VENTANA anti-MSH6 (SP93) Rabbit Monoclonal Primary Antibody

For use with the VENTANA MMR IHC Panel

REF 760-5092

08033676001

IVD Σ 50

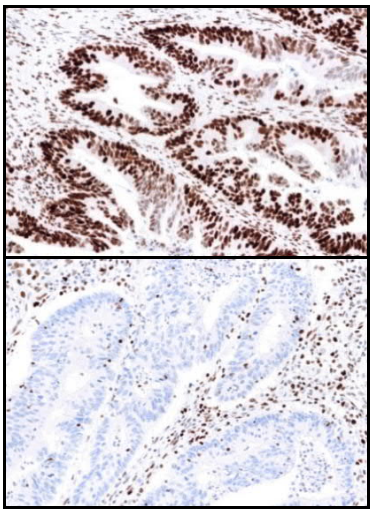

Figure 1. VENTANA anti-MSH6 (SP93) Rabbit Monoclonal Primary Antibody staining with Intact (top) or Loss (bottom) of expression in colon cancer tissue

INTENDED USE

VENTANA anti-MSH6 (SP93) Rabbit Monoclonal Primary Antibody (VENTANA anti-MSH6 (SP93) antibody) is intended for the qualitative detection of MSH6 protein in formalin-fixed, paraffin-embedded tissue sections. VENTANA- anti-MSH6 (SP93) antibody is ready to use on BenchMark ULTRA, XT and GX instruments with the OptiView DAB IHC Detection Kit and ancillary reagents.

VENTANA anti-MSH6 (SP93) antibody is part of the VENTANA MMR IHC Panel which includes VENTANA anti-MLH1 (M1) Mouse Monoclonal Primary Antibody, VENTANA anti-PMS2 (A16-4) Mouse Monoclonal Primary Antibody, VENTANA anti-MSH2 (G219-1129) Mouse Monoclonal Primary Antibody and VENTANA anti-BRAF V600E (VE1) Mouse Monoclonal Primary Antibody. The VENTANA MMR IHC Panel is indicated for the detection of mismatch repair protein deficiency as a test for the identification of individuals at risk for Lynch syndrome in patients diagnosed with colorectal cancer (CRC), and, with BRAF V600E status, as an aid to differentiate between sporadic and probable Lynch syndrome CRC in the absence of MLH1 protein expression.

These products should be interpreted by a qualified pathologist in conjunction with histological examination, relevant clinical information, and proper controls.

Intended for in vitro diagnostic (IVD) use.

SUMMARY AND EXPLANATION

Colorectal cancer is the third most common cancer and the fourth most prevalent cause of death in the world.[1] The majority of CRCs show chromosomal instability, however approximately 15% of cancers develop through an alternative pathway characterized by defective function of the DNA mismatch repair (MMR) system. As a consequence of the MMR deficiency, tumors exhibit microsatellite instability (MSI) resulting from the inability of MMR proteins to repair DNA replication errors.

CRCs with MMR defects are denoted as deficient MMR (dMMR) tumors. In contrast, CRCs with no MMR defects are denoted as proficient MMR (pMMR) tumors. The dMMR colorectal cancers are often poorly differentiated and frequently show proximal colon predominance, mucinous, medullary, or signet ring histologic features and increased numbers of tumor-infiltrating lymphocytes.[2,3] In general, MMR deficiency may be caused either by germline mutations in one of the MMR genes with subsequent loss of the corresponding normal allele through genetic or epigenetic mechanisms, somatic mutations in the alleles, or by epigenetic inactivation of the MLH1 gene through methylation.[4]

The four most commonly mutated MMR genes are MLH1, PMS2, MSH2, and MSH6. In normal cells, the MLH1 protein forms a complex (heterodimer) with the PMS2 protein, while the MSH2 protein forms a complex with the MSH6 protein.[5,6] When DNA mismatches occur, the MSH2/MSH6 heterodimer binds to the mismatched DNA, inducing a conformational change. The MLH1/PMS2 heterodimer binds the DNA-bound MSH2/MSH6 complex resulting in excision repair of the affected DNA.

The MLH1, PMS2, MSH2, and MSH6 proteins are clinically important MMR proteins encoded by genes that may be mutated in families with Lynch syndrome.[7,8] Carriers of these mutations have a high lifetime risk of developing colorectal and other cancers due to accumulation of DNA replication errors in proliferating cells. Lynch syndrome represents 1-6% of all CRCs. These tumors result from the inheritance of a germline autosomal dominant mutation in one of the four MMR genes, with MLH1 loss occurring in the majority of these Lynch syndrome associated CRCs.[5,9,10] More than 300 different mutations in the MMR family of proteins have been identified in patients with Lynch syndrome. The Lynch syndrome-associated tumor phenotype is generally characterized by immunohistochemical loss of expression in MMR proteins, particularly MLH1, PMS2, MSH2, and MSH6.[10-13] MMR IHC testing has been shown to be useful in the identification of the specific MMR gene in which either a germline or a somatic alteration is most likely to be found.[14]

As part of the VENTANA MMR IHC Panel, VENTANA anti-BRAF V600E (VE1) Mouse Monoclonal Primary Antibody (VENTANA anti-BRAF V600E (VE1) antibody) aids to differentiate sporadic and probable Lynch syndrome CRC in the absence of MLH1 protein expression.[15,16] In CRC, loss of MLH1 protein is frequently the result of hypermethylation of the MLH1 promoter and indicates a sporadic occurrence.[17] The presence of the BRAF V600E protein is tightly linked with hypermethylation of the MLH1 promoter. As a result, a positive staining result with VENTANA anti-BRAF V600E (VE1) antibody indicates sporadic CRC.

CLINICAL SIGNIFICANCE

Lynch syndrome was described in the 1960s and identified a link between the loss of MMR function and cancer.[18] Loss of MMR proteins (MLH1, PMS2, MSH2 or MSH6) may lead to MSI and a higher lifetime risk of not only CRC, but also cancers of the stomach, brain, pancreas, skin, endometrium and ovaries. Patients with Lynch syndrome have a 50-80% lifetime risk for CRC.[5,19,20] Lynch syndrome is unique from other hereditary cancer syndromes as direct testing on tumor tissue aids in the identification of patients at risk for Lynch syndrome and helps inform subsequent germline genetic testing. Families with Lynch syndrome benefit from advanced cancer screening protocols.

Various guidelines, including National Comprehensive Cancer Network (NCCN) guidelines, recommend that all CRCs should be screened for potential Lynch syndrome to identify patients and families that will benefit from further genetic testing and counseling.[18,21-24] Using the VENTANA MMR IHC Panel will aid in determining the MMR status of CRCs by classifying them as intact or loss for MMR protein expression. Detection of all four MMR proteins in the tumor indicates normal or intact MMR. Loss of MLH1 or MSH2 expression is almost invariably accompanied with the loss of its heterodimer partner, PMS2 or MSH6, respectively. However, loss of PMS2 or MSH6 does not lead to loss of MLH1 or MSH2. Loss of PMS2, MSH2, and/or MSH6 is consistent with probable Lynch Syndrome, and patients should be referred for additional testing and counseling consistent with clinical practice.

Loss of MLH1 protein may indicate a sporadic occurrence or potential Lynch syndrome. In 15% or more of sporadic CRC, loss of MLH1 protein is due to hypermethylation of the MLH1 promoter.[5,25,26] Importantly, the BRAF V600E mutation is observed in about two thirds of tumors with loss of MLH1 expression from MLH1 promoter hypermethylation. In contrast, the BRAF V600E mutation is very rarely observed in Lynch syndrome tumors.[25] Therefore, if the result of the VENTANA anti-MLH1 (M1) Mouse Monoclonal Primary Antibody (VENTANA anti-MLH1 (M1) antibody) indicates loss of MLH1 protein, VENTANA anti-BRAF V600E (VE1) antibody may stratify the tumor as sporadic or probable Lynch syndrome.[5,27] In CRC, loss of MLH1 protein with a BRAF V600E status of positive strongly indicates that the tumor is the result of a sporadic occurrence, virtually eliminating Lynch syndrome as the underlying cause of malignancy.[17,28] When loss of MLH1 protein is accompanied with a BRAF V600E status of negative, the MLH1 loss is consistent with a high probability of Lynch syndrome.[29]

PRINCIPLE OF THE PROCEDURE

VENTANA anti-MSH6 (SP93) antibody Rabbit Monoclonal Primary Antibody (VENTANA anti-MSH6 (SP93) antibody) is a rabbit monoclonal antibody produced against a synthetic peptide corresponding to an internal region of human MSH6 protein. The VENTANA anti-MSH6 (SP93) antibody binds to MSH6 protein in formalin-fixed paraffin-embedded (FFPE) tissue sections. The antibody can be localized using a haptenated secondary antibody followed by a multimer anti-hapten-HRP conjugate (OptiView DAB IHC Detection Kit, Cat. No. 760-700 / 06396500001). The specific antibody-enzyme complex is then visualized with a precipitating enzyme reaction product. Each step is incubated for a precise time and temperature. At the end of each incubation step, each BenchMark ULTRA, XT, and GX instrument washes the sections to stop the reaction and to remove unbound material that would hinder the desired reaction in subsequent steps. It also applies ULTRA LCS (ULTRA LCS (Predilute), Cat. No. 650-210 / 05424534001) or LCS (LCS predilute, Cat. No. 650-010 / 05264839001), which minimizes evaporation of the aqueous reagents from the specimen slide.

In addition to staining with VENTANA anti-MSH6 (SP93) antibody, a second slide should be stained with the rabbit monoclonal negative reagent, Rabbit Monoclonal Negative Control Ig (Cat. No. 790-4795 / 06683380001). The negative reagent control is used to assess background staining.

REAGENT PROVIDED

VENTANA anti-MSH6 (SP93) antibody contains sufficient reagent for 50 tests.

One 5 mL dispenser of VENTANA anti-MSH6 (SP93) antibody contains approximately 5 µg of a rabbit monoclonal antibody.

The antibody is diluted in 0.05M Tris-HCl with 1% carrier protein, and 0.10% ProClin 300, a preservative.

Total protein concentration of the reagent is approximately 10 mg/mL. Specific antibody concentration is approximately 1 µg/mL. There is no known nonspecific antibody reactivity observed in this product.

VENTANA anti-MSH6 (SP93) antibody is a recombinant rabbit monoclonal antibody produced as purified cell culture supernatant.

Refer to the appropriate VENTANA detection kit package insert for detailed descriptions of: (1) Principles of the Procedure, (2) Materials and Reagents Needed, but Not Provided, (3) Specimen Collection and Preparation for Analysis, (4) Quality Control Procedures, (5) Troubleshooting, (6) Interpretation of Results, and (7) General Limitations.

MATERIALS REQUIRED BUT NOT PROVIDED

Staining reagents, such as VENTANA detection kits and ancillary components, including negative and positive tissue control slides, are not provided.

The following reagents and materials are required for staining but are not provided:

1. Additional VENTANA MMR IHC Panel antibodies:
   - VENTANA anti-MLH1 (M1) Mouse Monoclonal Primary Antibody (Cat. No. 760-5091 / 08033668001)
   - VENTANA anti-PMS2 (A16-4) Mouse Monoclonal Primary Antibody (Cat. No. 760-5094 / 08033692001)
   - VENTANA anti-MSH2 (G219-1129) Mouse Monoclonal Primary Antibody (Cat. No. 760-5093 / 08033684001)
   - VENTANA anti-BRAF V600E (VE1) Mouse Monoclonal Primary Antibody (Cat. No. 760-5095 / 08033706001)
2. Rabbit Monoclonal Negative Control Ig (Cat. No. 790-4795 / 06683380001)
3. Microscope slides, positively charged
4. Bar code labels (appropriate for negative reagent control and primary antibody being tested)
5. Xylene (Histological grade)
6. Ethanol or reagent alcohol (Histological grade)
   - 100% solution: Undiluted ethanol or reagent alcohol
   - 95% solution: Mix 95 parts of ethanol or reagent alcohol with 5 parts of deionized water
   - 80% solution: Mix 80 parts of ethanol or reagent alcohol with 20 parts of deionized water
7. Deionized or distilled water
8. OptiView DAB IHC Detection Kit (Cat. No. 760-700 / 06396500001)
9. For VENTANA anti-PMS2 (A16-4) Mouse Monoclonal Primary Antibody, OptiView Amplification Kit (Cat. No. 760-099 / 06396518001 or Cat. No. 860-099 / 06718663001)
10. EZ Prep Concentrate (10X) (Cat. No. 950-102 / 05279771001)
11. Reaction Buffer Concentrate (10X) (Cat. No. 950-300 / 05353955001)
12. ULTRA LCS (Predilute) (Cat. No. 650-210 / 05424534001) or LCS (LCS predilute Cat. No. 650-010 / 05264839001)
13. ULTRA Cell Conditioning Solution (ULTRA CC1) (Cat. No. 950-224 / 05424569001) or Cell Conditioning 1 (CC1) Cat. No. 950-124 / 05279801001)
14. Hematoxylin II (Cat. No. 790-2208 / 05277965001)
15. Bluing Reagent (Cat. No. 760-2037 / 05266769001)
16. Permanent mounting medium (Permount Fisher Cat. No. SP15-500 or equivalent)
17. Cover glass (sufficient to cover tissue, such as VWR Cat. No. 48393-060)
18. Automated coverslipper (such as the Tissue-Tek SCA Automated Coverslipper)
19. Light microscope
20. Absorbent wipes

STORAGE

Upon receipt and when not in use, store at 2-8°C. Do not freeze.

To ensure proper reagent delivery and the stability of the antibody, replace the dispenser cap after every use and immediately place the dispenser in a refrigerator in an upright position.

Every antibody dispenser is expiration dated. When properly stored, the reagent is stable to the date indicated on the label. Do not use reagent beyond the expiration date.

SPECIMEN PREPARATION

Routinely processed, FFPE tissues are suitable for use with this primary antibody when used with OptiView DAB IHC Detection Kit and the BenchMark ULTRA, XT and GX instruments.

Sections should be cut approximately 4 µm thick and mounted on positively-charged glass slides. Fresh cut slides should be used for staining, as antigenicity of cut tissue sections may diminish over time. It is recommended that positive and negative controls be run simultaneously with unknown specimens.

For the VENTANA MMR IHC Panel, it is recommended tissue be fixed within 6 hours of excision in 10% neutral buffered formalin (NBF) for 6-24 hours on the basis of staining in tonsil. Acceptable staining was also achieved with fixation in Zinc formalin and Z-5 fixatives for 6-24 hours. Alcohol formalin (AFA), 95% Ethanol, and PREFER fixatives are not recommended for use with the VENTANA MMR IHC Panel as tissues demonstrate no or variable staining.

The amount of fixative used should be 15 to 20 times the volume of tissue. No fixative will penetrate more than 2 to 3 mm of solid tissue or 5 mm of porous tissue in a 24-hour period. Fixation can be performed at room temperature (15°–25°C).[30,31]

WARNINGS AND PRECAUTIONS

1. For in vitro diagnostic (IVD) use.
2. For Professional Use Only
3. ProClin 300 solution is used as a preservative in this reagent. It is classified as an irritant and may cause sensitization through skin contact. Take reasonable precautions when handling. Avoid contact of reagents with eyes, skin, and mucous membranes. Use protective clothing and gloves.
4. Materials of human or animal origin should be handled as biohazardous materials and disposed of with proper precautions.
5. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water.
6. Avoid microbial contamination of reagents as it may cause incorrect results.
7. Consult local and/or state authorities with regard to recommended method of disposal.
8. For supplementary safety information, refer to the product Safety Data Sheet and the Symbol and Hazard Guide located at www.ventana.com.

STAINING PROCEDURE

VENTANA anti-MSH6 (SP93) antibody has been developed for use on BenchMark ULTRA, XT, and GX instruments in combination with the OptiView DAB IHC Detection Kit, and ancillary reagents. Table 1 lists the staining protocol for use with VENTANA anti-MSH6 (SP93) antibody. The effect of varying time and temperature of the antigen retrieval on assay robustness is unknown. Thus, deviation from the recommended conditions for antigen retrieval provided in the listed protocol may invalidate expected results. Appropriate controls should be employed and documented. Users who deviate from the listed protocol must accept responsibility for interpretation of patient results.

The parameters for the automated procedures can be displayed, printed, and edited according to the procedure in the instruments Operator's Manual. Refer to the package insert for the OptiView DAB IHC Detection Kit for more details regarding IHC staining procedures.

Table 1. Staining Protocol for VENTANA anti-MSH6 (SP93) antibody with OptiView DAB IHC Detection Kit on a BenchMark ULTRA, XT, and GX instrument

Procedure Type	Method		
	BenchMark ULTRA Instrument	BenchMark XT Instrument	BenchMark GX Instrument
Deparaffinization	Selected	Selected	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, 64 minutes, 100°C	Cell Conditioning 1, 64 minutes	Cell Conditioning 1, 64 minutes
Pre-Primary Peroxidase Inhibitor	Selected	Selected	Selected
Antibody (Primary)	12 minutes, 36°C	12 minutes, 37°C	12 minutes, 37°C
OptiView HQ Linker	8 minutes (default)		
OptiView HRP Multimer	8 minutes (default)		
Counterstain	Hematoxylin II, 4 minutes		
Post Counterstain	Bluing, 4 minutes		

Deviation from the recommended conditions, especially for antigen retrieval, provided in the listed protocol may invalidate expected results. However, due to variation in tissue fixation and processing, as well as general lab instrument and environmental conditions, it may be necessary to increase or decrease the primary antibody incubation based on individual specimens, and reader preference. For further information on fixation variables, refer to "Immunohistochemistry: Principles and Advances".[32]

QUALITY CONTROL PROCEDURES

Negative Reagent Control

Ventana Medical Systems, Inc. strongly recommends a negative reagent control be used to stain an adjacent section of the patient specimen tissue on a separate slide from the VENTANA anti-MSH6 (SP93) antibody stained slide. A negative reagent control rabbit monoclonal antibody (Rabbit Monoclonal Negative Control Ig, Cat. No. 790-4795 / 06683380001), is recommended for use in place of the primary antibody to evaluate nonspecific staining. The staining parameters for the negative reagent control antibody should be the same as that for the primary antibody.

Positive Tissue Control

A positive tissue control must be run with every staining procedure performed. Optimal laboratory practice is to include a positive control section on the same slide as the patient tissue. This practice helps to identify a failure to apply primary antibody or other critical reagent to the patient test slide. A tissue with weak positive staining is more suitable for optimal quality control. The positive staining tissue components are used to confirm that the antibody was applied and the instrument functioned properly. This tissue may contain both positive and negative staining cells or tissue components and serve as both the positive and negative control tissue. Control tissues should be fresh autopsy, biopsy, or surgical specimens prepared or fixed as soon as possible in a manner identical to the test sections. Such tissues may monitor all steps of the procedure from tissue preparation through staining. Use of a tissue section fixed or processed differently from the test specimen will provide control for all reagents and method steps except fixation and tissue processing.

Known positive tissue controls should be utilized only for monitoring the correct performance of processed tissues and test reagents, not as an aid in determining a specific diagnosis of patient samples. If the positive tissue controls fail to demonstrate positive staining, results with the test specimens should be considered invalid.

CRC tissue with an MSH6 Clinical Status of intact, or normal colon tissue pre-qualified with VENTANA anti-MSH6 (SP93) antibody may be used as a positive tissue control. Normal colon will stain positive for MSH6 using the VENTANA anti-MSH6 (SP93) antibody. The positive tissue control should exhibit unequivocal nuclear staining in viable tumor and/or normal colon tissue elements. For all tissues, internal positive control cells (i.e. lymphocytes, fibroblasts, or normal epithelium in the vicinity of the tumor) should stain positive in the nucleus.

Negative Tissue Control

Since the MLH1, PMS2, MSH2, and MSH6 proteins are expressed in all tissues, a normal negative tissue control does not exist for these biomarkers. However, CRC tissue with a MSH6 Clinical Status of Loss pre-qualified with VENTANA anti-MSH6 (SP93) antibody may be used as a negative tissue control. The negative tissue control should be used only to monitor the correct performance of processed tissues, test reagents and instruments and not as an aid in formulating a specific diagnosis of patient samples.

Assay Verification

Prior to initial use of an antibody or staining system in a diagnostic procedure, the specificity of the antibody should be verified by testing on a series of tissues with known IHC performance characteristics representing tissues Intact for MSH6 Clinical Status. (Refer to the Quality Control Procedures previously outlined in this section of the product insert and to the Quality Control recommendations of the College of American Pathologists Laboratory Accreditation Program, Anatomic Pathology Checklist[33] or the CLSI Approved Guideline.[30])

STAINING INTERPRETATION / EXPECTED RESULTS

The cellular staining pattern for VENTANA anti-MSH6 (SP93) antibody is nuclear in actively proliferating cells. CRC stained with VENTANA anti-MSH6 (SP93) antibody is assigned a Clinical Status by a trained pathologist based on their evaluation of the presence or absence of specific nuclear staining in the tumor. A Clinical Status of Intact is assigned to cases with unequivocal nuclear staining in viable tumor cells, in the presence of acceptable internal positive controls (nuclear staining in lymphocytes, fibroblasts, or normal epithelium in the vicinity of the tumor). A Clinical Status of Loss is assigned to cases with unequivocal loss of nuclear staining or focal weak equivocal nuclear staining in the viable tumor cells in the presence of internal positive controls as shown in Table 2.

If unequivocal nuclear stain is absent in internal positive controls and/or background staining interferes with interpretation, the assay should be considered unacceptable and repeated. Punctate nuclear staining of tumor cells should be considered negative (Loss). In cases with focal tumor cell staining, the intensity of the nuclear staining should be at least that of the internal positive controls along with the confluent /continuous staining of the nuclei in a few epithelial glands or nests for the case to be given a Clinical Status of Intact. In the absence of these conditions, a Clinical Status of Loss is given to the case.

Table 2. Staining interpretation for VENTANA anti-MSH6 (SP93) antibody

Clinical Status	Description
Intact MSH6 Expression	Unequivocal nuclear staining in viable tumor cells, in the presence of internal positive controls (nuclear staining in lymphocytes, fibroblasts, or normal colonic epithelium in the vicinity of the tumor)
Loss of MSH6 Expression	Unequivocal loss of nuclear staining or focal weak equivocal nuclear staining in the viable tumor cells in the presence of internal positive controls

VENTANA anti-MSH6 (SP93) antibody stained cases are categorized as Intact or Loss according to the presence or absence of specific staining in the tumor.

SPECIFIC LIMITATIONS

Ventana Medical Systems, Inc. provides antibodies and reagents at optimal dilution for use when the provided instructions are followed. Deviation from the recommended conditions for antigen retrieval provided in the listed protocol may invalidate expected results. Appropriate controls should be employed and documented. Users who deviate from the listed protocol must accept responsibility for interpretation of patient results.

For the VENTANA MMR IHC Panel, it is recommended that tissue specimens be fixed within 6 hours after collection for 6-24 hours in 10% neutral buffered formalin. Acceptable staining is observed following fixation for 6-24 hours using zinc formalin or Z-5. It is not recommended to fix tissues with 95% alcohol, alcohol formalin acetic acid (AFA), or PREFER fixatives.

Some cases may be particularly challenging due to the following issues:

- Nonspecific background: Some specimens may exhibit nonspecific background staining for reasons that are not well understood. For this reason, evaluation of a VENTANA anti-MSH6 (SP93) antibody slide must include a comparison of the slide to the negative reagent control slide to determine the level of nonspecific background staining. Cytoplasmic staining, if present, should be disregarded in VENTANA anti-MSH6 (SP93) antibody IHC interpretation.
- Focal Staining: Some specimens may exhibit focal staining in the tumor cells and staining intensity may vary from weak to strong. Based on the VENTANA anti-MSH6 (SP93) antibody IHC scoring algorithm, focal weak equivocal nuclear staining in the viable tumor cells in the presence of internal positive controls should be categorized as Loss. The staining can vary in the level of intensity and this intensity may vary throughout the tumor; however, this does not impact MSH6 Clinical Status.
- Punctate Staining: Some specimens may exhibit discrete punctate staining within a few nuclei of the tumor; the staining intensity may vary from weak to strong. This staining pattern should be ignored and if a case has only this type of staining pattern, it should be given a Clinical Status of Loss.
- Tissue or Staining Artifact: Histologic artifacts originating from the sample processing and microtomy processes can also complicate the determination of VENTANA anti-MSH6 (SP93) antibody IHC Clinical Status. These artifacts may include, but are not limited to, fixation gradients and edge effects, DAB trapping, nuclear bubbling, lack of staining in some regions of the tissue, tearing or folding of the tissue, and loss of the tissue section. In some instances, repeat staining of new sections or acquisition of a new specimen may be required.

VENTANA ANTI-MSH6 (SP93) ANTIBODY PERFORMANCE CHARACTERISTICS

Analytical Specificity/Sensitivity

Analytical specificity and sensitivity were determined by staining multiple cases of normal and neoplastic human tissues with VENTANA anti-MSH6 (SP93) antibody. The results are listed in Table 3 and Table 4. Positive staining is nuclear unless otherwise specified. No unexpected staining was observed with VENTANA anti-MSH6 (SP93) antibody on the normal and neoplastic tissues. As expected, since mismatch repair is present in all actively proliferating cells, most normal and neoplastic tissues demonstrated positive staining.

Table 3. Analytical Specificity/Sensitivity of VENTANA anti-MSH6 (SP93) antibody Staining in FFPE Normal Tissues

Tissue	# positive / total cases	Tissue	# positive / total cases
Adrenal Gland	3/3	Lung	4/4
Bladder	3/3	Lymph node	3/3
Bone Marrow	3/3	Mesothelium	3/3
Ovary	5/5	Pancreas	3/3
Breast	3/3	Parathyroid Gland	3/3
Cerebellum	3/3	Peripheral Nerve	5/5
Cerebrum	3/3	Prostate	3/3
Cervix	3/3	Skeletal Muscle	3/3
Colon	3/3	Skin	3/3
Endometrium	2/3	Spleen	3/3
Esophagus	3/3	Stomach	3/3
Heart	3/3	Testis	3/3
Hypophysis	3/3	Thymus	3/3
Intestine	3/3	Thyroid	4/4
Kidney	3/3	Tongue/Salivary Gland	3/3
Liver	3/3	Tonsil	3/3

Note: Mismatch repair proteins such as MSH6 are present in all actively proliferating cells. For all tissues, positive/negative staining was determined for tissue specific elements in the presence of positive staining in normal control cells (lymphocytes, fibroblasts, and epithelial cells).

Table 4. Analytical Specificity/Sensitivity of VENTANA anti-MSH6 (SP93) antibody Staining in a Variety of FFPE Neoplastic Tissues

Pathology	# positive / total cases
Glioblastoma (Cerebrum)	1/1
Meningioma (Cerebrum)	1/1
Ependymoma (Cerebrum)	1/1
Oligodendroglioma (Cerebrum)	1/1
Serous adenocarcinoma (Ovary)	1/1
Adenocarcinoma (Ovary)	1/1
Pancreatic neuroendocrine neoplasm (Pancreas)	1/1
Seminoma (Testis)	2/2
Medullary carcinoma (Thyroid)	1/1
Papillary carcinoma (Thyroid)	1/1
Ductal carcinoma in situ (Breast)	1/1
Microinvasive ductal carcinoma (Breast)	1/1
Invasive ductal carcinoma (Breast)	1/1
B-cell lymphoma; NOS (Spleen)	1/1

Pathology	# positive / total cases
Small cell carcinoma (Lung)	1/1
Squamous cell carcinoma (Lung)	1/1
Adenocarcinoma (Lung)	1/1
Neuroendocrine carcinoma (Esophagus)	1/1
Adenocarcinoma (Esophagus)	1/1
Signet ring carcinoma (Stomach)	1/1
Adenocarcinoma (Small intestine)	1/1
Stromal sarcoma (small intestine)	1/1
Adenocarcinoma (Colon)	1/1
Adenocarcinoma (Rectum)	1/1
Gastrointestinal stromal tumor (GIST) (Rectum)	1/1
Hepatocellular carcinoma (Liver)	1/1
Hepatoblastoma (Liver)	1/1
Clear cell carcinoma (Kidney)	1/1
Adenocarcinoma (Prostate)	2/2
Adenocarcinoma (Uterus)	1/1
Clear cell carcinoma (Endometrium)	1/1
Squamous cell carcinoma (Cervix)	2/2
Embryonal rhabdomyosarcoma (Striated muscle)	1/1
Squamous cell carcinoma (Skin)	1/1
Neuroblastoma (Retroperitoneum)	1/1
Mesothelioma (Peritoneum)	1/1
B-cell lymphoma; NOS (Lymph node)	2/2
Hodgkin lymphoma (Lymph node)	1/1
Diffuse anaplastic large cell lymphoma	1/1
Leiomyosarcoma (Bladder)	1/1
Osteosarcoma	1/1
Spindle cell rhabdomyosarcoma (Peritoneum)	1/1
Leiomyosarcoma (Smooth muscle)	1/1

Note: Mismatch repair proteins such as MSH6 are present in all actively proliferating cells. For all tissues, positive/negative staining was determined for tumor cells in the presence of positive staining in normal control cells (lymphocytes, fibroblasts, and epithelial cells).

Within-Day Repeatability and Day-to-Day Precision

The repeatability and precision of VENTANA anti-MSH6 (SP93) antibody was evaluated on the BenchMark ULTRA instrument in combination with the OptiView DAB IHC Detection Kit.

Within-Day Repeatability was evaluated using 10 CRC specimens (5 Intact and 5 Loss for MSH6 expression). Five replicate slides from each of the CRC specimens were stained with VENTANA anti-MSH6 (SP93) antibody on a single BenchMark ULTRA instrument within a single day. Each VENTANA anti-MSH6 (SP93) antibody-stained slide was paired with a negative reagent control stained slide from the same case. All slide pairs were randomized, and then evaluated as Intact or Loss by a single pathologist blinded to the case diagnosis.

Day-to-Day Precision was also evaluated using 10 CRC specimens (5 Intact and 5 Loss for MSH6 expression). Replicate slides from each of the CRC specimens were stained with VENTANA anti-MSH6 (SP93) antibody on a BenchMark ULTRA instrument on each of 5 non-consecutive days. Each VENTANA anti-MSH6 (SP93) antibody-stained slide was paired with a negative reagent control stained slide from the same case. All slide pairs were randomized, and then evaluated as Intact or Loss by a single pathologist blinded to the case diagnosis.

None of the slides stained with the negative reagent control showed specific staining and background staining was ≤ 0.5. Using pooled data of all possible pairings, both Within-Day Repeatability and Day-to-Day Precision studies demonstrated 100% positive percent agreement (PPA), 100% negative percent agreement (NPA) and 100% overall percent agreement (OPA). A summary of the results can be found in Table 5.

Table 5. Within-Day Repeatability and Day-to-Day Precision of the VENTANA anti-MSH6 (SP93) antibody as Measured by Clinical Status (Intact or Loss)

Repeatability/ Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Within-Day Repeatability	Intact	PPA	25/25	100.0	(86.7,100.0)
	Loss	NPA	25/25	100.0	(86.7,100.0)
	Total	OPA	50/50	100.0	(92.9,100.0)
Day-to-Day Precision	Intact	PPA	50/50	100.0	(92.9, 100.0)
	Loss	NPA	50/50	100.0	(92.9, 100.0)
	Total	OPA	100/100	100.0	(96.3, 100.0)

Note: 95% CIs were calculated using the (Wilson) Score method.

BenchMark Instrument-to-Instrument Precision

BenchMark ULTRA Instrument-to-Instrument Precision of the VENTANA anti-MSH6 (SP93) antibody was determined by staining replicate slides of 10 CRC specimens (5 Intact and 5 Loss for MSH6 expression) across 3 BenchMark ULTRA instruments with VENTANA anti-MSH6 (SP93) antibody using the OptiView DAB IHC Detection Kit. Replicate slides from each of the CRC specimens were stained with VENTANA anti-MSH6 (SP93) antibody on 3 BenchMark ULTRA instruments.

Each VENTANA anti-MSH6 (SP93) antibody-stained slide was paired with a negative reagent control stained slide from the same case. All slide pairs were randomized, and then evaluated for Clinical Status (Intact or Loss) by a single pathologist blinded to the case diagnosis. None of the slides stained with the negative reagent control showed specific staining and background staining was ≤ 0.5.

For BenchMark ULTRA Instrument-to-Instrument Precision, pairwise comparisons of the Clinical Status of slides for each specimen were made between instruments and demonstrated 100% PPA, NPA, and OPA. A summary of the results can be found in Table 6.

Table 6. BenchMark ULTRA Instrument-to-Instrument Precision of the VENTANA anti-MSH6 (SP93) antibody as Measured by Clinical Status (Intact or Loss)

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Instrument-to-Instrument	Intact	PPA	30/30	100.0	(88.6,100.0)
	Loss	NPA	30/30	100.0	(88.6,100.0)
	Total	OPA	60/60	100.0	(94.0,100.0)

Note: 95% CIs were calculated using the (Wilson) Score method.

In addition, Instrument-to-Instrument Precision of the VENTANA anti-MSH6 (SP93) antibody was determined by staining replicate slides of 6 CRC specimens (4 Intact and 2 Loss for MSH6 expression) across 3 BenchMark XT and 3 BenchMark GX instruments with VENTANA anti-MSH6 (SP93) antibody using the OptiView DAB IHC Detection Kit.

There were 15 observations per case when pooling the 3 instruments together; the median for each case was determined from these 15 observations. Individual observations of that same case were deemed to be concordant with the median case signal intensity if they were within 0.5 signal intensity. For BenchMark XT and BenchMark GX Instrument-to-Instrument Precision, pairwise comparisons of stain intensity scores of tumor for each specimen were made and demonstrated 100% OPA between 3 BenchMark XT and 100% OPA between 3 BenchMark GX instruments. For all slides background staining was acceptable (≤ 0.5) on both the BenchMark XT or GX instruments.

BenchMark Platform Concordance

Concordance across the BenchMark ULTRA, XT and GX instruments for the VENTANA anti-MSH6 (SP93) antibody was determined by staining CRC specimens with VENTANA anti-MSH6 (SP93) antibody using the OptiView DAB IHC Detection Kit. All slides were evaluated for Clinical Status (Intact/Loss) by a single pathologist.

Pairwise comparisons of CRC specimens were made between platforms including GX to ULTRA (134 Intact and 20 Loss cases), GX to XT (133 Intact and 21 Loss cases) and ULTRA to XT (136 Intact and 20 Loss cases). All pairwise comparisons made between platforms demonstrated 100% average positive agreement (APA), average negative agreement (ANA), and OPA.

Reader Precision Studies

Within- and Between-Reader precision was evaluated on 20 CRC (11 Intact and 9 Loss cases) stained with VENTANA anti-MSH6 (SP93) antibody and the OptiView DAB IHC Detection Kit. Each VENTANA anti-MSH6 (SP93) antibody-stained slide was paired with a negative reagent control stained slide from the same case.

All slide pairs were randomized and evaluated by 3 pathologists for Intact or Loss MSH6 Clinical status. Pathologists were blinded to the case diagnosis. Following a four week washout period, the VENTANA anti-MSH6 (SP93) antibody-stained slides were re-randomized for a second evaluation of the MSH6 Clinical Status by each of the 3 pathologists. None of the slides stained with the negative reagent control showed specific staining and background staining was ≤ 0.5.

Within-Reader precision compared initial and final slide evaluations from a single pathologist providing 20 CRC slide comparisons per pathologist. Comparisons from the 3 pathologists were pooled and demonstrated 98.5% APA, 98.1% ANA, and 98.3% OPA for Within-Reader precision. A summary of the results can be found in Table 7.

Between-Reader precision compared all slide evaluations (20 CRC x 2 evaluations/case x 3 pathologists = 120 slide evaluations) to a modal case status for each CRC case. The results demonstrate 100% PPA, 98.1% NPA, and 99.2% OPA for Between-Reader precision. A summary of the results can be found in Table 7.

Table 7. Within-Reader and Between-Reader Precision of the VENTANA anti-MSH6 (SP93) antibody on CRC cases as Measured by MSH6 Clinical Status (Intact/Loss)

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Within-Reader	Intact	APA	66/67	98.5	(88.0,100.0)
	Loss	ANA	52/53	98.1	(83.3,100.0)
	Total	OPA	59/60	98.3	(85.0,100.0)
Between Reader	Intact	PPA	66/66	100.0	(94.5,100.0)
	Loss	NPA	53/54	98.1	(90.2,99.7)
	Total	OPA	119/120	99.2	(95.4,99.9)

Note: For Within-Reader, the APA and ANA 95% CIs were calculated using the Clopper-Pearson based method; the OPA 95% CI was calculated using the percentile bootstrap method. For Between-Reader, 95% CIs were calculated using the (Wilson) Score method.

Lot-to-Lot Precision

Lot-to-Lot Precision of VENTANA anti-MSH6 (SP93) antibody was determined by testing 3 production lots of the VENTANA anti-MSH6 (SP93) antibody each on triplicate slides of 10 CRC (5 Intact and 5 Loss for MSH6 expression) on a BenchMark ULTRA instrument using the OptiView DAB IHC Detection Kit.

Each VENTANA anti-MSH6 (SP93) antibody-stained slide was paired with a negative reagent control stained slide from the same case. Slide pairs were randomized, and evaluated by a single pathologist blinded to the case diagnosis and VENTANA anti-MSH6 (SP93) antibody lot number. None of the slides stained with the negative reagent control showed specific staining and background staining was ≤ 0.5.

For VENTANA anti-MSH6 (SP93) antibody Lot-to-Lot Precision, all slide evaluations were compared to a modal case status for each CRC case. The OPA between the VENTANA anti-MSH6 (SP93) antibody lots was 100%; demonstrating that VENTANA anti-MSH6 (SP93) antibody staining is reproducible across antibody lots.

A summary of the results for Lot-to-Lot Precision of the VENTANA anti-MSH6 (SP93) antibody is shown in Table 8.

Table 8. Lot-to-Lot Precision of the VENTANA anti-MSH6 (SP93) antibody as Measured by Clinical Status (Intact or Loss)

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Lot-to-Lot	Intact	PPA	45/45	100.0	(92.1,100.0)
	Loss	NPA	45/45	100.0	(92.1,100.0)
	Total	OPA	90/90	100.0	(95.9,100.0)

Note: 95% CIs were calculated using the (Wilson) Score method.

Inter-Laboratory Reproducibility Study

An Inter-Laboratory Reproducibility Study of the VENTANA MMR IHC Panel was completed to demonstrate reproducibility of each VENTANA MMR IHC Panel assay to determine Clinical Status. The study included 6 CRC tissue specimens (3 Intact and 3 Loss) for each MMR protein and 16 CRC tissue specimens (8 Positive and 8 Negative) for BRAF V600E run across 3 BenchMark ULTRA instruments on each of 5 non-consecutive days over 21 days at three external laboratories. Each antibody-stained slide was paired with an H&E and negative reagent control stained slides from the same case. All slide sets were randomized and evaluated by a total of 6 readers (2 readers/site) who were blinded to the MMR Clinical Status of the study set. Each of the 40 cases in the study had 30 observations across all days, sites, and readers. The modal case reference status was derived for each case based on the most often observed status of the 30 observations. The study included a total of 1200 observations for all five proteins. For all evaluable cases, the acceptability rate for morphology and background in this study was 100%. A summary of the pooled (all five proteins) agreement statistics between the modal case reference status and individual observations can be found in Table 9.

Table 9. Agreement between the VENTANA MMR IHC Panel and Modal Case Reference Status

Inter-Laboratory Reproducibility	Clinical Status	Agreement			
		Type	n/N	%	95% CI
All Proteins	Intact/Positive	PPA	598/600	99.8	(98.7,100.0)
	Loss/Negative	NPA	593/600	98.9	(97.4, 99.5)
	Total	OPA	1191/1200	99.4	(98.6, 99.7)

Note: Clinical Status is defined as Intact or Loss for protein expression for MMR proteins and Positive or Negative for BRAF V600E protein. 95% CIs were calculated using a generalized linear mixed model (GLMM) approach

In addition, pairwise comparisons were made Between-Site, Between-Day and Between-Reader for the VENTANA anti-MSH6 (SP93) antibody. For MSH6, this study set included a total of 180 observations. A summary of the results can be found in Table 10. The data indicate assay reproducibility across 5 days, 3 sites, and 6 readers.

Table 10. Inter-Laboratory Reproducibility Pairwise Agreement Rates for the VENTANA anti-MSH6 (SP93) antibody as Measured by Clinical Status (Intact or Loss)

Inter-Laboratory Reproducibility		Agreement			
		Type	n/N	%	95% CI
Between-Site (3 sites)		APA	360/364	98.9	(96.8,100.0)
		ANA	352/356	98.9	(96.6,100.0)
		OPA	356/360	98.9	(96.7,100.0)
Between-Day (5 non-consecutive days)	Site A	APA	120/120	100.0	(96.9,100.0)
		ANA	120/120	100.0	(96.9,100.0)
		OPA	120/120	100.0	(96.9,100.0)
	Site B	APA	120/124	96.8	(90.9,100.0)
		ANA	112/116	96.6	(88.9,100.0)
		OPA	116/120	96.7	(90.0,100.0)
	Site C	APA	120/120	100.0	(96.9,100.0)
		ANA	120/120	100.0	(96.9,100.0)
		OPA	120/120	100.0	(96.9,100.0)
Between-Reader (2 pathologists per site)		APA	90/91	98.9	(96.8,100.0)
		ANA	88/89	98.9	(96.6,100.0)
		OPA	89/90	98.9	(96.7,100.0)

Note: 95%CIs were calculated using the percentile bootstrap method; in instances where the point estimate was 100%, (Wilson) Score method was used.

Accuracy Study: Method Comparison of VENTANA MMR IHC Panel Results to Molecular Testing (DNA sequencing and MLH1 promoter hypermethylation)

A study was conducted to compare the performance of the VENTANA MMR IHC Panel to molecular testing including a comprehensive DNA sequencing colon panel for the identification of CRCs that (i) are MMR deficient (dMMR), and (ii) contain the BRAF V600E mutation. The DNA sequencing colon panel included genomic analysis of variants present in MMR genes (MLH1, PMS2, MSH2, MSH6, EPCAM), BRAF, and other genes important in carcinogenesis (e.g. PIK3CA, KRAS, NRAS, ERBB2, etc.). Sequencing included all exons, intronic and flanking sequences as well as large deletions, duplications, and mosaicism.

For the study, sequential CRC cases were stained by H&E and evaluated for indications of proper fixation and morphology including the presence of cellular elements (tumor and internal control cells). Each case was evaluated to determine if the specimen contained a minimum of 50% tumor content to provide sufficient representation of tumor cells in the sample as recommended for molecular testing. Following review, 105 sequential cases meeting these criteria were enrolled into the study. In addition, 13 CRC cases showing a Clinical status of Loss by IHC were included to ensure that Loss of each marker was represented in the study. Sections of all cases in the study were stained by IHC with the VENTANA MMR IHC Panel and appropriate negative reagent controls. Additional sections were subjected to the DNA sequencing colon panel. MLH1 promoter hypermethylation is one of the mechanisms which may lead to loss of MLH1 protein expression, and it is linked to sporadic CRC rather than potential Lynch syndrome diagnosis. Therefore, all MLH1 loss cases identified by IHC in the study were tested for hypermethylation of the MLH1 promoter.

In the final study set of 118 cases, the analysis included PPA and NPA for all markers pooled (i.e. all observations pooled) where molecular testing acted as the reference status for IHC comparison. The analysis included a comparison of MMR protein status (Intact / Loss) to molecular status defined as Normal (no pathogenic mutation(s), negative for MLH1 promoter hypermethylation, and wild-type BRAF (no V600E mutation)) or Abnormal (presence of pathogenic mutation(s), positive for MLH1 promoter hypermethylation, and/or positive for the BRAF V600E mutation). For this study, a pathogenic mutation within the tumor is defined as a germline or somatic mutation predicted to results in the loss of MMR protein expression. Point estimates were 99.4% PPA, 93.5% NPA and 98.8% OPA as shown in Table 11.

A pooled analysis comparing the four MMR IHC markers (without the VENTANA anti-BRAF V600E (VE1) antibody) to molecular testing results was also performed. Point estimates were 99.3% PPA, 89.7% NPA and 98.5% OPA as summarized in Table 12.

An additional analysis compared the four MMR IHC marker results to the molecular testing results for the MMR genes at the case level to include the status of all markers and create a dMMR/pMMR outcome for the two methods. This analysis is shown in Table 13, and exhibits an OPA of 97.4% between the two methods.

IHC MMR status and molecular testing MMR status were also compared for individual MMR markers within the study. The OPA of each MMR marker, when compared to the combined results of the DNA sequencing colon panel and MLH1 promoter hypermethylation testing, was 100.0% for VENTANA anti-MLH1 (M1) antibody, 99.1% for VENTANA anti-PMS2 (A16-4) Mouse Monoclonal Primary Antibody, 98.3% for VENTANA anti-MSH2 (G219-1129) Mouse Monoclonal Primary Antibody and 96.6% for VENTANA anti-MSH6 (SP93) Rabbit Monoclonal Primary Antibody.

BRAF V600E Clinical status in CRCs obtained by IHC using the VENTANA anti-BRAF V600E (VE1) antibody was also compared to BRAF mutational status results determined by DNA sequencing. The PPA, NPA, and OPA of IHC testing using the VENTANA anti-BRAF V600E (VE1) antibody using DNA sequencing as the reference all were 100% (Table 14). Additional testing was performed to verify the ability of the VENTANA anti-BRAF V600E (VE1) antibody to further stratify CRC cases showing a loss of MLH1 protein expression. Of the 23 positive BRAF V600E cases, 20 cases had loss of MLH1 protein by IHC and were positive for MLH1 promoter hypermethlyation. These data are consistent with the close association of BRAF V600E positive status with MLH1 promoter hypermethylation status. The remaining three cases were pMMR (intact for all MMR proteins). All BRAF V600E positive specimens were identified as sporadic CRC. The results verified that the VENTANA anti-BRAF V600E (VE1) antibody correctly identifies CRCs having the BRAF V600E mutation. The data also supported the use of VENTANA anti-BRAF V600E (VE1) antibody to differentiate between sporadic and probable Lynch syndrome CRC in the absence of MLH1 expression.

Table 11. Pooled Analysis for VENTANA MMR IHC Panel Agreement between IHC and Molecular Testing

Status* (Molecular/IHC)	Agreement			
	Type	n/N	%	95% CI
Normal/Intact	PPA	523/526	99.4	(98.7,100.0)
Abnormal/Loss	NPA	58/62	93.5	(87.1, 98.6)
Total	OPA	581/588	98.8	(98.0, 99.7)

*For IHC, MMR Status is Intact or Loss for protein expression. For this analysis, BRAF V600E negative and positive cases were included in Intact or Loss categories, respectively. Molecular testing indicates absence (Normal) or presence (Abnormal) of potential pathogenic mutations or MLH1 promoter hypermethylation. 95% CIs were calculated using the percentile bootstrap method.

Table 12. Pooled Analysis for four MMR IHC markers (without VENTANA anti-BRAF V600E (VE1) antibody) Agreement between IHC and Molecular Testing

Status* (Molecular/IHC)	Agreement			
	Type	n/N	%	95% CI
Normal/Intact	PPA	428/431	99.3	(98.4,100.0)
Abnormal/Loss	NPA	35/39	89.7	(79.4, 97.7)
Total	OPA	463/470	98.5	(97.3, 99.6)

*For IHC, Status is Intact or Loss for protein expression. Molecular testing indicates absence (Normal) or presence (Abnormal) of potential pathogenic mutations or MLH1 promoter hypermethylation. 95% CIs were calculated using the percentile bootstrap method.

Table 13. Agreement between the four MMR IHC markers and Molecular Testing Results for MMR Status (dMMR/pMMR)

MMR Status*	Agreement			
	Type	n/N	%	95% CI
pMMR	PPA	79/80	98.8	(93.3,99.8)
dMMR	NPA	35/37	94.6	(82.3, 98.5)
Total	OPA	114/117	97.4	(92.7, 99.1)

*For IHC, pMMR status for a case is represented by Intact status for all MMR proteins, while dMMR status is represented by Loss of one or more MMR proteins. For molecular testing, pMMR status is represented by the absence of pathogenic mutations or MLH1 promoter hypermethylation, while dMMR status is represented by the presence of pathogenic mutations or MLH1 promoter hypermethylation. 95% CIs were calculated using the (Wilson) Score method.

Table 14. Agreement between IHC using VENTANA anti-BRAF V600E (VE1) antibody and Molecular Testing

BRAF V600E Status (Molecular/IHC)	Agreement			
	Type	n/N	%	95% CI
Positive/Abnormal	PPA	23/23	100.0	(85.7, 100.0)
Negative/Normal	NPA	95/95	100.0	(96.1, 100.0)
Total	OPA	118/118	100.0	(96.8, 100.0)

Status for BRAF V600E was defined as Positive or Negative IHC results and Abnormal (presence of the V600E mutation) or Normal (wild-type BRAF) results by molecular testing. 95% CIs were calculated using the (Wilson) Score method.

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