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

For use with the VENTANA MMR IHC Panel

REF 760-5095

08033706001

IVD Σ 50

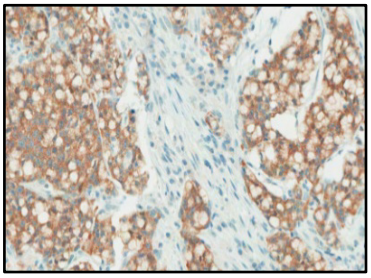

Figure 1. VENTANA anti-BRAF V600E (VE1) antibody staining of neoplastic cells in colon cancer tissue.

INTENDED USE

VENTANA anti-BRAF V600E (VE1) Mouse Monoclonal Primary Antibody (VENTANA anti-BRAF V600E (VE1) antibody) is intended for the qualitative detection of BRAF V600E protein in formalin-fixed, paraffin-embedded tissue sections. VENTANA anti-BRAF V600E (VE1) antibody is ready to use on BenchMark IHC/ISH instruments with the OptiView DAB IHC Detection Kit and ancillary reagents.

VENTANA anti-BRAF V600E (VE1) 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-MSH6 (SP93) Rabbit 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 cancer 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]

The *BRAF* gene is located on chromosome 7q34 and 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 and is detected in 12% of all CRC.[15,16]

As part of the VENTANA MMR IHC Panel, VENTANA anti-BRAF V600E (VE1) antibody aids to differentiate sporadic and probable Lynch syndrome CRC in the absence of MLH1 protein expression.[17,18] In CRC, loss of MLH1 protein is frequently the result of hypermethylation of the *MLH1* promoter and indicates a sporadic occurrence.[19] 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.

Apart from its role in the stratification of CRC, the *BRAF V600E* mutation is detected in approximately 8% of all solid tumors, including 43% of melanomas, 39% of papillary thyroid carcinomas, 12% of serous ovarian carcinomas, 2% of lung cancers, and in other cancers.[16] Furthermore, the *BRAF V600E* mutation has been recently described as a molecular marker of hairy cell leukemia.[20]

CLINICAL SIGNIFICANCE

Lynch syndrome was described in the 1960s and identified a link between the loss of MMR function and cancer.[21] 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.[5,22,23] 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.[21,24-27] 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.[5,28,29] 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.[28] 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.[5,30] 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.[21,31] 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.[32]

PRINCIPLE OF THE PROCEDURE

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).[32]

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 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 (Predilute) (Cat. No. 650-210 / 05424534001) or LCS (Predilute) (Cat. No. 650-010 / 05264839001), which minimizes evaporation of the aqueous reagents from the specimen slide.

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 phosphate buffer (pH 7.3) with carrier protein, Brij 35, and 0.05% ProClin 300, 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 method sheet for detailed descriptions of: Principle of the Procedure, Material and Methods, Specimen Collection and Preparation for Analysis, Quality Control Procedures, Troubleshooting, Interpretation of Results, and 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.

Not all products listed in the method sheet may be available in all geographies. Consult your local support representative.

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

1. Recommended control tissue
2. Microscope slides, positively charged
3. VENTANA anti-MLH1 (M1) Mouse Monoclonal Primary Antibody (Cat. No. 730-7159 / 09605584001 or Cat. No. 760-5091 / 08033668001)
4. VENTANA anti-PMS2 (A16-4) Mouse Monoclonal Primary Antibody (Cat. No. 730-7158 / 09607161001 or Cat. No. 760-5094 / 08033692001)
5. VENTANA anti-MSH2 (G219-1129) Mouse Monoclonal Primary Antibody (Cat. No. 730-7160 / 09607137001 or Cat. No. 760-5093 / 08033684001)
6. VENTANA anti-MSH6 (SP93) Rabbit Monoclonal Primary Antibody (Cat. No. 730-7161 / 09606769001 or Cat. No. 760-5092 / 08033676001)
7. Negative Control (Monoclonal) (Cat. No. 760-2014 / 05266670001)
8. Rabbit Monoclonal Negative Control Ig (Cat. No. 790-4795 / 06683380001)
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 (50 test) or Cat. No. 860-099 / 06718663001 (250 test))
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. LCS (Predilute) (Cat. No. 650-010 / 05264839001)
15. ULTRA Cell Conditioning Solution (ULTRA CC1) (Cat. No. 950-224 / 05424569001)
16. Cell Conditioning Solution (CC1) (Cat. No. 950-124 / 05279801001)
17. Hematoxylin II (Cat. No. 790-2208 / 05277965001)
18. Bluing Reagent (Cat. No. 760-2037 / 05266769001)
19. Permanent mounting medium
20. Cover glass
21. Automated coverslipper
22. General purpose laboratory equipment
23. BenchMark IHC/ISH instrument

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 the 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 BenchMark IHC/ISH instruments. The recommended tissue fixative is 10% neutral buffered formalin.[35]

For VENTANA anti-BRAF V600E (VE1) antibody, tissue fixation is recommended within 2 hours of excision in 10% neutral buffered formalin (NBF) for at least 12 hours on the basis of xenograft models generated from the A2058 (melanoma) and LS411N (CRC) human cell-lines, which are positive for BRAF V600E expression. However, fixation times of up to 72 hours in 10% NBF gave equivalent BRAF V600E staining results. Acceptable VENTANA anti-BRAF V600E (VE1) antibody staining was also achieved with fixation in Zinc formalin fixative for 12-72 hours.

Alcohol formalin (AFA), 95% Ethanol, Z-5, and PREFER fixatives are not recommended for use with VENTANA anti-BRAF V600E (VE1) antibody. Xenograft tissues fixed in alcohol formalin 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).[35,36]

Sections should be cut approximately 4 µm thick and mounted on positively charged slides. Slides should be stained immediately, as antigenicity of cut tissue sections may diminish over time. However, unstained CRC tissue slides stored at 5 ± 3°C or 30 ± 5°C for up to 8 weeks demonstrated similar VENTANA anti-BRAF V600E (VE1) antibody stain intensity compared to the tissue specimens prepared from the same block and stained with VENTANA anti-BRAF V600E (VE1) antibody on day 1. Ask your Roche representative for a copy of “Recommended Slide Storage and Handling” for more information.

WARNINGS AND PRECAUTIONS

1. For *in vitro* diagnostic (IVD) use.
2. For professional use only.
3. **CAUTION:** In the United States, Federal law restricts this device to sale by or on the order of a physician. (Rx Only)
4. Do not use beyond the specified number of tests.
5. 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.
6. 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.
7. 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.[37,38]
8. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water.
9. Avoid microbial contamination of reagents as it may cause incorrect results.
10. 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.
11. Consult local and/or state authorities with regard to recommended method of disposal.
12. Product safety labeling primarily follows EU GHS guidance. Safety data sheet available for professional user on request.

13. 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 IHC/ISH instruments in combination with the OptiView DAB IHC Detection Kit and ancillary reagents. Refer to Table 2 for recommended staining protocol.

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 instrument User Guide. Refer to the OptiView DAB IHC Detection Kit method sheet for more details regarding immunohistochemistry staining procedures.

For more details on the proper use of this device, refer to the inline dispenser method sheet associated with P/N 760-5095.

Table 2. Recommended staining protocol for VENTANA anti-BRAF V600E (VE1) antibody with OptiView DAB IHC Detection Kit on BenchMark IHC/ISH instruments.

Procedure Type	Method		
	GX	XT	ULTRA or ULTRA PLUS
Deparaffinization	Selected	Selected	Selected
Cell Conditioning (Antigen Unmasking)	CC1 64 minutes	CC1 64 minutes	ULTRA CC1 64 minutes, 100°C
Pre-Primary Peroxidase Inhibitor	Selected	Selected	Selected
Antibody (Primary)	28 minutes, 37°C	16 minutes, 37°C	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. 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."[36]

QUALITY CONTROL PROCEDURES

Negative Reagent Control

In addition to staining with VENTANA anti-BRAF V600E (VE1) antibody, a second slide should be stained with a mouse monoclonal negative reagent, Negative Control (Monoclonal). The negative reagent control is used to assess non-specific 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 helps identify any failures applying reagents to the slide. Tissue with weak positive staining is best suited for quality control. The positive staining tissue components are used to confirm that the antibody was applied and the instrument functioned properly. Control tissue may contain both positive and negative staining elements and serve as both the positive and negative control tissue. Control tissues should be fresh autopsy, biopsy, or surgical specimen, 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[39] 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 specimens stained with the VENTANA anti-BRAF V600E (VE1) antibody are 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

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-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, enterocytes, interstitial cells of testis, adrenal gland, 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 the pituitary gland. In addition, this antibody also stains cilia in lung.
- 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 Artifact: 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.

PERFORMANCE CHARACTERISTICS

ANALYTICAL PERFORMANCE

Staining tests for sensitivity, specificity, and precision were conducted and the results are listed below.

Sensitivity and Specificity

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. Sensitivity/Specificity of VENTANA anti-BRAF V600E (VE1) antibody was determined by testing FFPE normal tissues.

Tissue	# positive / total cases	Tissue	# positive / total cases
Cerebrum	0/3	Esophagus	0/3
Cerebellum *	1/3	Stomach	0/3
Adrenal gland	0/3	Small intestine *	2/4
Ovary	0/3	Colon *	5/12
Pancreas *	2/3	Liver	0/3
Lymph node	0/3	Tongue/salivary gland	0/3
Pituitary gland **	3/3	Kidney	0/3
Testis *	2/3	Prostate	0/3
Thyroid	0/3	Bladder	0/3
Breast	0/3	Parathyroid gland	0/3
Spleen	0/3	Endometrium	0/3
Tonsil	0/3	Cervix	0/3
Thymus	0/3	Skeletal muscle	0/3
Bone Marrow	0/3	Skin	0/3
Lung	0/3	Nerve	0/5
Heart	0/3	Mesothelium	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 the pituitary gland.

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. Sensitivity/Specificity of VENTANA anti-BRAF V600E (VE1) antibody was determined by testing 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
Neuroendocrine neoplasm (Pancreas)	0/1
Seminoma (Testis)	0/2
Medullary carcinoma (Thyroid)	0/1
Papillary carcinoma (Thyroid)	21/28
Ductal carcinoma in situ (Breast)	0/1
Microinvasive ductal carcinoma (Breast)	0/1
Invasive ductal carcinoma (Breast)	3/131
B-cell lymphoma; NOS (Spleen)	0/1
Small cell carcinoma (Lung)	0/7
Squamous cell carcinoma (Lung)	0/90
Adenocarcinoma (Lung)	1/73
Neuroendocrine carcinoma (Esophagus)	0/1
Adenocarcinoma (Esophagus)	0/1
Signet-ring cell carcinoma (Stomach)	0/1
Adenocarcinoma (Small intestine)	0/1
Stromal sarcoma (Small intestine)	1/1
Adenocarcinoma (Colon)	64/234
Gastrointestinal stromal tumor (GIST) (Colon)	0/1
Adenocarcinoma (Rectum)	0/1
GIST (Rectum)	0/1
Melanoma (Rectum)	0/1

Pathology	# positive / total cases
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
Embryonal rhabdomyosarcoma (Striated muscle)	0/1
Melanoma	10/24
Basal cell carcinoma (Skin)	0/1
Squamous cell carcinoma (Skin)	0/1
Neuroblastoma (Retroperitoneum)	0/1
Mesothelioma (Peritoneum)	0/1
Spindle cell rhabdomyosarcoma (Peritoneum)	0/1
B-cell lymphoma; NOS (Lymph node)	0/2
Hodgkin lymphoma (Lymph node)	1/1
Urothelial carcinoma (Bladder)	0/1
Leiomyosarcoma (Bladder)	0/1
Osteosarcoma (Bone)	0/1
Leiomyosarcoma (Smooth muscle)	0/1

Precision

Performance Characteristics on BenchMark ULTRA Instrument

Within-Run Repeatability and Between-Day Intermediate Precision

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

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 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 sets were randomized, and then evaluated as Positive or Negative by a single pathologist blinded to the case diagnosis.

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 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 sets 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-Run Repeatability and Between-Day Intermediate 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. BenchMark ULTRA instrument Within-Run Repeatability and Between-Day Intermediate 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-Run 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)
Between-Day Intermediate 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.

Between-Instrument Intermediate Precision

BenchMark ULTRA 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 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 sets 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 Between-Instrument Intermediate 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 Between-Instrument Intermediate 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
Between-Instrument Intermediate Precision	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.

In addition, Between-Instrument Intermediate Precision of the VENTANA anti-BRAF V600E (VE1) antibody was determined by staining replicate slides of 6 specimens (2 CRC negative and 2 CRC and 2 Thyroid cancer positive for BRAF V600E Clinical Status) across 3 BenchMark XT and 3 BenchMark GX instruments with VENTANA anti-BRAF V600E (VE1) 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 Between-Instrument Intermediate Precision, pairwise comparisons of stain intensity scores of slides for each specimen were made and demonstrated 98.9% OPA between 3 BenchMark XT and 100% OPA 3 between BenchMark GX instruments. None of the slides stained with a negative reagent control showed specific staining, and background staining was ≤ 0.5 on both the BenchMark XT and BenchMark GX instruments.

BenchMark IHC/ISH Instrument Concordance

Concordance across the BenchMark IHC/ISH instruments for the VENTANA anti-BRAF V600E (VE1) antibody was determined by staining CRC specimens, thyroid cancer and melanoma specimens with VENTANA anti-BRAF V600E (VE1) antibody using the OptiView DAB IHC Detection Kit. All slides were evaluated for Clinical Status (Positive/Negative) by a single pathologist.

Pairwise comparisons of Clinical Status for 228 total specimens (177 CRC, 27 thyroid cancers and 24 melanomas) were made between BenchMark GX to BenchMark ULTRA instruments and BenchMark XT to BenchMark ULTRA instruments. BenchMark GX to BenchMark ULTRA instruments demonstrated 98.0% average positive agreement (APA), 99.0% average negative agreement (ANA), and 98.7% OPA. BenchMark XT to BenchMark ULTRA instruments demonstrated 96.6% APA, 98.4% ANA, and 97.8% OPA. For BenchMark GX to BenchMark XT instruments, pairwise comparisons of 230 total specimens (179 CRC, 27 thyroid cancers and 24 melanomas) were made between platforms. BenchMark GX to BenchMark XT instruments demonstrated 98.7% APA, 99.4% ANA, and 99.1% OPA.

Reader Precision Studies

Within- and Between-Reader precision was evaluated on 20 CRC specimens (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 an H&E and a negative reagent control stained slide from the same case.

All slide sets were randomized and evaluated by 3 pathologists for Positive or Negative BRAF V600E Clinical Status. Pathologists were blinded to the case diagnosis. Following a two-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 pathologists were pooled and demonstrated 100% APA, 100% ANA, and 100% 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)
	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 sets 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 mixed model (GLMM) approach.

In addition, pairwise comparisons were made Between-Site, Between-Day, and Between-Reader for the 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: 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 result in the loss of MMR protein expression. Point estimates were 99.4% PPA, 93.5% NPA, and 98.8% OPA as shown in Table 14.

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 15.

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 16 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 17). 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 hypermethylation. 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 14. 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 15. 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 16. 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 17. 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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NOTE: A point (period/stop) is always used in this document as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

The summary of safety and performance can be found here:

https://ec.europa.eu/tools/eudamed

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
UDI	Unique Device Identification

REVISION HISTORY

Rev	Updates
F	Updates to the Materials Required But Not Provided section in the form of additional P/N's for 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-MSH6 (SP93) Rabbit Monoclonal Primary Antibody.

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