterized to have the potential to predispose to FAP. In addition, ~20% of germ line mutations include gross deletions, insertions, and complex rearrangements.

3.7 Mutation spectrum, prevalence, and ethnic association of common mutations

Mutations in the *APC* gene have been observed in all ethnic groups.

3.7.1 *APC* gene mutation spectrum

The *APC* gene coding region consists of 15 exons, with exon 15 individually representing ~6.5 kb. The 5′ coding region of exon 15 includes a mutation cluster region, which is a common site of somatic mutations arising in tumors. Two recurrent mutations at codons 1061 and 1309 (located in the 5′ part of exon 15) account for ~30% of germ line *APC* mutations (http://perso.curie.fr/Thierry.Soussi/APC.html; http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html; and http://life2.tau.ac.il/GeneDis/Tables/APC/APC.html). Apart from these recurrent mutations, there is a high degree of allelic heterogeneity in the *APC* gene, giving rise to FAP disease. The vast majority of mutations found in the *APC* gene represent truncating

mutations (caused by small deletions, 46%; small insertions, 10%; and nonsense mutations, 28%). Missense mutations (3%) and gross alterations (single- and multiexon deletions and duplications) (13%) have also been reported (http://perso.curie.fr/Thierry.Soussi/APC.html; http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html; and http://life2.tau.ac.il/GeneDis/Tables/APC/APC.html). Recent data suggest that gross alterations in the *APC* gene may have been underreported initially, with up to 20% of FAP families potentially carrying a gross alteration. Mutations contributing to classic FAP occur between exon 5 and the 5′ portion of exon 15, whereas those associated with AFAP tend to cluster in the extreme 5′ portion of the gene (exons 1–4) and the 3′ portion of exon 15. There is limited correlation between the site of truncating mutations on the APC gene and the associated phenotype. Mutations that cluster in the region of codons 1250–1400 are associated with early-onset and severe polyposis, whereas mutations located 3′ to codon 1400 frequently correlate with osteomas, dental changes, and desmoids. This phenotype/genotype correlation is quite complex, which may be explained by variable interference of different mutant APC proteins with wild-type function, in addition to potential modifier genes. The new mutation rate for FAP has been reported to be as high as 20%.[82] Although genetic risk can be evaluated through mutation testing, refined correlations between specific mutations and clinical phenotypes remain limited and do not provide any guidance for the clinical management of patients with FAP disease.

3.7.2 p.I1307K Missense mutation in the *APC* gene

This mutation does not lead to classic FAP, but it carries an increased (10–20%)[1] lifetime risk of developing colon cancer. It is estimated that 6% of all individuals of Ashkenazi Jewish ancestry carry the p.I1307K mutation. Genetic testing for the p.I1307K mutation is an option for individuals of Ashkenazi Jewish ancestry with a family history of colon cancer or polyps, a personal history of colon cancer or polyps, or a heightened concern for colon cancer. Early screening has been recommended for individuals who test positive for the p.I1307 missense mutation.[83,84] The test is not appropriate for individuals who are not of Ashkenazi Jewish ancestry.

3.7.3 p.E1317Q Missense mutation

This mutation has been suggested to be associated with a predisposition to colon adenomas and/or colon cancer; however, there is no consensus, and the role of the p.E1317Q variant in colon cancer is uncertain.[85]

3.8 Testing criteria

Testing for FAP should be considered for individuals with the following[12]:

1. Presence of 100 or more polyps;
2. Autosomal dominant inheritance;
3. Possible additional findings, such as congenital hypertrophy of retinal pigment epithelium, osteomas, supernumerary teeth, odontomas, desmoids, epidermoid cysts, duodenal and other small-bowel adenomas, gastric fundic gland polyps.

Testing for AFAP should be considered for individuals with the following:

1. Presence of <100 adenomas (average 30 polyps; Note: Individuals with 100 or more polyps occurring at older ages (35–40 years or older) may be found to have AFAP.);
2. Frequent right-sided distribution of polyps;
3. Adenomas and cancers at an age older than that for classic FAP and other gastrointestinal manifestations

3.9 Algorithm for testing

A suggested algorithm for FAP/AFAP syndrome testing is shown in **Figure 2**. It is recommended that FAP testing be performed using full sequencing of the *APC* gene. If no mutation is detected, then testing for large gene rearrangements should be performed.

3.10 Sensitivity and specificity

Comprehensive analysis of the entire *APC* gene is necessary for diagnostic testing of FAP. A mutation is detected in ~80% of patients with a clinical diagnosis of FAP, with DNA sequencing detecting 87% of point mutations and small insertions or deletions.[86] The remaining 10–15% of mutations are gross deletions and duplications, which can be detected by MLPA, Southern blot, or real-time quantitative PCR analysis.

3.11 Diagnostic versus predictive testing

Molecular testing for mutations in the *APC* gene is used for diagnostic and presymptomatic testing. Positive results are considered diagnostic rather than predictive because the penetrance of a mutation is virtually 100%.

3.12 Prenatal testing

This test can be used for prenatal diagnosis in both amniotic fluid cells and chorionic villus samples. Prior knowledge of the pathogenic familial mutation detected in the affected individual is required for prenatal testing. Several laboratories offer prenatal testing. Refer to GeneTests (http://www.genetests.org).

3.13 Guidelines

3.13.1 Methodological considerations

All general guidelines for PCR and DNA sequencing in the ACMG Standards and Guidelines for Clinical Genetics Laboratories apply (http://www.acmg.net). The following additional details are specific for *APC* gene testing. For this test, there are two valid methods with different strengths and weaknesses.

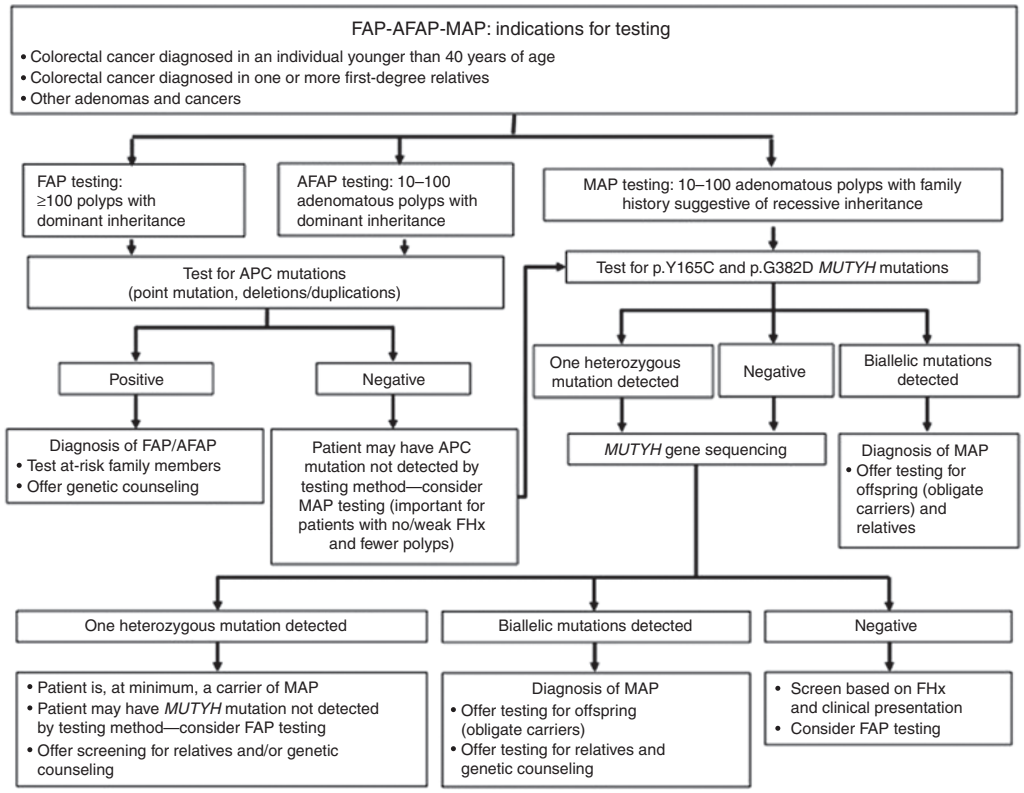

Figure 2 FAP-AFAP-MAP: indications of mapping. AFAP, attenuated adenomatous polyposis; FAP, familial adenomatous polyposis; FHx, family history; MAP, MYH-associated polyposis.

3.13.2 Detection of point mutations in the *APC* gene: methods
3.13.2.1 PCR method. The PCR method is described in section 2.13.7.1 (refs. 87–90).

3.13.2.2 Primer design. Primer design is described in section 2.13.7.2.

3.13.2.3 PCR product. PCR product is described in section 2.13.7.3.

3.13.2.4 Scanning. In the *APC* gene, the large majority of mutations are point mutations. Direct DNA sequencing of all 15 coding exons of the *APC* gene is considered the gold standard for mutation detection. However, many different approaches have been described to identify mutations in the *APC* gene. In the past, several clinical laboratories used the RNA-based protein truncation test, also known as the in vitro synthesized protein assay, which has a sensitivity ranging from 70 to 90%, but the protein truncation test approach has disadvantages, including decreased RNA stability in blood lymphocytes, assay artifacts, and an inability to detect nontruncating mutations. In addition, not all laboratories actually characterize (i.e., sequence) the putative mutation implicated by a protein truncation test alteration. More recently, a DNA-based protein truncation test has been published, and some laboratories continue to use it.[91,92] Other, less popular methods include scanning methods, followed by limited sequencing of aberrant fragments, as described in section 2.13.8. However, none of these methods has detection sensitivity as high as that of direct sequencing, which is a standard method in most clinical laboratories. Refer to ACMG Standards and Guidelines for Clinical Genetics Laboratories, Section G19, for technical details.

3.13.2.5 Sanger sequencing. Sanger sequencing is described in section 2.13.9.1.

3.13.2.6 NGS. This process is described in section 2.13.9.2.

3.13.3 Methods for detection for of large rearrangements
Gene-targeted CGH arrays can also be used, as described in section 2.13.10.

3.13.4 Interpretation
Refer to section 2.14.3 for interpretation.

4 MYH-ASSOCIATED POLYPOSIS

MYH-associated polyposis (MAP) is caused by biallelic mutations in the *MUTYH* gene. It is characterized by an increased lifetime risk of CRC. The penetrance of CRC in individuals with biallelic germ line *MUTYH* mutations is high but incomplete at age 60 years. MAP is estimated to account for 0.7% of all CRC cases and up to 2% of familial or early-onset CRC cohorts in which affected individuals have a low number (<15–20) of adenomas.[93–96]

4.1 Gene symbol/chromosome locus, OMIM, and transcript number

MUTYH (OMIM 604933 (608456), NM_001048171.1) is the gene symbol recognized by the HUGO nomenclature committee (http://www.gene.ucl.ac.uk/nomenclature/). The gene

has also been referred to as *MYH*. The chromosome locus is 1p32.1–1p34.3.

4.2 Other loci

None.

4.3 Brief clinical description

The *MUTYH* gene has been implicated in predisposition to the multiple adenoma phenotype. MAP has been described as an autosomal recessive form of FAP associated with susceptibility to CRC.[95,97] MAP is characterized by multiple colorectal adenomas and a high risk of CRC. The polyp burden of individuals affected with MAP is variable, and although the data are limited, current evidence suggests that biallelic mutations can be found in up to 30% of patients with 15–100 adenomas and in ~7% of patients with >100 adenomas.[3] Biallelic mutations have been identified in patients with no detectable polyposis.

4.4 Mode of inheritance

Given the autosomal recessive nature of the disease, family histories are usually unremarkable. With the absence of family history in MAP and the high rate of de novo mutations in AFAP and FAP, analysis of both *MUTYH* and *APC* gene mutations should be considered for patients with multiple adenomatous polyps.

4.5 Gene description/normal gene product

The human MutY homolog, *MUTYH*, is a human base excision repair gene involved in preventing 8-oxo-dG-induced mutagenesis. *MUTYH* contains 16 exons encoding a protein of 535 amino acids. *MUTYH*, an adenine-specific DNA glycosylase, removes adenine residues mispaired with 8-oxo-dG or guanine.

4.6 Mutational mechanism/abnormal gene product

MAP is caused by the deficiency or absence of the MUTYH protein. MUTYH repairs DNA by removing adenine residues that are mispaired with 8-oxoguanine during replication of oxidized DNA. Tumors from patients with biallelic *MUTYH* mutations have an excess of somatic mutations (guanine-to-thymine transversions) in the *APC* gene.

4.7 MYH transcript and mutation nomenclature

Multiple transcripts are produced from *MUTYH,* which has complicated the nomenclature used to describe mutations identified in the gene. The two major transcripts are hMYHα1 (NM_012222.2) and hMYHα3 (NM_001048171.1), encoding polypeptides of 546 and 535 amino acids, respectively. The hMYHα3 transcript is 33 nucleotides shorter than the hMYHα1 transcript and results from alternative splicing of exon 3, which eliminates 11 amino acids from the 5′ end of exon 3 (GMIAECPGAPA). All other codons, and therefore amino acids, are identical between the two isoforms. Although most publications use the hMYHα3 variant when naming mutations, some reports use the full-length transcript (hMYHα1). When reporting results for *MUTYH* testing, or when comparing reports from different laboratories, it is imperative to note which transcript has been used to name the alteration(s) found in the gene. **Table 2** illustrates this concept with the two most common alterations and the nomenclature used to describe them by isoform.

4.8 Mutation spectrum, prevalence, and ethnic association of common mutations

Most major ethnic groups seem to have mutations in the *MUTYH* gene. There appear to be a number of founder mutations common to specific ethnic groups, such as p.Y90X in Pakistani patients and p.E499X in Indian patients; several studies have examined control groups for the two common missense mutations, with a prevalence of 0–2% established in different ethnic populations. Other specific mutations have been reported in the Japanese, Italian, Portuguese, and Finnish populations.[1,98–100]

Although a number of mutations in the *MUTYH* gene have been documented, two missense mutations, p.Y165C and p.G382D, account for 70–80% of mutant alleles in the Northern European population, with a third mutation, 1395delGGA, accounting for ~25% of mutant alleles in persons of a Southern European (Mediterranean) background.[101] The common missense mutations in *MUTYH*, p.Y165C and p.G382D, have a well-established effect on glycosylase function in experimental systems. More recently, additional missense changes have been reported. Deletions and frameshift and nonsense mutations that are likely to be pathogenic have also been reported.

4.9 Testing criteria

Testing for MAP should be considered for individuals with the following criteria:

1. CRC diagnosed in an individual younger than 40 years of age;
2. The presence of 10 or more adenomatous polyps in the absence of a germ line *APC* gene mutation;
3. 3.Family history of colon cancer consistent with an autosomal recessive inheritance. This includes colon cancers with or without polyps.

4.10 Algorithm for testing

A suggested algorithm for MAP syndrome testing is shown in **Figure 2**. It is recommended that testing for MAP be first performed for the two most common mutations, p.Y165C and p.G382D, before proceeding to full sequencing of the *MUTYH* gene. If one mutation is detected, then full *MUTYH* sequencing should be performed. If the two common mutations are not detected, then full sequencing of the *MUTYH* gene can be considered depending on the clinical presentation and family history.

Table 2 Isoforms of MUTYH and mutation nomenclature for the common mutations

hMYHα1	hMYHα3
p.Y176C	p.Y165C
p.G393D	p.G382D

4.11 Sensitivity and specificity

Targeted mutation analysis for p.Y165C and p.G382D covers ~80% of mutations in the *MUTYH* gene in the North European and Caucasian population. Sequence analysis of the entire coding region of the *MUTYH* gene can detect up to 99% of mutations. Heterozygous single-exon and multiexon deletions and duplications cannot be detected by Sanger sequencing.[95,97,101–104]

4.12 Diagnostic versus predictive testing

Molecular testing for mutations in the *MUTYH* gene is used for confirmative diagnosis and carrier detection. More than 100 mutations have been described in the *MUTYH* gene (Human Gene Mutation Database, http://www.hgmd.org). If heterozygosity for only one of the common mutations is detected in a diagnostic case or no mutation is detected, sequencing of the *MUTYH* gene should be considered.

4.13 Carrier testing

Approximately 1–2% of the general population is predicted to carry a *MUTYH* mutation. Individuals who are heterozygous for *MUTYH* mutations may be at risk—depending on the status of their reproductive partner—of having a child with this recessive condition.

4.14 Guidelines

4.14.1 Definition of mutation categories

The majority of the mutations detected in the *MUTYH* gene are point mutations, which include nonsense, missense, and small insertion/deletion mutations. Only one intragenic deletion has been described in the *MUTYH* gene.[105]

4.14.2 Methodological considerations

All general guidelines for PCR and DNA sequencing in the ACMG Standards and Guidelines for Clinical Genetics Laboratories apply (http://www.acmg.net). The following additional details are specific for *MUTYH* gene testing. For this test, there are two valid methods with different strengths and weaknesses.

4.14.3 Detection of point mutations in the *MUTYH* gene: methods

4.14.3.1 PCR method. The PCR method is described in section 2.13.7.1 (refs. 87–90).

4.14.3.2 Primer design. Primer design is described in section 2.13.7.2.

4.14.3.3 PCR product. PCR product is described in section 2.13.7.3.

4.14.3.4 Targeted detection of p.Y165C and p.G382D. Restriction-fragment-length polymorphism is a PCR/restriction enzyme digestion–based technique that allows for the discrimination of gene variants by producing genotype-specific banding patterns on native agarose or acrylamide gels; it is the most commonly used method for detection of the p.Y165C and p.G382D mutations. Briefly, the region surrounding the mutation to be interrogated is amplified by PCR. The PCR product is then digested with a restriction enzyme, *BseXI* or *Bgl II*, specific for the p.Y165C and G382 mutations, respectively. The digested products are then electrophoresed on a native polyacrylamide gel to detect the mutant allele. The digested product produces a specific pattern if only wild-type sequence is present and a different but specific pattern if only mutant sequence is present. A properly designed assay must allow for the recognition of heterozygous individuals as well. A restriction-fragment-length polymorphism (RFLP) gel for mutation detection of p.Y165C for heterozygous samples using the *BseXI* and *Bgl II* restriction enzymes gives fragments of 105, 83, and 61 bp, and for homozygous samples, it yields fragments of 105 and 61 bp.

4.13.3.5 Other technologies. Other technologies, such as denaturing high-pressure liquid chromatography, pyrosequencing, and allele-specific primer extension, can also be used for detecting p.Y165C and p.G382D mutations.

4.13.3.6 Limitations. Most of the targeted methods are extremely sensitive and can detect >99% of the mutations in the *MUTYH* gene. The presence of unknown SNPs in close proximity to the mutation may lead to a false-negative result.

4.13.3.7 Sanger sequencing. Sanger sequencing is described in section 2.13.9.1.

4.13.3.8 NGS. This process is described in section 2.13.9.2.

4.14.4 Methods for detection of large gene rearrangements

No large deletions or duplications have been reported in the *MUTYH* gene.

4.14.5 Interpretation

Refer to section 2.14.3 for interpretation.

5 SAMPLE REPORTS

Sample reports are available in the **Supplementary Appendix** online. Sample report templates are also available in publications by Scheuner et al.[106,107]

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

ACKNOWLEDGMENT

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DISCLOSURE

A.G., M.F., R.M., W.S., and M.H. are employed by fee-for-service laboratories performing next-generation sequencing. Two individuals serve on advisory boards or in other capacities for companies providing next-generation sequencing services (M.H.: GenomeQuest, RainDance, Ingenuity, Oxford Gene Technology, and InVitae; M.F.: SoftGenetics and SVBio). The University of Utah and W.S. (and two coinventors) have a pending patent to use BRAF mutation status in microsatellite-stable tumors as a predictor of poor prognosis. The patent rights have been licensed by Ventana.

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Patient Name:	Jane D.	
ID number:	057398468	Patient Information
DOB:	04/05/2007	Race/Ethnicity:
Gender:	Female	Family History:
Ordering Physician:	Dr. Good	Personal History:
Date of Report:	June 15, 2010	

Test Performed: Microsatellite Instability Testing (MSI) and Immunohistochemistry (IHC)

Indication for testing: Colorectal cancer. Rule out Lynch syndrome.

Specimen Type: Blood and FFPE block

Result: MSI- High, IHC- loss of MSH2

Interpretation:

Microsatellite Instability Analysis (MSI):
Comparative analysis of DNA samples extracted from blood (DNA#) and tumor (DNA#) was performed using the methodology described. More than 40% of the markers analyzed were found to be unstable for this patient. Our analysis is consistent with an MSI-High (MSI-H) result in this patient's tumor DNA.

Immunohistochemistry (IHC) Analysis:
The paraffin-embedded tumor tissue was forwarded to the Department of Pathology for immunohistochemical analysis for MLH1, MSH2, MSH6, and PMS2 proteins. The report of immunohistochemical analysis received on / / is attached.

Summary:
Collective data on this patient's tumor sample suggest a defect in the expression of the *MSH2* gene. We recommend analysis of the *MSH2* gene to assess mutations in the coding region. Genetic counseling is recommended.

Method:MSI was performed using a PCR-based assay to analyze five microsatellite markers (NCI panel) followed by fluorescent fragment analysis. IHC was performed for all four MMR proteins: MLH1, MSH2, MSH6, and PMS2.

This report was reviewed and approved by:

_____ _____

John Doe, M.D., Ph.D. Date

Director, The Laboratory

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Patient Name:	Jane D.	
ID number:	057398468	Patient and Family Information
DOB:	04/05/2007	Race/Ethnicity:
Gender:	Female	Family History:
Ordering Physician:	Dr. Good	Personal history:
Date of Report:	June 15, 2010	

Test Performed: Lynch Syndrome: *MSH2* Gene Sequence Analysis

Indication for testing: Possible diagnosis of Lynch syndrome
Specimen Type: Blood

Result: No mutation detected

Interpretation:

A sample from this individual was referred to our laboratory for molecular testing for hereditary non-polyposis colon cancer (HNPCC). HNPCC is an autosomal dominant disorder characterized by an increased risk of colon and other cancers and is associated with tumors exhibiting MSI. HNPCC is caused by a germline mutation in a mismatch repair gene (*MLH1, MSH2, MSH6*, and *PMS2*). Mutations in *MLH1* and *MSH2* account for about 90% of mutations in families with HNPCC. A single mutation in one copy of the *MSH2* gene is associated with disease.

Sequence analysis did not detect any mutations in the *MSH2* gene. This analysis will not detect large deletions, large duplications, mutations in the promoter or other regulatory regions. Some intronic mutations will not be detected by this assay. These results must be interpreted in the context of this individual's clinical features. Genetic counseling is recommended.

Variants: A list of sequence variants unrelated to disease identified in this individual is available upon request (1)

Methodology: PCR was used to amplify the 16 coding exons and immediate flanking regions of the *MSH2* gene. The PCR products were sequenced in the forward and reverse directions. Reporting of intronic changes, if detected, is limited to the 20 nucleotides flanking exonic sequence. Nucleotide numbering is based on GenBank accession number NM_000251.1; nucleotide 1 corresponds to the A of the start codon ATG.

Reference: 1. www.ncbi.nlm.nih.gov/snp

NOTE: The interpretation of nucleotide changes is based on our current understanding of the *MSH2* gene. These interpretations may change over time as more information about these genes becomes available. Possible diagnostic errors include sample mix-ups, genetic variants that

interfere with analysis, and other sources.

This report was reviewed and approved by:

_____ _____

John Doe, M.D., Ph.D. Date

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Patient Name:	Jane D.	
ID number:	057398468	Patient and Family Information
DOB:	04/05/2007	Race/Ethnicity:
Gender:	Female	Family History:
Ordering Physician:	Dr. Good	Personal history:
Date of Report:	June 15, 2010	

Test Performed: Lynch syndrome: *MSH2* Gene Sequence Analysis

Indication for testing: Tumor from patient demonstrates absence of protein expression for both MSH2 and MSH6. Test for the presence of a mutation in the *MSH2* gene.

Specimen Type: Blood

Result: **Mutation detected**. One copy of an IVS5+3A>T splice site mutation was detected in the *MSH2* gene of this individual.

Interpretation:

A sample from this individual was referred to our laboratory for molecular testing for hereditary non-polyposis colon cancer (HNPCC). HNPCC is an autosomal dominant disorder characterized by an increased risk of colon and other cancers and is associated with tumors exhibiting MSI. HNPCC is caused by a germline mutation in a mismatch repair gene (*MLH1, MSH2, MSH6*, and *PMS2*). Mutations in *MLH1* and *MSH2* account for about 90% of mutations in families with HNPCC. A single mutation in one copy of the *MSH2* gene is associated with disease. Information provided to us indicates that this individual has colon cancer and that immunohistochemistry (IHC) testing is suggestive of a *MSH2* gene mutation.

Sequence analysis of the entire coding region of the *MSH2* gene identified an IVS5+3A>T mutation in intron 5. The IVS5+3A>T mutation has been reported as a mutation in patients with colon cancer and found to result in aberrant splicing of the *MSH2* RNA (1,2). These results must be interpreted in the context of the individual's clinical features. Genetic counseling is recommended for this individual and for other family members at risk for carrying this mutation.

Since we have documented the presence of a mutation in this affected family member, testing for at-risk individuals in this family is possible. The Laboratory offers targeted mutation analysis for family members at risk for carrying the mutations identified in this individual. For more

information on targeted testing, please visit http://XXXX or call (XXX) XXX-XXXX to speak with the laboratory genetic counselor.

Variants: A list of sequence variants unrelated to disease identified in this individual is available upon request (3)

Methodology: PCR was used to amplify the 16 coding exons and immediate flanking regions of the *MSH2* gene. The PCR products were sequenced in the forward and reverse directions. Reporting of intronic changes, if detected, is limited to the 20 nucleotides flanking exonic sequence. Nucleotide numbering is based on GenBank accession number NM_000251.1; nucleotide 1 corresponds to the A of the start codon ATG.

References:

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2. Auclair *et al.* 2006. *Hum. Mutat*. 27: 145-154.
3. ww.ncbi.nlm.nih.gov/snp

NOTE: The interpretation of nucleotide changes is based on our current understanding of the *MSH2* gene. These interpretations may change over time as more information about these genes becomes available. Possible diagnostic errors include sample mix-ups, genetic variants that interfere with analysis, and other sources.

This report was reviewed and approved by:

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Patient Name:	Jane D.	
ID number:	057398468	Patient and Family Information
DOB:	04/05/2007	Race/Ethnicity:
Gender:	Female	Family History:
Ordering Physician:	Dr. Good	Personal history:
Date of Report:	June 15, 2010	

Test Performed: Familial Adenomatous Polyposis (FAP): *APC* Gene Sequence Analysis

Indication for testing: Possible diagnosis of familial adenomatous polyposis (FAP). Test for the presence of a mutation in the *APC* gene.

Specimen Type: Blood

Result: No mutation detected

Interpretation:

A sample from this individual was sent to our laboratory for molecular testing for familial adenomatous polyposis (FAP). *APC*-associated polyposis conditions result from a mutation in the *APC* gene and cause a predisposition for colon cancer. Disorders in this category include familial adenomatous polyposis (FAP), attenuated FAP (AFAP), Gardner syndrome, and Turcot syndrome. Information provided to us indicates that this individual has a personal history of colon polyps and family history of colon cancer.

These results decrease the likelihood but do not rule out the diagnosis of FAP. Some individuals who have a diagnosis of FAP and involvement of the *APC* gene may have a mutation that is not identified by the methods described above (e.g. promoter mutations, etc.). Genetic counseling is recommended.

Colorectal cancer surveillance should be tailored based on the patient's clinical presentation. Genetic counseling is recommended.

Variants: A list of sequence variants unrelated to disease identified in this individual is available upon request (1)

Method:

PCR was used to amplify the 15 coding exons and immediate flanking regions of the *APC* gene. The PCR products were sequenced in the forward and reverse directions. Nucleotide numbering is based on GenBank accession number NM_000038.4; nucleotide 1 corresponds to the A of the start codon ATG.

Reference: 1. www.ncbi.nlm.nih.gov/snp

This report was reviewed and approved by:

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Patient Name:	Jane D.	
ID number:	057398468	Patient and Family Information
DOB:	04/05/2007	Race/Ethnicity:
Gender:	Female	Family History:
Ordering Physician:	Dr. Good	Personal history:
Date of Report:	June 15, 2010	

Test Performed: Familial Adenomatous Polyposis (FAP): *APC* Gene Sequence Analysis

Indication for testing: Possible diagnosis of familial adenomatous polyposis (FAP). Test for the presence of a mutation in the *APC* gene.

Specimen Type: Blood

Result: Mutation detected. One copy of a c.646C>T (p.R216X) nonsense mutation was detected in the *APC* gene of this individual.

Interpretation: Consistent with diagnosis of Familial Adenomatous Polyposis (FAP)

A sample from this individual was sent to our laboratory for molecular testing for familial adenomatous polyposis (FAP). *APC*-associated polyposis conditions result from a mutation in the *APC* gene and cause a predisposition for colon cancer. Disorders in this category include familial adenomatous polyposis (FAP), attenuated FAP (AFAP), Gardner syndrome, and Turcot syndrome. Information provided to us indicates that this individual has a personal history of colon polyps and family history of colon cancer.

Sequence analysis of the entire coding region of the *APC* gene identified a c.646C>T (p.R216X) nonsense mutation in exon 6. The c.646C>T mutation is predicted to result in the replacement of the codon for the amino acid arginine with a premature translation stop at codon 216 (p.R216X). The c.646C>T (p.R216X) mutation has been reported to be associated with FAP and is of a type predicted to cause disease (1).

This result must be interpreted in the context of the individual's clinical features. Genetic counseling is recommended for this individual and for other family members at risk for carrying this mutation.

The Laboratory offers targeted mutation analysis for family members at risk for carrying the mutations identified in this individual. For more information on custom diagnostic testing, please visit http://XXX or call (XXX) XXX-XXXX to speak with the laboratory genetic counselor.

Variants: A list of sequence variants unrelated to disease identified in this individual is available upon request (2)

Method:

PCR was used to amplify the 15 coding exons and immediate flanking regions of the *APC* gene. The PCR products were sequenced in the forward and reverse directions. Nucleotide numbering is based on GenBank accession number NM_000038.4; nucleotide 1 corresponds to the A of the start codon ATG.

References:

1. Lamlum et al., (1999) Nat Med 5: 1071-1075
2. www.ncbi.nih.gov/dbSNP

NOTE: The interpretation of nucleotide changes is based on our current understanding of the *APC* gene. These interpretations may change over time as more information about these genes becomes available. Possible diagnostic errors include sample mix-ups, genetic variants that interfere with analysis, and other sources.

This report was reviewed and approved by:

_____ _____

John Doe, M.D., Ph.D. Date

Director, The Molecular Laboratory

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www._____.com

Patient Name:	Jane D.	
ID number:	057398468	Patient and Family Information
DOB:	04/05/2007	Race/Ethnicity:
Gender:	Female	Family History:
Ordering Physician:	Dr. Good	Personal history:
Date of Report:	June 15, 2010	

Test Performed: Familial Adenomatous Polyposis (FAP): *APC* Gene Deletion/Duplication Analysis

Indication for testing: Possible diagnosis of familial adenomatous polyposis (FAP). Test for the presence of a mutation in the *APC* gene.

Specimen Type: Blood

Result: Mutation detected. A deletion mutation in the *APC* gene encompassing exon 1 to exon 5 was detected.

Interpretation: Consistent with diagnosis of Familial Adenomatous Polyposis (FAP)

A sample from this individual was sent to our laboratory for molecular testing for familial adenomatous polyposis (FAP). *APC*-associated polyposis conditions result from a mutation in the *APC* gene and cause a predisposition for colon cancer. Disorders in this category include familial adenomatous polyposis (FAP), attenuated FAP (AFAP), Gardner syndrome, and Turcot syndrome. No clinical information was provided to our laboratory for this individual. Previous sequence analysis performed in our laboratory did not detect a mutation in the *APC* gene of this individual (see report xxx).

A deletion mutation encompassing exon 1 to exon 5 with approximate genomic breakpoints between nucleotide positions g.111,991,287 and g.111,929,529 upstream of exon 1 and g.112,117,348 in intron 5 was detected in this individual. These results must be interpreted in the context of this individual's clinical features. Genetic counseling is recommended.

This result must be interpreted in the context of the individual's clinical features. Genetic counseling is recommended for this individual and for other family members at risk for carrying this mutation.

Since we have documented the presence of a mutation in this affected family member, testing for at-risk individuals in this family is possible. The Laboratory offers targeted mutation analysis for family members at risk for carrying the mutations identified in this individual. For more information on targeted testing, please visit http://XXX or call (XXX) XXX-XXXX to speak with the laboratory genetic counselor.

Method:

A DNA sample extracted from the blood sample was analyzed using a comparative genomic hybridization (CGH) array custom designed for analyzing the *APC* gene.

Note: Direct analysis of the *APC* gene deletions is highly accurate. Possible diagnostic errors include sample mix-ups, genotyping errors and rare genetic variants that interfere with analysis and other sources. Genomic coordinate numbering is based on GRCh37/hg19.

This report was reviewed and approved by:

_____ _____

John Doe, M.D., Ph.D. Date

Director, The Molecular Laboratory

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www._____.com

Patient Name:	Jane D.	
ID number:	057398468	Patient and Family Information
DOB:	04/05/2007	Race/Ethnicity:
Gender:	Female	Family History:
Ordering Physician:	Dr. Good	Personal history:
Date of Report:	June 15, 2010	

Test Performed: MUTYH-Associated Polyposis (MAP): *MUTYH* Gene-Targeted Mutation Analysis

Indication for testing: Patient has early-onset rectal cancer and previously tested negative for mutations in the *APC* gene. Test for the presence of bi-allelic mutations in the *MUTYH* gene.

Specimen Type: Blood

Result: No mutation detected.

Interpretation:

A sample from this individual was referred to our laboratory for molecular testing for mutations associated with *MYH*-associated polyposis (MAP). Mutations in the *MYH* gene account for a proportion of individuals with a clinical diagnosis of familial adenomatous polyposis (FAP) or attenuated FAP (AFAP) who do not have a detectable *APC* gene mutation. In these individuals, the polyp burden ranges from only a few to the hundreds typical of classic FAP. Information provided to us indicates that this individual had early onset colon cancer.

These results decrease the likelihood but do not rule out the possibility that the patient's rectal cancer is due to bi-allelic mutations in the *MUTYH* gene. Based on published information, the p.Y165C and p.G382D mutations account for approximately 80% of the mutations detected within the *MUTYH* gene. Genetic counseling is recommended.

Method:
A XXXX method was used to test DNA for the presence of the p.Y165C and p.G382D mutations in the *MUTYH* gene.

This report was reviewed and approved by:
_____ _____

John Doe, M.D., Ph.D. Date
Director, The Molecular Laboratory

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Patient Name:	Jane D.	
ID number:	057398468	Patient and Family Information
DOB:	04/05/1977	Race/Ethnicity:
Gender:	Female	Family History:
Ordering Physician:	Dr. Good	Personal history:
Date of Report:	June 15, 2010	

Test Performed: MUTYH-Associated Polyposis (MAP): *MYH* Gene-Targeted Mutation Analysis

Indication for testing: Patient has multiple adenomatous polyps. Test for the presence of bi-allelic mutations in the *MUTYH* gene.

Specimen Type: Blood

Result: Two copies of the p.Y165C mutation were identified.

Interpretation: Consistent with diagnosis of MUTYH-Associated Polyposis (MAP)

A sample from this individual was referred to our laboratory for molecular testing for mutations associated with *MYH*-associated polyposis (MAP). Mutations in the *MYH* gene account for a proportion of individuals with a clinical diagnosis of familial adenomatous polyposis (FAP) or attenuated FAP (AFAP) who do not have a detectable *APC* gene mutation. In these individuals, the polyp burden ranges from only a few to the hundreds typical of classic FAP. Information provided to us indicates that this individual had early onset colon cancer.

Results suggest that the multiple adenomas in this individual are caused by bi-allelic mutations within the *MUTYH* gene. Additionally, this individual is at increased risk for developing colon cancer and other extracolonic manifestations associated with MUTYH-associated polyposis (MAP). Appropriate screening procedures should be considered.

Identification of a mutation for this individual means that predictive testing can be performed for at-risk family members. Additionally, because this is an autosomal recessive disorder, carrier testing should be offered to the reproductive partner of XXXX. Genetic counseling is recommended.

Method:

A XXXX method was used to test DNA for the presence of the p.Y165C and p.G382D mutations in the *MUTYH* gene.

This report was reviewed and approved by:

_____ _____

John Doe, M.D., Ph.D. Date

Director, The Molecular Laboratory

Supplemental Table 1: PCR Primers for Amplification of *MLH1* Gene

Primer Set	Size (bp)	Primer Name	Primer Sequence	Comments	Method	GC addition
MLH1_Ex1	267	MLH1-1F-2	TGT AAA ACG ACG GCC AGT agg tga ttg gct gaa ggc ac		Stepdown	
		MLH1-1R-2	CAG GAA ACA GCT ATG ACC gcc cgt taa gtc gta gcc ct			
MLH1_Ex2	257	MLH1-2F-2	TGT AAA ACG ACG GCC AGT atg tac att aga gta gtt gca gac tga taa		Stepdown	
		MLH1-2R-2	CAG GAA ACA GCT ATG ACC agt ttc cag aac aga gaa agg tcc			
MLH1_Ex3	277	MLH1-3F-2	TGT AAA ACG ACG GCC AGT caa gaa aat ggg aat tca aag aga t		Stepdown	
		MLH1-3R-2	CAG GAA ACA GCT ATG ACC cta aca aat gac aga caa tgt cat cac			
MLH1_Ex4	257	MLH1-4F-2	TGT AAA ACG ACG GCC AGT cct ttg gtg agg tga cag tgg		Stepdown	
		MLH1-4R-2	CAG GAA ACA GCT ATG ACC cag gat tac tct gag acc tag gca a			
MLH1_Ex5	263	MLH1-5F-2	TGT AAA ACG ACG GCC AGT ttt tcc cct tgg gat tag tat cta tc		Stepdown	
		MLH1-5R-2	CAG GAA ACA GCT ATG ACC ccc tga aaa ctt aga agc aat ttt att t			
MLH1_Ex6	271	MLH1-6F-2	TGT AAA ACG ACG GCC AGT gga cat ctt ggg ttt tat ttt caa g		Stepdown	Add GC
		MLH1-6R-2	CAG GAA ACA GCT ATG ACC tgt tca atg tat gag cac tag aac aca			
MLH1_Ex7_8	453	MLH1-7/8F-2	TGT AAA ACG ACG GCC AGT ggg ctc tga cat cta gtg tgt gtt		Stepdown	
		MLH1-7/8R-2	CAG GAA ACA GCT ATG ACC aaa ata atg tga tgg aat gat aaa cca			
MLH1_Ex9	277	MLH1-9F-2	TGT AAA ACG ACG GCC AGT tct gat tct ttt gta atg ttt gag ttt tg		Stepdown	
		MLH1-9R-2	CAG GAA ACA GCT ATG ACC cat aaa att ccc tgt ggg tgt ttc			
MLH1_Ex10	287	MLH1-10F-2	TGT AAA ACG ACG GCC AGT ctg agg tga ttt cat gac ttt gtg t		Stepdown	
		MLH1-10R-2	CAG GAA ACA GCT ATG ACC gag gag agc ctg ata gaa cat ctg t			
MLH1_Ex11	317	MLH1-11F-2	TGT AAA ACG ACG GCC AGT gtg ggc ttt ttc tcc ccc t		Stepdown	
		MLH1-11R-2	CAG GAA ACA GCT ATG ACC ctc tca cgt ctg gcc gg			
MLH1_Ex12a	561	MLH1_Ex12Fa-2	TGT AAA ACG ACG GCC AGT CTCCATTTGGGGACCTGTAT		Stepdown	Add GC
		MLH1-12R-2	CAG GAA ACA GCT ATG ACC gtt tta tta cag aat aaa gga ggt agg ctg			
MLH1_Ex12b	619	MLH1_Ex12Fa-2	TGT AAA ACG ACG GCC AGT CTCCATTTGGGGACCTGTAT		Stepdown	Add GC
		MLH1_Ex12Ra-2	CAG GAA ACA GCT ATG ACC GGCAAGTCAGGCAGAGAGAA			
MLH1_Ex13	318	MLH1-13F-2	TGT AAA ACG ACG GCC AGT cca aaa tgc aac cca caa aat t		Stepdown	
		MLH1-13R-2	CAG GAA ACA GCT ATG ACC aac ctt ggc agt tga ggc c			
MLH1_Ex14	307	MLH1-14F-2	TGT AAA ACG ACG GCC AGT ggt gtc tct agt tct ggt gcc tg		Stepdown	
		MLH1-14R-2	CAG GAA ACA GCT ATG ACC tgc ctg tgc tcc ctg ga			
MLH1_Ex15	239	MLH1-15F-2	TGT AAA ACG ACG GCC AGT ccc att ttg tcc caa ctg gtt		Stepdown	
		MLH1-15R-2	CAG GAA ACA GCT ATG ACC gag agc tac tat ttt cag aaa cga tca g			
MLH1_Ex16	328	MLH1-16F-2	TGT AAA ACG ACG GCC AGT tgg gaa ttc agg ctt cat ttg		Stepdown	
		MLH1-16R-2	CAG GAA ACA GCT ATG ACC gca ccc ggc tgg aaa tt			
MLH1_Ex17	257	MLH1-17F-2	TGT AAA ACG ACG GCC AGT gca ctg gag aaa tgg gat ttg		Stepdown	
		MLH1-17R-2	CAG GAA ACA GCT ATG ACC cct cca gca cac atg cat g			
MLH1_Ex18	278	MLH1-18F-2	TGT AAA ACG ACG GCC AGT agt ctg tga tct ccg ttt aga atg ag		Stepdown	
		MLH1-18R-2	CAG GAA ACA GCT ATG ACC ttg tat gag gtc ctg tcc tag tcc t			
MLH1_Ex19	324	MLH1-19F-2	TGT AAA ACG ACG GCC AGT cat cag cca gga cac cag tg		Stepdown	
		MLH1-19R-2	CAG GAA ACA GCT ATG ACC cgg aat aca gag aaa gaa gaa cac a			

Supplemental Table 2: PCR Primers for Amplification of *MSH2* Gene

Primer Set	Size (bp)	Primer Name	Primer Sequence	Comments	Method	GC addition
MSH2_Ex1	321	MSH2-1Fa-2	CTG CT TGT AAA ACG ACG GCC AGT tcg cgc att ttc ttc aac c		Stepdown	Add GC
		MSH2-1Ra-2	CTG CT CAG GAA ACA GCT ATG ACC gtc cct ccc cag cac g			
MSH2_Ex2a	337	MSH2-2F-2	TGT AAA ACG ACG GCC AGT gaa gtc cag cta ata cag tgc ttg a		Stepdown	
		MSH2-2R-2	CAG GAA ACA GCT ATG ACC aaa cac aat taa att ctt cac att ttt att tt			
MSH2_Ex2b	270	MSH2-2Fseq-2	TGT AAA ACG ACG GCC AGT aag gag caa aga atc tgc aga g		Stepdown	
		MSH2-2R-2	CAG GAA ACA GCT ATG ACC aaa cac aat taa att ctt cac att ttt att tt			
MSH2_Ex3	467	MSH2-3Fa-2	CTG CT TGT AAA ACG ACG GCC AGT aga gtt tgg att ttt cct ttt tgc		Stepdown	
		MSH2-3Ra-2	CTG CT CAG GAA ACA GCT ATG ACC tca tgt caa tta aag agc ctt tcc			
MSH2_Ex4	352	MSH2-4F-2	TGT AAA ACG ACG GCC AGT ttc att ttt gct ttt ctt att cct ttt		Stepdown	
		MSH2-4R-2	CAG GAA ACA GCT ATG ACC ata tga cag aaa tat cct tct aaa aag tca cta t			
MSH2_Ex5a	321	MSH2-5F-2	TGT AAA ACG ACG GCC AGT act gga tcc agt ggt ata gaa atc ttc		Stepdown	
		MSH2-5R-2	CAG GAA ACA GCT ATG ACC gct tct tca gta tat gtc aat gaa aac a			
MSH2_Ex5b	238	MSH2-5F-2	TGT AAA ACG ACG GCC AGT act gga tcc agt ggt ata gaa atc ttc		Stepdown	
		MSH2-5Rseq-2	CAG GAA ACA GCT ATG ACC acc tga aaa agg tta agg gct ct			
MSH2_Ex6	287	MSH2-6F-2	TGT AAA ACG ACG GCC AGT gcg tag taa ggt ttt cac taa tga gc		Stepdown	
		MSH2-6R-2	CAG GAA ACA GCT ATG ACC cat gtg ggt aac tgc agg tta ca			
MSH2_Ex7	367	MSH2-7F-2	TGT AAA ACG ACG GCC AGT tga gac tta cgt gct tag ttg ata aat tt		Stepdown	
		MSH2-7R-2	CAG GAA ACA GCT ATG ACC gca cat tgc caa gta tat att gta tga g			
MSH2_Ex8	311	MSH2-8F-2	TGT AAA ACG ACG GCC AGT tga tgc ttg ttt atc tca gtc aaa att		Stepdown	
		MSH2-8R-2	CAG GAA ACA GCT ATG ACC aat cta caa act ttc tta aag tgg cct t			
MSH2_Ex9	253	MSH2-9Fa-2	CTG CT TGT AAA ACG ACG GCC AGT gtc ttt acc cat tat tta tag gat ttt gtc a		Stepdown	
		MSH2-9Ra-2	CTG CT CAG GAA ACA GCT ATG ACC gta tag aca aaa gaa tta ttc caa cct cc			
MSH2_Ex10	310	MSH2-10F-2	TGT AAA ACG ACG GCC AGT att gaa aaa tgg tag tag gta ttt atg gaa		Stepdown	
		MSH2-10R-2	CAG GAA ACA GCT ATG ACC cac atc atg tta gag cat tta ggg a			
MSH2_Ex11	285	MSH2-11Fa-2	CTG CT TGT AAA ACG ACG GCC AGT ata tgt ttc acg tag tac aca ttg ctt cta		Stepdown	
		MSH2-11Ra-2	CTG CT CAG GAA ACA GCT ATG ACC tca aat atc atg att ttt ctt ctg tta cc			
MSH2_Ex12	402	MSH2-12F-2	TGT AAA ACG ACG GCC AGT aat gta ttt tta cgg ctt ata tct gtt tat tat t		Stepdown	
		MSH2-12R-2	CAG GAA ACA GCT ATG ACC aaa caa aac gtt acc ccc aca			
MSH2_Ex13	412	MSH2-13Fa-2	CTG CT TGT AAA ACG ACG GCC AGT tat gtc agt gta aac cta cgc gat t		Stepdown	
		MSH2-13Ra-2	CTG CT CAG GAA ACA GCT ATG ACC tca cag gac aga gac ata cat ttc tat ct			
MSH2_Ex14	488	MSH2-14F-2	TGT AAA ACG ACG GCC AGT tgt ggc ata tcc ttc cca atg		Stepdown	
		MSH2-14R-2	CAG GAA ACA GCT ATG ACC aat aat tta tac taa ctt aga ata agg caa tta ctg at			
MSH2_Ex15	347	MSH2-15F-2	TGT AAA ACG ACG GCC AGT tac ata aat tgc tgt ctc ttc tca tgc		Stepdown	
		MSH2-15R-2	CAG GAA ACA GCT ATG ACC aaa aac ctt cat ctt agt gtc ctg ttt			
MSH2_Ex16	266	MSH2-16Fnew-2	TGT AAA ACG ACG GCC AGT taa tta cta atg gga cat tca cat gtg t		Stepdown	
		MSH2-16Rnew-2	CAG GAA ACA GCT ATG ACC tac ctt cat tcc att act ggg att t			

Supplemental Table 3: PCR Primers for Amplification of PMS2 Gene
Primers for Genomic PCR, Long-Range PCR and Allele Specific PCR to Distinguish Between Active and Pseudogene are Given

PMS2 Genomic PCR

Primer Set	Size (bp)	Primer Name	Primer Sequence	Comments	Method	GC addition
PMS2_Ex06	504	PMS2_g_Ex06-F	TGT AAA ACG ACG GCC AGT TGCTTCCCTTGATTTGTGCGATGAT	genomic	Stepdown	
		PMS2_g_Ex06-R	CAG GAA ACA GCT ATG ACC TGAGGCAGGAGAATTGCTTGAATCT			
PMS2_Ex06b	315	PMS2_g_Ex06b-F	TGT AAA ACG ACG GCC AGT TGTTGTAACTTGAGCTGTGTAATTC	genomic	Stepdown	
		PMS2_g_Ex06b-R	CAG GAA ACA GCT ATG ACC CTGGAAGGGACAATGGAAAC			
PMS2_Ex07b	245	PMS2_g_Ex07b-F	TGT AAA ACG ACG GCC AGT GCGCTTGTAATGTCAATAGCTTG	genomic	Stepdown	
		PMS2_g_Ex07b-R	CAG GAA ACA GCT ATG ACC TCAGGATAAAATGTTCAATTGTAGTTC			
PMS2_Ex07c	364	PMS2_g_Ex07-F	TGT AAA ACG ACG GCC AGT ACCCACGAGTTTGACATTGCAGTGA	genomic	Stepdown	
		PMS2_g_Ex07b-R	CAG GAA ACA GCT ATG ACC TCAGGATAAAATGTTCAATTGTAGTTC			
PMS2_Ex08	414	PMS2_g_Ex08-F	TGT AAA ACG ACG GCC AGT AGATTTGGAGCACAGATACCCGTGA	genomic	Stepdown	
		PMS2_1_Ex08-R	CAG GAA ACA GCT ATG ACC TGCGGTAGACTTCTGTAAATGCACA			
PMS2_Ex10	719	PMS2_g_Ex10-F	TGT AAA ACG ACG GCC AGT AGCCCTTCCGTATTTTGTCTATTCA	genomic	Stepdown	
		PMS2_g_Ex10-R	CAG GAA ACA GCT ATG ACC GCTTTAGAAGCTGTTTGTACACTGT			
PMS2_Ex10b	276	PMS2_g_Ex10b-F	TGT AAA ACG ACG GCC AGT TGAGACGCTGTCTGAAAATA	genomic	Stepdown	
		PMS2_g_Ex10b-R	CAG GAA ACA GCT ATG ACC GCTTTAGAAGCTGTTTGTAC			

PMS2 Long-range PCR

Primer Set	Size (bp)	Primer Name	Primer Sequence	Comments	Method	GC addition
PMS2_LR-1	9,964	PMS2_LR-1-F	Biotin-ACGTCGAAAGCAGCCAATGGGAGTT	Exon-01-05	PMS-LR	
		PMS2_LR-1-R	CTTCCACCTGTGCATACCACAGGCT			
PMS2_LR-2b	1,618	PMS2_LR-2b-F	Biotin-TTGCTTGTAATCTGCCAGATGTGGT	Exon-09	PMS-LR	
		PMS2_LR-2b-R	ATCTACTTTCTCCCTTGGTTGACAT			
PMS2_LR-3	8,812	PMS2_LR-3-F	Biotin-GCGTTGATATCAATGTTACTCCAGA	Exon-11-12	PMS-LR	
		PMS2_LR-3-R	AGTAGTCAGGGTAAAACATTCCAGT			
PMS2_LR-4	9,804	PMS2_LR-4-F	Biotin-AAAATTAGTCAGACTTGATGGTGTG	Exon-13-15	PMS-LR	
		PMS2_LR-4-R	CCTTCCATCTCCAAAACCAGCAAGA			

PMS2 Allele-specific PCR

Primer Set	Size (bp)	Primer Name	Primer Sequence	Comments	Method	GC addition
PMS2_Ex01	475	PMS2_1_Ex01-F	TGT AAA ACG ACG GCC AGT ACGTCGAAAGCAGCCAATGGGAGTT	PMS2_LR-1	Stepdown	
		PMS2_1_Ex01-R	CAG GAA ACA GCT ATG ACC CAGGTAGAAAGGAAATGCATTCAGT			
PMS2_Ex01b	221	PMS2_1_Ex01b-F	TGT AAA ACG ACG GCC AGT CTGGAGGGAACTTTCCCAGT	PMS2_LR-1	Stepdown	
		PMS2_1_Ex01b-R	CAG GAA ACA GCT ATG ACC CAACACTGAGGTCGCCACT			
PMS2_Ex02	455	PMS2_1_Ex02-F	TGT AAA ACG ACG GCC AGT ACAGTGTTGAGTCATTTCCCACAGT	PMS2_LR-1	Stepdown	
		PMS2_1_Ex02-R	CAG GAA ACA GCT ATG ACC TTCTTAGCATAACACCTGCCTGGCA			
PMS2_Ex02b	224	PMS2_Exon02b-F	TGT AAA ACG ACG GCC AGT TGTTTCTTGTAACTGATTTCTC	PMS2_LR-1	Stepdown	
		PMS2_Exon02b-R	CAG GAA ACA GCT ATG ACC CTTAACTACAACAACATTCACAG			
PMS2_Ex03b	225	PMS2_Exon03b_F	TGT AAA ACG ACG GCC AGT CTGATAGCATGGGTCCGTTT	PMS2_LR-1	Stepdown	
		PMS2_Exon03b_R	CAG GAA ACA GCT ATG ACC TTGCATTTCCCAAGACAGTG			
PMS2_Ex04b	261	PMS2_Exon04b_F	TGT AAA ACG ACG GCC AGT CACTGTCTTGGGAAATGCAA	PMS2_LR-1	Stepdown	
		PMS2_Exon04b_R	CAG GAA ACA GCT ATG ACC GTTTCTCTAAGGGGTCAAGTGAG			
PMS2_Ex05	540	PMS2_1_Ex05-F	TGT AAA ACG ACG GCC AGT CTTGATTATCTCAGAGGGATCGTCA	PMS2_LR-1	Stepdown	
		PMS2_1_Ex05-R	CAG GAA ACA GCT ATG ACC TCTCACTGTGTTGCCCAGTCCTAAT			

Supplemental Table 4: PCR Conditions for Genomic PCR, Stepdown PCR and Allele Specific PCR

Primer Set	Size (bp)	Primer Name	Primer Seq	Comments	Method	GC addition
MSH6_Ex1	523	MSH6 exon1F-2	TGT AAA ACG ACG GCC AGT tgt tga ttg gcc act ggg		Stepdown	Add GC
		MSH6 exon1Rnew-2	CAG GAA ACA GCT ATG ACC caa ccc cct gtg cga gcc tc			
MSH6_Ex2a	387	MSH6 exon2Fnew-2	TGT AAA ACG ACG GCC AGT taa ctg cct tta agg aaa ctt gac ca		Stepdown	
		MSH6 exon2Rnew-2	CAG GAA ACA GCT ATG ACC tca tat aga aaa aag tct gcc tgt ctg			
MSH6_Ex2b	315	MSH6 exon2Fnew-2	TGT AAA ACG ACG GCC AGT taa ctg cct tta agg aaa ctt gac ca		Stepdown	
		MSH6 exon2Rseq-2	CAG GAA ACA GCT ATG ACC tgg cag tag tga ctc tta cct gta tat g			
MSH6_Ex3	343	MSH6 exon3Fnew-2	TGT AAA ACG ACG GCC AGT ctg gtc ttg aac tgc tgg gat		Stepdown	
		MSH6 exon3R-2	CAG GAA ACA GCT ATG ACC ccc ctt tct tcc ccc atc			
MSH6_Ex4a	521	MSH6 exon4-1Fnew-2	TGT AAA ACG ACG GCC AGT tgc acg ggt acc att ata aag tca		Stepdown	
		MSH6 exon4-1R-2	CAG GAA ACA GCT ATG ACC gta ttc ttg gtt tct gat gaa atg cta g			
MSH6_Ex4b	456	MSH6 exon4-2F-2	TGT AAA ACG ACG GCC AGT gaa gga aac gcc ctc agc		Stepdown	
		MSH6 exon4-2R-2	CAG GAA ACA GCT ATG ACC cag ttg cct ttc atg aat acc ag			
MSH6_Ex4c	456	MSH6 exon4-3F-2	TGT AAA ACG ACG GCC AGT cca cat gga tgc tct tat tgg a		Stepdown	
		MSH6 exon4-3R-2	CAG GAA ACA GCT ATG ACC tca tct gaa aac tga cct atg aaa aac t			
MSH6_Ex4d	456	MSH6 exon4-4F-2	TGT AAA ACG ACG GCC AGT ttt gtt gat act tca ctg gga aag tt		Stepdown	
		MSH6 exon4-4R-2	CAG GAA ACA GCT ATG ACC ctc ctg atc aat aag gca ttt ttt g			
MSH6_Ex4e	456	MSH6 exon4-5F-2	TGT AAA ACG ACG GCC AGT ctc tag gtg gtt gtg tct tct acc tc		Stepdown	
		MSH6 exon4-5R-2	CAG GAA ACA GCT ATG ACC tga gta gcc tct caa gat ctg gaa			
MSH6_Ex4f	595	MSH6 exon4-6F-2a	TGT AAA ACG ACG GCC AGT CCC CAC TCT GTA ACC ATT ATG CTA		Stepdown	Add GC
		MSH6 exon4-6R-2	CAG GAA ACA GCT ATG ACC gtc cta cag cca att ctg ttg c			
MSH6_Ex4g	441	MSH6 exon4-7F-2	TGT AAA ACG ACG GCC AGT agc ctc ctg gaa tac cta gag aaa c		Stepdown	Add GC
		MSH6 exon4-7R-2	CAG GAA ACA GCT ATG ACC act tat ttt tag gga taa tat aca gct ggc			
MSH6_Ex4h		MSH6_Ex4-4aF	TGT AAA ACG ACG GCC AGT GCACGAGTGGAACAGACTGAGA		Stepdown	Add GC
		MSH6_Ex4-4R	CAG GAA ACA GCT ATG ACC ctc ctg atc aat aag gca ttt ttt g			
MSH6_Ex4i		MSH6_Ex4-5aF	TGT AAA ACG ACG GCC AGT AAAGCTAAGTGATGGCATTGGG		Stepdown	Add GC
		MSH6_Ex4-5R	CAG GAA ACA GCT ATG ACC tga gta gcc tct caa gat ctg gaa			
MSH6_Ex4j		MSH6_Ex4-7aF	TGT AAA ACG ACG GCC AGT GACCATGAAAAGGCTCGAAA		Stepdown	Add GC
		MSH6_Ex4-7R	CAG GAA ACA GCT ATG ACC act tat ttt tag gga taa tat aca gct ggc			
MSH6_Ex5	422	MSH6 exon5F-2	TGT AAA ACG ACG GCC AGT cac tta ggc tga taa aac ccc c		Stepdown	
		MSH6 exon5R-2	CAG GAA ACA GCT ATG ACC gta tgt tat tcc taa tgt cac aaa tga ctt t			
MSH6_Ex6	286	MSH6 exon6F-2	TGT AAA ACG ACG GCC AGT aag aca aaa gtt tat gaa act gtt act acc a		Stepdown	
		MSH6 exon6R-2	CAG GAA ACA GCT ATG ACC aga agc aaa tat ctt tta tca cat cta aat g			
MSH6_Ex7a	284	MSH6 exon7F-2	TGT AAA ACG ACG GCC AGT taa cct aga aga tga att tat gta ata tga ttt g		Stepdown	
		MSH6 exon7Rnew-2	CAG GAA ACA GCT ATG ACC ttc aga taa tct tct ata aaa atg gtt att tgt			
MSH6_Ex7b	141	MSH6 exon7Fseq-2	TGT AAA ACG ACG GCC AGT aag gtg aaa gta cat ttt ttg ttg aat ta		Stepdown	
		MSH6 exon7Rseq-2	CAG GAA ACA GCT ATG ACC tta atg tct tac cta att cat cca ca			
MSH6_Ex7c	226	MSH6 exon7F-2	TGT AAA ACG ACG GCC AGT taa cct aga aga tga att tat gta ata tga ttt g		Stepdown	
		MSH6_Ex7Rc-2	CAG GAA ACA GCT ATG ACC TCT TCA AAT GAG AAG TTT AAT GTC TTA C			
MSH6_Ex7d	172	MSH6 Ex7dF	TGT AAA ACG ACG GCC AGT AGCA*CATTCTCTGGTGCTTGTGG*		Stepdown	Add GC
		MSH6 Ex7dR	CAG GAA ACA GCT ATG ACC tctgtgccacaatggtgagt			
MSH6_Ex7e	302	MSH6 exon7F-2	TGT AAA ACG ACG GCC AGT taa cct aga aga tga att tat gta ata tga ttt g		Stepdown	Add GC
		MSH6 Ex7dR	CAG GAA ACA GCT ATG ACC tctgtgccacaatggtgagt			
MSH6_Ex8a	306	MSH6 exon8Fa-2	CTG CT TGT AAA ACG ACG GCC AGT tga gtt act tcc tta tgc ata ttt tac t		Stepdown	
		MSH6 exon8Ra-2	CTG CT CAG GAA ACA GCT ATG ACC aat att agc gat aca tgt gct aga a			

MSH6_Ex8b	331	MSH6 exon8F-2-2	CTG CT TGT AAA ACG ACG GCC AGT tgt tgc ttt tct gtc cta gca ttt t		Stepdown	
		MSH6 exon8R-2-2	CTG CT CAG GAA ACA GCT ATG ACC gtg ccc tct caa aaa acc gaa			
MSH6_Ex9	353	MSH6 exon9Fnew-2	TGT AAA ACG ACG GCC AGT tgc tag cac atg tat cgc taa tat t		Stepdown	
		MSH6 exon9Rnew-2	CAG GAA ACA GCT ATG ACC gca tca tcc ctt ccc ctt tta			
MSH6_Ex10a	331	MSH6 exon10F-2	TGT AAA ACG ACG GCC AGT gaa ggg atg atg cac tat gaa aaa		Stepdown	
		MSH6 exon10R-2	CAG GAA ACA GCT ATG ACC gta gaa ggt aga taa gaa tta aaa ggg ttt aat tt			
MSH6_Ex10b	258	MSH6 exon10Fseq-2	TGT AAA ACG ACG GCC AGT ggc tag tga aag gtc aac tgt aga tgc		Stepdown	
		MSH6 exon10R-2	CAG GAA ACA GCT ATG ACC gta gaa ggt aga taa gaa tta aaa ggg ttt aat tt			

sequencing primer	MSH6_Ex7Fseq-2	TTTTTGTTGAATTAAGTGAAACTGC	for common indel in 3' intronic

Supplemental Table 3: PCR Primers for Amplification of PMS2 Gene
Primers for Genomic PCR, Long-Range PCR and Allele Specific PCR to Distinguish Between Active and Pseudogene are Given

PMS2_Ex09	279	PMS2_2b_Ex09-F	TGT AAA ACG ACG GCC AGT CCTTCTAAGAACATGCTGGTTGGTT	PMS2_LR-2b	Stepdown	
		PMS2_2b_Ex09-R	CAG GAA ACA GCT ATG ACC ATCTCATTCCAGTCATAGCAGAGCT			
PMS2-11c	575	PMS2_3_Ex11c-F	TGT AAA ACG ACG GCC AGT CGTCCTCTCACCATTTCAGG	PMS2_LR-3	Stepdown	
		PMS2_3_Ex11c-R	CAG GAA ACA GCT ATG ACC TTTGAATGGCAGTCCACATC			
PMS2-11d	635	PMS2_3_Ex11d-F	TGT AAA ACG ACG GCC AGT GAGGTGGAGAAGGACTCGG	PMS2_LR-3	Stepdown	
		PMS2_3_Ex11d-R	CAG GAA ACA GCT ATG ACC tgcgcaacagagcaagac			
PMS2_Ex12	493	PMS2_3_Ex12-F	TGT AAA ACG ACG GCC AGT GCCAAGATTGTGCCATTGCACTGTA	PMS2_LR-3	Stepdown	
		PMS2_3_Ex12-R	CAG GAA ACA GCT ATG ACC AGTAGATACAAGGTCTTGCTGTGTT			
PMS2_Ex12b	379	PMS2_3_Ex12b-F	TGT AAA ACG ACG GCC AGT CCTGGGTGACAGAACGAGAC	PMS2_LR-3	Stepdown	
		PMS2_3_Ex12b-R	CAG GAA ACA GCT ATG ACC TCAATTTGAGGGGGAGTCTG			
PMS2_Ex13	372	PMS2_4_Ex13-F	TGT AAA ACG ACG GCC AGT GTGACACTTAGCTGAGTAGTGTTGT	PMS2_LR-4	Stepdown	
		PMS2_4_Ex13-R	CAG GAA ACA GCT ATG ACC ATGTTAGCCAGGCTGGTCTCAAACT			
PMS2_Ex14	473	PMS2_4_Ex14-F	TGT AAA ACG ACG GCC AGT GGTCTGTATCTCCTGACCTCATGAT	PMS2_LR-4	Stepdown	
		PMS2_4_Ex14-R	CAG GAA ACA GCT ATG ACC GCACGTAGCTCTCTGTGTAAAATGA			
PMS2_Ex15	522	PMS2_4_Ex15-F	TGT AAA ACG ACG GCC AGT GCTGAGATCTAGAACCTAGGCTTCT	PMS2_LR-4	Stepdown	
		PMS2_4_Ex15-R	CAG GAA ACA GCT ATG ACC ACACACGAGCGCATGCAAACATAGA			
PMS2_Ex15b	209	PMS2_4_Ex15b-F	TGT AAA ACG ACG GCC AGT CGTTGAACCATTGTGTCTCAC	PMS2_LR-4	Stepdown	
		PMS2_4_Ex15b-R	CAG GAA ACA GCT ATG ACC TCTGCGATAAAACCAATTATTCC			
PMS2_Ex15c	272	PMS2_4_Ex15c-F	TGT AAA ACG ACG GCC AGT GCTTCTGAAGAGCTGCCATT	PMS2_LR-4	Stepdown	
		PMS2_4_Ex15c-R	CAG GAA ACA GCT ATG ACC TGTCTTTCAAAACATAAAAATCTGC			