VENTANA anti-BRAF V600E (VE1) Mouse Monoclonal Primary Antibody

For use with the VENTANA MMR IHC Panel

REF 790-5095

07862270001

IVD Σ 50

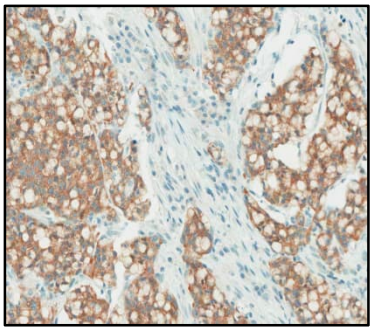

Figure 1. VENTANA anti-BRAF V600E (VE1) Mouse Monoclonal Primary Antibody staining of neoplastic cells in colon cancer tissue.

INTENDED USE

The VENTANA MMR IHC Panel is a qualitative immunohistochemistry (IHC) test intended for use in the light microscopic assessment of mismatch repair (MMR) proteins (MLH1, PMS2, MSH2, and MSH6) and BRAF V600E protein in formalin-fixed, paraffin-embedded colorectal cancer (CRC) tissue sections. The OptiView DAB IHC Detection Kit is used with MLH1, MSH2, MSH6 and BRAF V600E, and the OptiView DAB IHC Detection Kit with OptiView Amplification Kit is used for PMS2 detection. The VENTANA MMR IHC Panel is intended for use on the BenchMark ULTRA and BenchMark ULTRA PLUS instruments. The VENTANA MMR IHC Panel 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, VENTANA anti-MSH6 (SP93) Rabbit Monoclonal Primary Antibody, and VENTANA anti-BRAF V600E (VE1) Mouse Monoclonal Primary Antibody.

The VENTANA MMR IHC Panel is indicated in patients diagnosed with colorectal cancer (CRC) to detect mismatch repair (MMR) proteins deficiency as an aid in the identification of probable Lynch syndrome and to detect BRAF V600E protein as an aid to differentiate between sporadic CRC and probable Lynch syndrome.

Results from the VENTANA MMR IHC Panel should be interpreted by a qualified pathologist in conjunction with histological examination, relevant clinical information, and proper controls.

The clinical performance of this device to guide treatment of MMR deficient patients has not been established.

Intended for in vitro diagnostic (IVD) use. Prescription Use Only.

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 immunohisto-chemical 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 potential 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 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 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.[18,28] 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

The BRAF gene located on chromosome 7q34 encodes a cytoplasmic serine-threonine kinase that acts downstream of the mitogen-activated protein kinase (MAPK) signaling pathway. Oncogenic mutations in the *BRAF* gene, all within the kinase domain, constitutively activate the MAPK signaling pathway resulting in increased cell proliferation and apoptosis resistance. The most common of all activating *BRAF* mutations (T1799A point mutation) results in a substitution of valine (V) to glutamic acid (E) at position 600 of the amino acid sequence.[30] The *BRAF V600E* mutation was detected in approximately

8% of all solid tumors, including 43% of melanomas, 39% of papillary thyroid carcinomas, 12% of serous ovarian carcinomas, 12% of colorectal adenocarcinomas, 2% of lung cancers, and in other cancers.[31] Furthermore, the *BRAF V600E* mutation has been recently described as a molecular marker of hairy cell leukemia.[32]

The VENTANA anti-BRAF V600E (VE1) antibody is a mouse monoclonal antibody (clone VE1) produced against a synthetic peptide representing the BRAF mutated amino acid sequence from amino acid 596 to 606 (GLATEKSRWSG). This mutation-specific antibody exhibits a cytoplasmic staining pattern. This antibody differentiates the *V600E* mutation in the BRAF protein from the wild type BRAF protein and other mutated BRAF proteins.[33,34]

In the context of mismatch repair (MMR) IHC testing for potential Lynch syndrome, the identification of the BRAF V600E mutation in MLH1 loss cases is indicative of sporadic colorectal cancer (CRC).[29]

VENTANA anti-BRAF V600E (VE1) antibody binds specifically to the BRAF V600E mutant 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, the BenchMark ULTRA or BenchMark ULTRA PLUS 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), which minimizes evaporation of the aqueous reagents from the specimen slide.

In addition to staining with VENTANA anti-BRAF V600E (VE1) antibody, a second slide should be stained with a mouse monoclonal negative reagent, such as Negative Control (Monoclonal) (Cat. No. 760-2014 / 05266670001). The negative reagent control is used to assess background staining.

MATERIAL PROVIDED

VENTANA anti-BRAF V600E (VE1) antibody contains sufficient reagent for 50 tests.

One 5 mL dispenser of VENTANA anti-BRAF V600E (VE1) antibody contains approximately 60 µg of mouse monoclonal antibody.

The antibody is diluted in 0.1M phosphate buffer (pH 7.3) with 0.3% carrier protein, 0.05% Brij 35, and 0.05% ProClin300, a preservative.

Specific antibody concentration is approximately 12 µg/mL.

VENTANA anti-BRAF V600E (VE1) antibody is a mouse 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) 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. Recommended control tissue
2. Additional VENTANA MMR IHC Panel antibodies:
   - VENTANA anti-MLH1 (M1) Mouse Monoclonal Primary Antibody (Cat. No. 790-5091 / 07862237001)
   - VENTANA anti-PMS2 (A16-4) Mouse Monoclonal Primary Antibody (Cat. No. 790-5094 / 07862261001)
   - VENTANA anti-MSH2 (G219-1129) Mouse Monoclonal Primary Antibody (Cat. No. 790-5093 / 07862253001)
   - VENTANA anti-MSH6 (SP93) Rabbit Monoclonal Primary Antibody (Cat. No. 790-5092 / 07862245001)
3. Negative Control (Monoclonal) (Cat No. 760-2014 / 05266670001)
4. Microscope slides, positively charged
5. Bar code labels (appropriate for negative reagent control and primary antibody being tested)
6. Xylene (Histological grade)
7. 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
8. Deionized or distilled water
9. OptiView DAB IHC Detection Kit (Cat. No. 760-700 / 06396500001)
10. For VENTANA anti-PMS2 (A16-4) Mouse Monoclonal Primary Antibody, OptiView Amplification Kit (Cat. No. 760-099 / 06396518001 or Cat. No. 860-099 / 06718663001)
11. EZ Prep Concentrate (10X) (Cat. No. 950-102 / 05279771001)
12. Reaction Buffer Concentrate (10X) (Cat. No. 950-300 / 05353955001)
13. ULTRA LCS (Predilute) (Cat. No. 650-210 / 05424534001)
14. ULTRA Cell Conditioning Solution (ULTRA CC1) (Cat. No. 950-224 / 05424569001)
15. Hematoxylin II (Cat. No. 790-2208 / 05277965001)
16. Bluing Reagent (Cat. No. 760-2037 / 05266769001)
17. Permanent mounting medium (Permount Fisher Cat. No. SP15-500 or equivalent)
18. Cover glass (sufficient to cover tissue, such as VWR Cat. No. 48393-060)
19. Automated coverslipper (such as the Tissue-Tek SCA Automated Coverslipper)
20. Light microscope
21. Absorbent wipes

STORAGE AND STABILITY

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

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.

Routinely processed FFPE tissues are suitable for use with this primary antibody when used with OptiView DAB IHC Detection Kit and the BenchMark ULTRA or BenchMark ULTRA PLUS instrument. The recommended tissue fixative is 10% neutral buffered formalin.[35]

It is recommended that positive and negative controls be run simultaneously with unknown specimens.

WARNINGS AND PRECAUTIONS

1. For in vitro diagnostic (IVD) use.
2. For professional use only.
3. Do not use beyond the specified number of tests.
4. 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.
5. Positively charged slides may be susceptible to environmental stresses resulting in inappropriate staining. Ask your Roche representative for more information on how to use these types of slides.
6. Materials of human or animal origin should be handled as biohazardous materials and disposed of with proper precautions. In the event of exposure, the health directives of the responsible authorities should be followed.[36,37]
7. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water.
8. Avoid microbial contamination of reagents as it may cause incorrect results.
9. For further information on the use of this device, refer to the BenchMark IHC/ISH instrument User Guide, and instructions for use of all necessary components located at navifyportal.roche.com.
10. Consult local and/or state authorities with regard to recommended method of disposal.
11. Product safety labeling primarily follows EU GHS guidance. Safety data sheet available for professional user on request.

12. To report suspected serious incidents related to this device, contact the local Roche representative and the competent authority of the Member State or Country in which the user is established.

This product contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Table 1. Hazard information.

Hazard	Code	Statement
Warning 	H317	May cause an allergic skin reaction.
	P261	Avoid breathing mist or vapours.
	P272	Contaminated work clothing should not be allowed out of the workplace.
	P280	Wear protective gloves.
	P333 + P313	If skin irritation or rash occurs: Get medical advice/ attention.
	P362 + P364	Take off contaminated clothing and wash it before reuse.
	P501	Dispose of contents/ container to an approved waste disposal plant.

This product contains CAS # 55965-84-9, reaction mass of: 5-chloro-2-methyl-2H-isothiazol-3-one and 2-methyl-2H-isothiazol-3-one (3:1)

STAINING PROCEDURE

VENTANA anti-BRAF V600E (VE1) antibody has been developed for use on BenchMark ULTRA and BenchMark ULTRA PLUS instruments in combination with OptiView DAB IHC Detection Kit, and ancillary reagents. Table 2 lists the staining protocol for use with VENTANA anti-BRAF V600E (VE1) 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 on staining is unknown and 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.

For more details on the proper use of this device, refer to the inline dispenser method sheet associated with P/N 790-5095.

Table 2. Recommended Staining Protocol for VENTANA anti-BRAF V600E (VE1) antibody with OptiView DAB IHC Detection Kit on a BenchMark ULTRA or BenchMark ULTRA PLUS instrument

Procedure Type	Method
Deparaffinization	Selected
Cell Conditioning (Antigen Unmasking)	Cell Conditioning 1, 64 minutes, 100°C
Pre-Primary Peroxidase Inhibitor	Selected
Antibody (Primary)	16 minutes, 36°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".[38]

QUALITY CONTROL PROCEDURES

Negative Reagent Control

In addition to staining with VENTANA anti-BRAF V600E (VE1) antibody, a second slide should be stained with the appropriate negative reagent control. Negative Control Monoclonal, Cat. No. 760-2014 / 05266670001, is recommended for use in place of the primary antibody to evaluate nonspecific staining. The staining protocol 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.

An appropriate positive tissue control would be a pre-qualified case of CRC that is positive for the VENTANA anti-BRAF V600E (VE1) antibody. The positive tissue control should exhibit, cytoplasmic staining of any intensity in viable tumor cells above background.

Negative Tissue Control

A negative tissue control would be a pre-qualified case of CRC that is negative with the VENTANA anti-BRAF V600E (VE1) antibody. The negative tissue control should be used only to monitor 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 positive and negative for the *BRAF* V600E mutation. (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[37] CAP or the CLSI Approved Guideline.[40])

STAINING INTERPRETATION / EXPECTED RESULTS

The cellular staining pattern for VENTANA anti-BRAF V600E (VE1) antibody is cytoplasmic in tumor cells. CRC stained with the VENTANA anti-BRAF V600E (VE1) antibody is assigned a Clinical Status by a trained pathologist based on their evaluation of the presence or absence of specific cytoplasmic staining in the tumor. A Clinical Status of Positive is assigned to cases with unequivocal cytoplasmic staining of any intensity in viable tumor cells above background. A Clinical Status of Negative is assigned to cases with no staining or equivocal cytoplasmic staining in viable tumor cells. Nuclear staining, weak to strong staining of isolated viable tumor cells, and/or small tumor clusters should be considered negative.

SPECIFIC LIMITATIONS

Ventana Medical Systems, Inc. provides antibodies and reagents at optimal dilution for use when the provided instructions are followed. Deviation from the listed protocol on staining is unknown and may invalidate expected results. Users are cautioned against the use of acidic buffers for antigen retrieval as these buffers can result in suboptimal staining that is difficult to interpret.[41] Users who deviate from the listed protocol must accept responsibility for interpretation of patient results.

VENTANA anti-BRAF V600E (VE1) antibody has been cleared for use on BenchMark ULTRA and BenchMark ULTRA PLUS instruments with the OptiView DAB IHC Detection Kit and is not cleared with any other detection methods or automated staining instruments.

VENTANA anti-BRAF V600E (VE1) antibody stained cases are categorized as Positive or Negative according to the presence or absence of staining over the entire tumor area. The staining can vary in the level of intensity and this intensity may vary throughout the tumor; however, this does not impact BRAF V600E Clinical Status.

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

- VENTANA anti-BRAF V600E (VE1) antibody was found to occasionally exhibit weak cytoplasmic and nuclear staining in smooth muscle, Purkinje cells of cerebellum, normal colon epithelial cells, interstitial cells of testis, pituitary gland, acinar structures of pancreas, glandular cells of intestine, and some tumor cells; however, such cases should not be considered as positive for BRAF V600E.42 In addition, this antibody showed moderate staining in neuroendocrine cells in hypophysis.
- Nonspecific background: Some specimens may exhibit nonspecific background staining for reasons that are not well understood. Therefore, evaluation of a VENTANA anti-BRAF V600E (VE1) antibody stained slide should include a comparison of this slide to the negative reagent control stained slide to determine the level of nonspecific background staining. Nuclear staining in tumor cells is sometimes observed; however, the significance of this is not understood.
- Tissue or Staining Artifacts: Histologic artifacts originating from the sample processing and microtomy processes can also complicate the determination of VENTANA anti-BRAF V600E (VE1) antibody 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.
- All assays might not be registered on every instrument. Please contact your local Roche representative for more information.

VENTANA ANTI-BRAF V600E (VE1) ANTIBODY PERFORMANCE CHARACTERISTICS

Analytical Sensitivity/Specificity

Analytical sensitivity and specificity were determined by staining multiple cases of normal and neoplastic human tissues with VENTANA anti-BRAF V600E (VE1) antibody. The results are listed in Table 3 and Table 4. No unexpected staining was observed with VENTANA anti-BRAF V600E (VE1) antibody on normal and neoplastic tissues, with the exceptions of those stated in the SPECIFIC LIMITATIONS section.

Table 3. Analytical Sensitivity/Specificity of VENTANA anti-BRAF V600E (VE1) antibody Staining in FFPE Normal Tissues

Tissue	# positive / total cases	Tissue	# positive / total cases
Adrenal Gland	0/3	Lung	0/3
Bladder	0/3	Lymph node	0/3
Bone Marrow	0/3	Mesothelium	0/3
Ovary	0/3	Pancreas	2/3*
Breast	0/3	Parathyroid Gland	0/3
Cerebellum	1/3*	Peripheral Nerve	0/5
Cerebrum	0/3	Prostate	0/3
Cervix	0/3	Skeletal Muscle	0/3
Colon	5/12*	Skin	0/3
Endometrium	0/3	Spleen	0/3
Esophagus	0/3	Stomach	0/3
Heart	0/3	Testis	2/3*
Hypophysis	3/3**	Thymus	0/3
Intestine	2/4*	Thyroid	0/3
Kidney	0/3	Tongue/Salivary Gland	0/3
Liver	0/3	Tonsil	0/3

* Weak cytoplasmic and nuclear staining in Purkinje cells of cerebellum, smooth muscle and epithelial cells of normal colon, glandular cells of intestine, acinar structures of pancreas, and interstitial cells of testis.

** Moderate staining observed in neuroendocrine cells in hypophysis.

For all tissues, positive/negative staining was determined for tissue specific elements and such cases should not be considered as positive for BRAF V600E Clinical Status.[16]

Table 4. Analytical Sensitivity/Specificity of VENTANA anti-BRAF V600E (VE1) antibody staining in a variety of FFPE neoplastic tissues.

Pathology	# positive / total cases
Glioblastoma (Cerebrum)	0/1
Meningioma (Cerebrum)	0/1
Ependymoma (Cerebrum)	0/1
Oligodendroglioma (Cerebrum)	0/1
Serous adenocarcinoma (Ovary)	0/1
Adenocarcinoma (Ovary)	0/1
Pancreatic neuroendocrine neoplasm (Pancreas)	0/1
Seminoma (Testis)	0/2
Medullary carcinoma (Thyroid)	0/1
Papillary carcinoma (Thyroid)	1/1
Ductal carcinoma in situ (Breast)	0/1
Microinvasive ductal carcinoma (Breast)	0/1
Invasive ductal carcinoma (Breast)	1/1
B-cell lymphoma; NOS (Spleen)	0/1
Small cell carcinoma (Lung)	0/1
Squamous cell carcinoma (Lung)	0/1
Neuroendocrine carcinoma (Esophagus)	0/1
Adenocarcinoma (Esophagus)	0/1
Signet ring carcinoma (Stomach)	0/1
Adenocarcinoma (Small intestine)	0/1
Stromal sarcoma (Small intestine)	1/1
Adenocarcinoma (Colon)	0/1
Gastrointestinal stromal tumor (GIST) (Colon)	0/1
Adenocarcinoma (Rectum)	0/1
Gastrointestinal stromal tumor (GIST) (Rectum)	0/1
Melanoma (Rectum)	0/1
Hepatocellular carcinoma (Liver)	0/1
Hepatoblastoma (Liver)	0/1
Clear cell carcinoma (Kidney)	0/1
Adenocarcinoma (Prostate)	0/2
Adenocarcinoma (Uterus)	0/1
Clear cell carcinoma (Uterus)	0/1
Squamous cell carcinoma (Cervix)	0/2

Pathology	# positive / total cases
Embryonal rhabdomyosarcoma (Striated muscle)	0/1
Squamous cell carcinoma (Skin)	0/1
Basal cell carcinoma (Skin)	0/1
Neuroblastoma (Retroperitoneum)	0/1
Mesothelioma (Peritoneum)	0/1
B-cell lymphoma; NOS (Lymph node)	0/2
Hodgkin's lymphoma (Lymph node)	1/1
Transitional cell carcinoma (Bladder)	0/1
Leiomyosarcoma (Bladder)	0/1
Osteosarcoma	0/1
Spindle cell rhabdomyosarcoma (Peritoneum)	0/1
Leiomyosarcoma (Smooth muscle)	0/1

Performance Characteristics on BenchMark ULTRA Instrument

Within-Day Repeatability and Day-to-Day Precision

The repeatability and precision of VENTANA anti-BRAF V600E (VE1) antibody was evaluated on the BenchMark ULTRA instrument with the OptiView DAB IHC Detection Kit.

Within-Day Repeatability was evaluated using 10 CRC specimens (5 Positive and 5 Negative for BRAF V600E Clinical Status). Five replicate slides from each of the CRC specimens were stained with VENTANA anti-BRAF V600E (VE1) antibody on a single BenchMark ULTRA instrument within a single day. Each VENTANA anti-BRAF V600E (VE1) 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 Positive or Negative by a single pathologist blinded to the case diagnosis.

Day-to-Day Precision was also evaluated using 10 CRC specimens (5 Positive and 5 Negative for BRAF V600E Clinical Status). Replicate slides from each of the CRC specimens were stained with VENTANA anti-BRAF V600E (VE1) antibody on a BenchMark ULTRA instrument on each of 5 non-consecutive days. In addition, a single slide from each case was stained with negative reagent control. Each VENTANA anti-BRAF V600E (VE1) 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 Positive or Negative 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 are provided; as shown in Table 5.

Table 5. BenchMark ULTRA Instrument Within-Day Repeatability and Day-to-Day Precision of the VENTANA anti-BRAF V600E (VE1) antibody as Measured by Clinical Status (Positive/Negative)

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

Instrument-to-Instrument Precision

BenchMark ULTRA Instrument-to-Instrument Precision of the VENTANA anti-BRAF V600E (VE1) antibody was determined by staining replicate slides of 10 CRC specimens (5 Positive and 5 Negative for BRAF V600E Clinical Status) across 3 BenchMark ULTRA instruments with the VENTANA anti-BRAF V600E (VE1) antibody using the OptiView DAB IHC Detection Kit. In addition, a single slide from each case was stained with a negative reagent control.

Each VENTANA anti-BRAF V600E (VE1) 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 (Positive/Negative) 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 demonstrating 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-BRAF V600E (VE1) antibody as Measured by Clinical Status (Positive/Negative)

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Instrument-to-Instrument	Positive	PPA	30/30	100.0	(88.6,100.0)
	Negative	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.

Reader Precision Studies

Within- and Between-Reader Precision was evaluated on 20 CRCs (10 cases positive for the BRAF V600E mutation and 10 cases negative for the BRAF V600E mutation) stained with VENTANA anti-BRAF V600E (VE1) antibody using the OptiView DAB IHC Detection Kit on a BenchMark ULTRA instrument. Each VENTANA anti-BRAF V600E (VE1) 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 Positive or Negative BRAF V600E Clinical Status. Pathologists were blinded to the case diagnosis. Following a four week washout period, the VENTANA anti-BRAF V600E (VE1) antibody-stained slides were re-randomized for a second evaluation of the BRAF V600E 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 pathologist were pooled and demonstrated 100% average positive agreement (APA), 100% average negative agreement (ANA), and 100% overall percent agreement (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, NPA, and 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-BRAF V600E (VE1) antibody on CRC Cases as Measured by BRAF V600E Clinical Status (Positive/Negative)

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Within-Reader	Positive	APA	60/60	100.0	(93.9,100.0)
	Negative	ANA	60/60	100.0	(93.9,100.0)
	Total	OPA	60/60	100.0	(94.0,100.0)
Between Reader	Positive	PPA	60/60	100.0	(94.0,100.0)
	Negative	NPA	60/60	100.0	(94.0,100.0)

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
	Total	OPA	120/120	100.0	(96.9,100.0)

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 the VENTANA anti-BRAF V600E (VE1) antibody was determined by testing 3 production lots of the VENTANA anti-BRAF V600E (VE1) antibody on triplicate slides of 10 CRC cases (5 Positive and 5 Negative for the BRAF V600E mutation) on a BenchMark ULTRA instrument using the OptiView DAB IHC Detection Kit.

Each VENTANA anti-BRAF V600E (VE1) antibody-stained slide was paired with a negative reagent control stained slide from the same case. All slide pairs were randomized and evaluated by a single pathologist blinded to the case diagnosis and VENTANA anti-BRAF V600E (VE1) 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-BRAF V600E (VE1) antibody Lot-to-Lot Precision, the BRAF V600E Clinical Status obtained from each slide evaluation was compared to a modal case status for that case. The OPA, PPA, and NPA for the VENTANA anti-BRAF V600E (VE1) antibody lots were 100% demonstrating that VENTANA anti-BRAF V600E (VE1) antibody staining is reproducible across antibody lots.

A summary of the results for VENTANA anti-BRAF V600E (VE1) antibody Lot-to-Lot Precision is shown in Table 8.

Table 8. Lot-to-Lot Precision of the VENTANA anti-BRAF V600E (VE1) antibody as Measured by Clinical Status (Positive/Negative)

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Lot-to-Lot	Positive	PPA	45/45	100.0	(92.1,100.0)
	Negative	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 slide 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 model (GLMM) approach.

In addition, pairwise comparisons were made Between-site, Between-day and Between-reader for VENTANA anti-BRAF V600E (VE1) antibody. For BRAF V600E, this study set included a total of 480 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-BRAF V600E (VE1) antibody as Measured by Clinical Status (Positive or Negative)

Inter-Laboratory Reproducibility		Agreement			
		Type	n/N	%	95% CI
Between-Site (3 sites)		APA	960/972	98.8	(97.2,100.0)
		ANA	936/948	98.7	(97.0,100.0)
		OPA	948/960	98.8	(97.1,100.0)
Between-Day (5 non-consecutive days)	Site A	APA	320/320	100.0	(98.8,100.0)
		ANA	320/320	100.0	(98.8,100.0)
		OPA	320/320	100.0	(98.8,100.0)
	Site B	APA	320/320	100.0	(98.8,100.0)
		ANA	320/320	100.0	(98.8,100.0)
		OPA	320/320	100.0	(98.8,100.0)
	Site C	APA	320/332	96.4	(92.0,100.0)
		ANA	296/308	96.1	(90.4,100.0)
		OPA	308/320	96.3	(91.3,100.0)
Between-Reader (2 pathologists per site)		APA	242/243	99.6	(98.8,100.0)
		ANA	236/237	99.6	(98.7,100.0)
		OPA	239/240	99.6	(98.8,100.0)

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

Performance Characteristics on BenchMark ULTRA PLUS Instrument

Within-Day Repeatability and Day-to-Day Precision

Within-Day (Within-Run) Repeatability was evaluated using 10 CRC specimens (5 Positive and 5 Negative for BRAF V600E Clinical Status). Five replicate slides from each of the CRC specimens were stained with VENTANA anti-BRAF V600E (VE1) antibody on a single BenchMark ULTRA PLUS instrument within a single day. Each VENTANA anti-BRAF V600E (VE1) 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 Positive or Negative by a single pathologist blinded to the case diagnosis.

Day-to-Day (Between-Day) Intermediate Precision was also evaluated using 10 CRC specimens (5 Positive and 5 Negative for BRAF V600E Clinical Status). Replicate slides from each of the CRC specimens were stained with VENTANA anti-BRAF V600E (VE1) antibody on a BenchMark ULTRA PLUS instrument on each of 5 non-consecutive days. In addition, a single slide from each case was stained with negative reagent control. Each VENTANA anti-BRAF V600E (VE1) 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 Positive or Negative 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 is provided; as shown in Table 11.

Table 11. BenchMark ULTRA PLUS Instrument Within-Day Repeatability and Day-to-Day Precision of the VENTANA anti-BRAF V600E (VE1) antibody as Measured by Clinical Status (Positive/Negative)

Repeatability/Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Within-Day Repeatability	Positive	PPA	25/25	100.0	(86.7,100.0)
	Negative	NPA	25/25	100.0	(86.7,100.0)
	Total	OPA	50/50	100.0	(92.9,100.0)
Day to Day Precision	Positive	PPA	50/50	100.0	(92.9,100.0)
	Negative	NPA	48/48	100.0	(92.6,100.0)
	Total	OPA	98/98	100.0	(96.2,100.0)

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

BenchMark ULTRA PLUS Instrument-to-Instrument Precision

BenchMark ULTRA PLUS Instrument-to-Instrument (Between-Instrument) Intermediate Precision of the VENTANA anti-BRAF V600E (VE1) antibody was determined by staining replicate slides of 10 CRC specimens (5 Positive and 5 Negative for BRAF V600E Clinical Status) across 3 BenchMark ULTRA PLUS instruments with the VENTANA anti-BRAF V600E (VE1) antibody using the OptiView DAB IHC Detection Kit. In addition, a single slide from each case was stained with a negative reagent control.

Each VENTANA anti-BRAF V600E (VE1) 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 (Positive/Negative) 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 PLUS Instrument-to-Instrument Precision, pairwise comparisons of the Clinical Status of slides for each specimen were made between instruments demonstrating 100% PPA, NPA, and OPA. A summary of the results can be found in Table 12.

Table 12. BenchMark ULTRA PLUS Instrument-to-Instrument Precision of the VENTANA anti-BRAF V600E (VE1) antibody as Measured by Clinical Status (Positive/Negative)

Precision	Clinical Status	Agreement			
		Type	n/N	%	95% CI
Instrument-to-Instrument	Positive	PPA	30/30	100.0	(88.6,100.0)
	Negative	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.

Between Platform Concordance for BenchMark ULTRA PLUS and BenchMark ULTRA

A study was conducted to compare the staining performance of VENTANA anti-BRAF V600E (VE1) antibody, using the OptiView DAB IHC Detection Kit, on the BenchMark ULTRA PLUS instrument versus the BenchMark ULTRA instrument. One hundred twenty (120) colorectal carcinoma tissue cases (14 positive for BRAF V600E, 13 negative for BRAF V600E, 93 MMR all-comers) were stained, and the stained slides were evaluated by a pathologist who determined the diagnostic status. The overall percent agreement was 98.3%. All tissues stained with VENTANA anti-BRAF V600E (VE1) antibody had acceptable morphology and background staining. A summary of the results is provided; as shown in Table 13.

Table 13. Agreement of the VENTANA anti-BRAF V600E (VE1) antibody on a BenchMark ULTRA PLUS instrument compared to a BenchMark ULTRA instrument as Measured by Clinical Status (Positive/Negative)

Between Platform Concordance	Agreement			
	Type	n/N	%	95% CI
ULTRA to ULTRA PLUS	PPA	34/35	97.1	(85.5, 99.5)
	NPA	84/85	98.8	(93.6, 99.8)
	OPA	118/120	98.3	(94.1, 99.5)

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

Accuracy Study: Concordance of VENTANA MMR IHC Panel Results to DNA Sequencing Results

A study was conducted to compare the performance of the VENTANA MMR IHC Panel to a comprehensive DNA sequencing colon panel for the identification of CRCs that result from potential Lynch syndrome. 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, 150 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 DNA sequencing. Of the 150 sequential CRC cases, 7 cases were excluded from the study set due to insufficient viable tumor (inadequate cellularity or lack of tumor content), 3 cases due to misclassification as CRC, and 1 due to clerical error. The remaining sequential study set cases were sectioned and a second H&E evaluation of bracketing slides was completed to ensure tissue integrity and tumor were represented through all sections, until sufficient cases (minimum of 100 cases) were enrolled into the study. Two cases were removed from the study due to lack of sufficient viable tumor content throughout the block and 1 was not evaluated due to clerical error. Following review, the sequential study set included 111 cases meeting the selection criteria and were enrolled into the study. The remaining 25 sequential CRC cases were not evaluated and not enrolled into the study. In addition, an enrichment study set of 15 CRC cases showing a Clinical status of Loss by IHC were included to ensure that Loss of each protein was represented in the study. Tissue sections of all cases in the study were stained by IHC with the VENTANA MMR IHC Panel and appropriate negative reagent controls. Additional tissue sections from each case were subjected to the DNA sequencing colon panel. Of the 126 enrolled cases, 7 cases were excluded from analyses due to failure of sequencing.

In the final study set of 119 cases (including one case that failed to produce IHC results due to tissue loss), the analysis compared the results of the VENTANA MMR IHC Panel to those for DNA sequencing at the case level, where DNA sequencing acted as the reference status for IHC comparison as shown in Table 14. For IHC, the MMR status (Intact / Loss) was stratified by BRAF V600E status, and for DNA sequencing, results were characterized by the presence or absence of potential pathogenic mutations. For this study, a pathogenic mutation within the tumor is defined as a germline or somatic mutation predicted to result in the loss of MMR protein expression. Point estimates for this comparison were 77.8% PPA, 97.0% NPA and 94.1% OPA.

IHC MMR status and DNA sequencing status were compared separately for individual MMR proteins within the study in Table 16. For MLH1 and PMS2 loss cases, results were stratified by BRAF V600E. The OPA of each MMR protein, when compared to the results of the DNA sequencing colon panel, was 95.8% for VENTANA anti-MLH1 (M1) antibody, 94.1% 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. Out of three cases containing a potential pathogenic mutation in the *MLH1* gene, all were MLH1 loss cases by IHC. One of these cases was also BRAF V600E positive suggesting sporadic CRC. It is likely given the variable allelic frequency that the pathogenic mutation identified in the tumor does not represent a germline mutation, but an acquired mutation in this tumor.

Four of the eight cases that contained a potential pathogenic mutation affecting MSH6 expression demonstrated MSH6 Intact status by IHC. Of these, two contained *POLE* mutations which variably affect the expression of MMR proteins[34,35,38] and do not represent Lynch syndrome mutations. One case demonstrated MSH6 IHC staining in a small portion of the tumor and was designated Intact, but DNA sequencing showed several mutations in the *MSH6 gene which likely result from somatic mutations.* An analysis of the VENTANA MMR IHC Panel and DNA sequencing results was also performed for the sequential (Table 17) and enrichment (Table 18) study sets at the case level. In addition, an evaluation of the IHC results for each MMR protein and DNA sequencing within the sequential and enrichment study sets are presented in Table 19 and Table 20, respectively.

The VENTANA anti-BRAF V600E (VE1) antibody is included in the VENTANA MMR IHC panel for the stratification of CRC cases showing a loss of MLH1 protein expression to sporadic or likely Lynch syndrome cancers. Of the 24 BRAF V600E IHC positive cases in this study, 20 cases had loss of MLH1 protein by IHC. The remaining four cases were pMMR (intact for all MMR proteins). All 24 BRAF V600E positive specimens were identified as sporadic CRC. Thus the data support the use of VENTANA anti-BRAF V600E (VE1) antibody to differentiate between sporadic and probable Lynch syndrome CRC in the absence of MLH1 expression. In addition, BRAF V600E Clinical status in CRC obtained by IHC using the VENTANA anti-BRAF V600E (VE1) antibody was also compared to *BRAF* mutational status results determined by DNA sequencing. For 23 BRAF V600E IHC positive cases (one case failed to yield DNA sequencing results), the PPA, NPA, and OPA of the VENTANA anti-BRAF V600E (VE1) antibody based IHC testing using DNA sequencing results as the reference all were 100%. These results verified that the VENTANA anti-BRAF V600E (VE1) antibody correctly identifies CRC having the *BRAF V600E* mutation.

Table 14. Evaluation of the VENTANA MMR IHC Panel and DNA Sequencing Results at the case level – ALL CASES

A) Comparison of VENTANA MMR IHC Panel and DNA Sequencing Results

VENTANA MMR IHC Panel Results		DNA Sequencing Results			
		Pathogenic Mutation	No Pathogenic Mutation	Invalid	Total
MMR Loss	BRAF V600E +	1	19	0	20
	BRAF V600E -	14	3	1	18
MMR Intact	BRAF V600E +	0	3	1	4
	BRAF V600E -	2	76	5	83
Invalid		1	0	0	1
Total		18	101	7	126

Note: Invalids are defined as failure to produce results by IHC and/or DNA sequencing.

Table 15. B) Agreement between VENTANA MMR IHC Panel and DNA Sequencing Results

Agreement			
Type	n/N	%	95% CI
PPA	14/18	77.8	(54.8, 91.0)
NPA	98/101	97.0	(91.6, 99.0)
OPA	112/119	94.1	(88.4, 97.1)

Note: Only Invalids resulting from a failure by IHC are included in the analysis. 95% CIs were calculated using the (Wilson) Score method.

Note: The association between the test results and the final diagnosis with respect to potential Lynch Syndrome is an estimate because the study was enriched with potential Lynch syndrome positive cases.

Table 16. Evaluation of the VENTANA MMR IHC Panel and DNA Sequencing Results at the Individual MMR Protein Level – ALL CASES

A) Comparison of Individual MMR Protein Status and DNA Sequencing Results

IHC Results		DNA Sequencing Results		
		Pathogenic Mutation	No Pathogenic Mutation	Total
MLH1 Loss	BRAF V600E +	1*	19	20
	BRAF V600E -	2	4	6
MLH1 Intact		0	92	92
Total		3	115	118
PMS2 Loss	BRAF V600E +	0	20	20
	BRAF V600E -	3	7	10
PMS2 Intact		0	88	88
Total		3	115	118
MSH2 Loss		3	2	5
MSH2 Intact		0	113	113
Total		3	115	118
MSH6 Loss		4	0	4
MSH6 Intact		4	110	114
Total		8	110	118

*Variant allele frequency indicates this is a rare MLH1 (p.K196Nfs*6) somatic mutation event and not a germline mutation.

B) Agreement between Individual MMR Protein and DNA Sequencing Results

Agreement				
Protein	Type	n/N	%	95% CI
MLH1	PPA	2/3	66.7	(20.8, 93.9)
	NPA	111/115	96.5	(91.4, 98.6)
	OPA	113/118	95.8	(90.5, 98.2)
PMS2	PPA	3/3	100.0	(43.9, 100.0)
	NPA	108/115	93.9	(88.0, 97.0)
	OPA	111/118	94.1	(88.3, 97.1)
MSH2	PPA	3/3	100.0	(43.9,100.0)
	NPA	113/115	98.3	(93.9,99.5)
	OPA	116/118	98.3	(94.0,99.5)
MSH6	PPA	4/8	50.0	(21.5,78.5)
	NPA	110/110	100.0	(96.6,100.0)
	OPA	114/118	96.6	(91.6, 98.7)

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

Table 17. Evaluation of the VENTANA MMR IHC Panel and DNA Sequencing Results at the Case Level – SEQUENTIAL STUDY SET

A) Comparison of VENTANA MMR IHC Panel and DNA Sequencing Results

VENTANA MMR IHC Panel Results		DNA Sequencing Results			
		Pathogenic Mutation	No Pathogenic Mutation	Invalid	Total
MMR Loss	BRAF V600E +	1	18	0	19
	BRAF V600E -	4	2	0	6
MMR Intact	BRAF V600E +	0	3	1	4
	BRAF V600E -	1	76	5	82
Invalid		0	0	0	0
Total		6	99	6	111

Note: Invalids are defined as failure to produce results by IHC and/or DNA sequencing.

B) Agreement of VENTANA MMR IHC Panel and DNA Sequencing Results

Agreement			
Type	n/N	%	95% CI
PPA	4/6	66.7	(30.0, 90.3)
NPA	97/99	98.0	(92.9, 99.4)
OPA	101/105	96.2	(90.6, 98.5)

Note: Only Invalids resulting from a failure by IHC are included in the analysis. 95% CIs were calculated using the (Wilson) Score method.

Table 18. Evaluation of the VENTANA MMR IHC Panel and DNA Sequencing Results at the Case Level – ENRICHMENT STUDY SET

A) Comparison of VENTANA MMR IHC Panel and DNA Sequencing Results

VENTANA MMR IHC Panel Results		DNA Sequencing Results			
		Pathogenic Mutation	No Pathogenic Mutation	Invalid	Total
MMR Loss	BRAF V600E +	0	1	0	1
	BRAF V600E -	10	1	1	12
MMR Intact	BRAF V600E +	0	0	0	0
	BRAF V600E -	1	0	0	1
Invalid		1	0	0	1
Total		12	2	1	15

Note: Invalids are defined as failure to produce results by IHC and/or DNA sequencing.

B) Agreement of VENTANA MMR IHC Panel and DNA Sequencing Results

Agreement			
Type	n/N	%	95% CI
PPA	10/12	83.3	(55.2, 95.3)
NPA	1/2	50.0	(9.5, 90.5)
OPA	11/14	78.6	(52.4, 92.4)

Agreement			
Type	n/N	%	95% CI

Note: Only Invalids resulting from a failure by IHC are included in the analysis. 95% CIs were calculated using the (Wilson) Score method.

Table 19. Evaluation of MMR protein and DNA Sequencing Results at the Individual MMR Protein Level – SEQUENTIAL STUDY SET

A) Comparison of Individual MMR Protein Status and DNA Sequencing Results

IHC Results		DNA Sequencing Results		
		Pathogenic Mutation	No Pathogenic Mutation	Total
MLH1 Loss	BRAF V600E +	1	18	19
	BRAF V600E -	1	4	5
MLH1 Intact		0	81	81
Total		2	103	105
PMS2 Loss	BRAF V600E +	0	19	19
	BRAF V600E -	0	5	5
PMS Intact		0	81	81
Total		0	105	105
MSH2 Loss		1	0	1
MSH2 Intact		0	104	104
Total		1	104	105
MSH6 Loss		0	0	0
MSH6 Intact		3	102	105
Total		3	102	105

B) Agreement of Individual MMR Protein Status and DNA Sequencing Results

Agreement				
Protein	Type	n/N	%	95% CI
MLH1	PPA	1/2	50.0	(9.5, 90.5)
	NPA	99/103	96.1	(90.4, 98.5)
	OPA	100/105	95.2	(89.3, 97.9)
PMS2	PPA	n.e.	n.e.	n.e.
	NPA	100/105	95.2	(89.3, 97.9)
	OPA	100/105	95.2	(89.3, 97.9)
MSH2	PPA	1/1	100.0	(20.7,100.0)
	NPA	104/104	100.0	(96.4, 100.0)
	OPA	105/105	100.0	(96.5, 100.0)
MSH6	PPA	0/3	0.0	(0.0, 56.1)
	NPA	102/102	100.0	(96.4,100.0)
	OPA	102/105	97.1	(91.9, 99.0)

Note: 95% CIs were calculated using the (Wilson) Score method. n.e.= not estimable.

Table 20. Evaluation of MMR protein and DNA Sequencing Results at the Individual MMR Protein Level – ENRICHMENT STUDY SET

A) Comparison of Individual MMR Protein Status and DNA Sequencing Results

IHC Results		DNA Sequencing Results		
		Pathogenic Mutation	No Pathogenic Mutation	Total
MLH1 Loss	BRAF V600E +	0	1	1
	BRAF V600E -	1	0	1
MLH1 Intact		0	11	11
Total		1	12	13
PMS2 Loss	BRAF V600E +	0	1	1
	BRAF V600E -	3	2	5
PMS2 Intact		0	7	7
Total		3	10	13
MSH2 Loss		2	2	4
MSH2 Intact		0	9	9
Total		2	11	13
MSH6 Loss		4	0	4
MSH6 Intact		1	8	9
Total		5	8	13

B) Agreement of Individual MMR Protein Status and DNA Sequencing Results

Agreement				
Protein	Type	n/N	%	95% CI
MLH1	PPA	1/1	100.0	(20.7, 100.0)
	NPA	12/12	100.0	(75.8, 100.0)
	OPA	13/13	100.0	(77.2, 100.0)
PMS2	PPA	3/3	100.0	(43.9, 100.0)
	NPA	8/10	80.0	(49.0, 94.3)
	OPA	11/13	84.6	(57.8, 95.7)
MSH2	PPA	2/2	100.0	(34.2,100.0)
	NPA	9/11	81.8	(52.3, 94.9)
	OPA	11/13	84.6	(57.8, 95.7)
MSH6	PPA	4/5	80.0	(37.6, 96.4)
	NPA	8/8	100.0	(67.6,100.0)
	OPA	12/13	92.3	(66.7, 98.6)

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

REFERENCES

1. Yuan L, Chi Y, Chen W, Chen X, Wei P, et al. Immunohistochemistry and microsatellite instability analysis in molecular subtyping of colorectal carcinoma based on mismatch repair competency. Int J Clin Exp Med. 2015;8(11):20988-21000.
2. Geiersbach KB, Samowitz WS. Microsatellite instability and colorectal cancer. Arch Pathol Lab Med. 2011;135(10):1269-1277.
3. Wright CL, Stewart ID. Histopathology and mismatch repair status of 458 consecutive colorectal carcinomas. Am J Surg Pathol. 2003;27(11):1393-1406.
4. Tiwari AK, Roy HK, Lynch HT. Lynch syndrome in the 21st century: clinical perspectives. QJM. 2016;109(3):151-158.
5. Buza N, Ziai J, Hui P. Mismatch repair deficiency testing in clinical practice. Expert Rev Mol Diagn. 2016;16(5):591-604.
6. Silva FCC, Torrezan GT, Ferreira JRO, Oliveira LP, Begnami M, et al. Germline Mutations in MLH1 Leading to Isolated Loss of PMS2 Expression in Lynch Syndrome: Implications for Diagnostics in the Clinic. Am J Surg Pathol. 2017;41(6):861-864.
7. Boyer JC, Umar A, Risinger JI, Lipford JR, Kane M, et al. Microsatellite instability, mismatch repair deficiency, and genetic defects in human cancer cell lines. Cancer Res. 1995;55(24):6063-6070.
8. Lawes DA, Pearson T, Sengupta S, Boulos PB. The role of MLH1, MSH2 and MSH6 in the development of multiple colorectal cancers. Br J Cancer. 2005;93(4):472-477.
9. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. N Engl J Med. 2003;348(10):919-932.
10. Peltomaki P. Role of DNA mismatch repair defects in the pathogenesis of human cancer. J Clin Oncol. 2003;21(6):1174-1179.
11. Lynch HT, Smyrk T. Hereditary nonpolyposis colorectal cancer (Lynch syndrome). An updated review. Cancer. 1996;78(6):1149-1167.
12. Caldes T, Godino J, Sanchez A, Corbacho C, De la Hoya M, et al. Immunohistochemistry and microsatellite instability testing for selecting MLH1, MSH2 and MSH6 mutation carriers in hereditary non-polyposis colorectal cancer. Oncol Rep. 2004;12(3):621-629.
13. Shia J, Klimstra DS, Nafa K, Offit K, Guillem JG, et al. Value of immunohistochemical detection of DNA mismatch repair proteins in predicting germline mutation in hereditary colorectal neoplasms. Am J Surg Pathol. 2005;29(1):96-104.
14. Cunningham JM, Tester DJ, Thibodeau SN. Mutation detection in colorectal cancers : direct sequencing of DNA mismatch repair genes. Methods Mol Med. 2001;50:87-98.
15. Domingo E, Laiho P, Ollikainen M, Pinto M, Wang L, et al. BRAF screening as a low-cost effective strategy for simplifying HNPCC genetic testing. J Med Genet. 2004;41(9):664-668.
16. Jin M, Hampel H, Zhou X, Schunemann L, Yearsley M, et al. BRAF V600E mutation analysis simplifies the testing algorithm for Lynch syndrome. Am J Clin Pathol. 2013;140(2):177-183.
17. Deng G, Bell I, Crawley S, Gum J, Terdiman JP, et al. BRAF mutation is frequently present in sporadic colorectal cancer with methylated hMLH1, but not in hereditary nonpolyposis colorectal cancer. Clin Cancer Res. 2004;10(1 Pt 1):191-195.
18. Giardiello FM, Allen JI, Axilbund JE, Boland CR, Burke CA, et al. Guidelines on Genetic Evaluation and Management of Lynch Syndrome: A Consensus Statement by the US Multi-Society Task Force on Colorectal Cancer. Diseases of the Colon & Rectum. 2014;57(8):1025-1048.
19. Egoavil C, Alenda C, Castillejo A, Paya A, Peiro G, et al. Prevalence of Lynch syndrome among patients with newly diagnosed endometrial cancers. PLoS One. 2013;8(11):e79737.
20. Connell LC, Mota JM, Braghiroli MI, Hoff PM. The Rising Incidence of Younger Patients With Colorectal Cancer: Questions About Screening, Biology, and Treatment. Curr Treat Options Oncol. 2017;18(4):23.
21. Provenzale D, Gupta S, Ahnen DJ, Bray T, Cannon JA, et al. Genetic/Familial High-Risk Assessment: Colorectal Version 1.2016, NCCN Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw. 2016;14(8):1010-1030.
22. Balmana J, Balaguer F, Cervantes A, Arnold D, Group EGW. Familial risk-colorectal cancer: ESMO Clinical Practice Guidelines. Ann Oncol. 2013;24 Suppl 6:vi73-80.
23. Evaluation of Genomic Applications in P, Prevention Working G. Recommendations from the EGAPP Working Group: genetic testing strategies in newly diagnosed individuals with colorectal cancer aimed at reducing morbidity and mortality from Lynch syndrome in relatives. Genet Med. 2009;11(1):35-41.
24. Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst. 2004;96(4):261-268.
25. Parsons MT, Buchanan DD, Thompson B, Young JP, Spurdle AB. Correlation of tumour BRAF mutations and MLH1 methylation with germline mismatch repair

(MMR) gene mutation status: a literature review assessing utility of tumour features for MMR variant classification. J Med Genet. 2012;49(3):151-157.
26. Shia J. Evolving approach and clinical significance of detecting DNA mismatch repair deficiency in colorectal carcinoma. Semin Diagn Pathol. 2015;32(5):352-361.
27. Thiel A, Heinonen M, Kantonen J, Gylling A, Lahtinen L, et al. BRAF mutation in sporadic colorectal cancer and Lynch syndrome. Virchows Arch. 2013;463(5):613-621.
28. Toon CW, Chou A, DeSilva K, Chan J, Patterson J, et al. BRAFV600E immunohistochemistry in conjunction with mismatch repair status predicts survival in patients with colorectal cancer. Mod Pathol. 2014;27(5):644-650.
29. Koinuma K, Shitoh K, Miyakura Y, Furukawa T, Yamashita Y, et al. Mutations of BRAF are associated with extensive hMLH1 promoter methylation in sporadic colorectal carcinomas. Int J Cancer. 2004;108(2):237-242.
30. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, et al. Mutations of the BRAF gene in human cancer. Nature. 2002;417(6892):949-954.
31. Vakiani E, Solit DB. KRAS and BRAF: drug targets and predictive biomarkers. J Pathol. 2011;223(2):219-229.
32. Tiacci E, Schiavoni G, Forconi F, Santi A, Trentin L, et al. Simple genetic diagnosis of hairy cell leukemia by sensitive detection of the BRAF-V600E mutation. Blood. 2012;119(1):192-195.
33. Capper D, Preusser M, Habel A, Sahm F, Ackermann U, et al. Assessment of BRAF V600E mutation status by immunohistochemistry with a mutation-specific monoclonal antibody. Acta Neuropathol. 2011;122(1):11-19.
34. Capper D, Berghoff AS, Magerle M, Ilhan A, Wohrer A, et al. Immunohistochemical testing of BRAF V600E status in 1,120 tumor tissue samples of patients with brain metastases. Acta Neuropathol. 2012;123(2):223-233.
35. Carson FL, Cappellano C. Histotechnology; A Self-Instructional Text, 5th edition. American Society for Clinical Pathology Press; 2020, 2022.
36. Occupational Safety and Health Standards: Occupational exposure to hazardous chemicals in laboratories. (29 CFR Part 1910.1450). Fed. Register.
37. Directive 2000/54/EC of the European Parliament and Council of 24 June 2020 on the protection of workers from risks related to exposure to biological agents at work.
38. Roche PC, Hsi ED, Firfer BL. Immunohistochemistry: Principles and Advances. Manual of Molecular and Clinical Laboratory Immunology, 7th Edition: American Society of Microbiology; 2006.
39. Rabinovitch A. The College of American Pathologists laboratory accreditation program. Accreditation and Quality Assurance. 2002;7(11):473-476.
40. CSLI. Quality Assurance for Design Control and Implementation of Immunohistochemistry Assays: Approved Guideline-Second Edition. CLSI document I/LA28-A2 (ISBN 1-56238-745-6). CLSI, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA, 2011.
41. Kuan SF, Navina S, Cressman KL, Pai RK. Immunohistochemical detection of BRAF V600E mutant protein using the VE1 antibody in colorectal carcinoma is highly concordant with molecular testing but requires rigorous antibody optimization. Hum Pathol. 2014;45(3):464-472.
42. Day F, Muranyi A, Singh S, Shanmugam K, Williams D, et al. A mutant BRAF V600E-specific immunohistochemical assay: correlation with molecular mutation status and clinical outcome in colorectal cancer. Target Oncol. 2015;10(1):99-109.
43. Haraldsdottir S, Hampel H, Tomsic J, Frankel WL, Pearlman R, et al. Colon and endometrial cancers with mismatch repair deficiency can arise from somatic, rather than germline, mutations. Gastroenterology. 2014;147(6):1308-1316 e1301.
44. Jansen AM, van Wezel T. Combined mismatch repair and POLE/POLD1 defects explain unresolved suspected Lynch syndrome cancers. 2016;24(7):1089-1092.
45. Kane DP, Shcherbakova PV. A common cancer-associated DNA polymerase epsilon mutation causes an exceptionally strong mutator phenotype, indicating fidelity defects distinct from loss of proofreading. Cancer Res. 2014;74(7):1895-1901.

Symbols

Ventana uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard (for USA: see elabdoc.roche.com/symbols for more information).

GTIN	Global Trade Item Number
Rx only	For USA: Caution: Federal law restricts this device to sale by or on the order of a physician.

REVISION HISTORY

Rev	Updates
E	Updates to Warnings and Precautions section. Updated to current template.

INTELLECTUAL PROPERTY

For USA: Rx only

CONTACT INFORMATION

Ventana Medical Systems, Inc.
1910 E. Innovation Park Drive
Tucson, AZ 85755
USA
+1 520 887 2155
+1 800 227 2155 (USA)

www.roche.com