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ihcDirect™ Mart-1 pHRP Staining Kit: Anti-Human Mart-1 (Clone A103)

K41009-030 300 tissue stains*
K41009-010 100 tissue stains*
K41009-005 50 tissue stains*

Intended Use: For In Vitro Diagnostic Use

Polymerized horseradish peroxidase (pHRP)-labeled anti Mart-1 (Melan A) antibody (Mart-1 pHRP) is intended for laboratory use to qualitatively identify by light microscopy the presence of Melan A in sections of formalin-fixed, paraffin-embedded and/or cryostat tissues using immunohistochemistry (IHC) test methods. The clinical interpretation of any staining or its absence should be complemented by morphological studies using proper controls and should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist/physician. This conjugate has been pre-diluted and optimized for IHC use without further dilution.

Summary and Explanation:

The Mart-1 antibody recognizes Melan A, a protein with 118 amino acids, which is a melanocyte differential antigen. It is present in melanocytes of normal skin, retina, nevi, and in more than 85% of melanomas (Orchard GE, 1998, Kageshita T, 1997). This antibody does not stain normal or tumor tissues from non-melanocyte lineage. The key component in this kit is polymerized horseradish peroxidase (pHRP) labeled mouse anti-human Mart-1 monoclonal antibody (clone A103). The ihc Blocker provided in the kit used prior to applying pHRP-Mart-1 conjugate will reduce background staining. Chromogen 3,3'diaminoben-zidine (DAB) is used in this kit.

Principle of Procedure:

The ready-to-use ihcDirect Mart-1 pHRP antibody conjugate is directly applied to pretreated tissue sections, where it binds to Melan A. A DAB working solution is then applied to the tissue. The Mart-1 antibody-linked pHRP catalyzes the DAB to form a visible brown color product which precipitates at the site of Melan A location. The specimen may then be counterstained with hematoxylin and a coverslip applied. Results are viewed and interpreted using a light microscope. Volumes are based upon 100 µl antibody per tissue. This IHC kit may be performed either manually or on an open automatic IHC staining system.

Reagents Provided:

Kit Part No.	Σ	Description
K41009-005*	50*	5ml size ihcDirect Mart-1 ready-to-use antibody conjugate, ihc Blocker and equivalent volume of ihc DAB and ihc DAB Diluent.
K41009-010*	100*	10ml size ihcDirect Mart-1 ready-to-use antibody conjugate, ihc Blocker and equivalent volume of ihc DAB and ihc DAB Diluent.
K41009-030*	300*	2 X 15ml size ihcDirect Mart-1 ready-to-use antibody conjugate, ihc Blocker and equivalent volume of ihc DAB and ihc DAB Diluent.

* At estimated volume of 100 µl of antibody conjugate per tissue

Immunogen	Clone	Species	IgClass	Protein Conc.
Recombinant Mart-1	A103	Mouse	IgG1	10 mg/ml

Mart-1 antibody is mouse monoclonal antibody to Melan-A purified from ascites. HRP is extracted from horseradish plant. The ihc Blocker contains normal goat serum and 1% BSA in a proprietary buffer system with 0.01% Thimerosal as preservative.

The ihc DAB Diluent contains hydrogen peroxide and some other non-hazardous materials at a reportable concentration. The ihc DAB (chromogen) contains 3,3'diaminobenzidine which is dissolved in a proprietary buffer system with no hazardous chemicals at a reportable concentration. This reagent is light sensitive. For best results, minimize time vial is open. Keep away from light.

Mart-1 pHRP Kit Components for 5ml, 10ml and 30ml Test Kits:

Kit Components	Component Part Numbers	Sizes
Mart-1 pHRP	H31009-(R###) (005, 010, 015)	5ml, 10ml, 15ml
ihc Blocker	C30005-(###ML) (005, 010, 015)	5ml, 10ml, 15ml
ihc DAB Diluent	C30004-(###ML) (005, 010, 015)	5ml, 10ml, 15ml
ihc DAB	C30003-(###UL), (150, 300, 450)	150µl, 300µl, 450µl

Note: *The K41009-030, 30ml kit size uses DAB Kit, K500001-030 which contains 2 each of ihc DAB (15ml) and ihc DAB Diluent(450µl).*

Materials Needed but Not Provided:

The following reagents/supplies may be required in staining but are not provided:

1. Frozen tissue fixative (ihc Fixative) or reagent grade acetone
2. Positive and negative control tissues
3. Microscope slides, positively charged
4. Staining jars, baths or processing tools
5. ihc Wash Buffer (PBS-T)
6. Pipettor and pipet tips
7. Timer
8. Antigen retrieval buffer (when using FFPE tissues)
9. Peroxide blocker (optional)
10. Instruments used for tissue pretreatment, such as water bath, or pressure cooker or microwave oven (when using FFPE tissues)
11. Hematoxylin
12. Xylene
13. Ethanol
14. Mounting medium
15. Cover slips
16. Light microscope (40 - 400x)

Novodiax Bulk Reagent Formulations:

1. ihc Fixative, (375ml of methyl alcohol, 100ml of 37% formaldehyde and 25ml of glacial acetic acid).
2. ihc Wash Buffer (PBS-T), (10 mM phosphate buffer, pH7.2, 150 mM NaCl, 0.05% Tween-20).
3. ihc Antigen Retrieval Buffer (10mM Citric buffer, pH 6.0, 0.02% Tween 20).

Storage and Handling

The kit should be stored at 2-8°C. Do not freeze. DAB working solution should be made prior to use and is stable at 2-8°C during the day the reagents are made. This kit is suitable for use until expiry date when stored at 2-8°C. Do not use the product after expiration date stamped on vial unless dating extension information is provided by Novodiax. If reagents are stored under any conditions other than those specified in the package insert, they must be verified by the user.

Positive and negative controls should be run simultaneously with patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures or a problem with the antibody is suspected, contact Novodiax Support at 888-439-2716, ext. 1 or at 1 (510) 342-3043 ext. 1 immediately.

Specimen Preparation:

Paraffin Sections: Tissues routinely processed, neutral buffered 10% formalin-fixed are suitable for use prior to paraffin embedding. Consult references (Kiernan, 1981; Sheehan & Hrapchak, 1980). Variable results may occur as a result of prolonged fixation. Each section should be cut to the appropriate thickness (approximately 4-5 µm) and placed on a positively charged glass slide. Slides containing the tissue section may be baked for at least one hour but not exceeding 24 hours in a 58-60^{O}C±5^{0}C oven. Osseous tissues should be decalcified prior to tissue processing to facilitate tissue cutting and prevent damage to microtome blades (Kiernan, 1981: Sheehan & Hrapchak, 1980).

Cryostat Sections: Frozen tissue is sectioned to the appropriate thickness (approximately 5 µm) and placed on a positively charged glass slide. Tissues should be fixed in either the Novodiax ihc Fixative or reagent grade acetone for 30-seconds-to-1-minute. Reagent grade acetone may be kept cold, e.g. at cryostat temperatures or at room temperature. Following fixation, tissues may be stored in PBS-T for as long as a day.

Treatment of Tissues Prior to Staining: Pretreatment is tissue dependent and should be performed as suggested in the staining procedure sections.

Warnings and Precautions:

1. The Mart-1 pHRP antibody conjugate is pre-diluted. Further dilution may reduce signal intensity or false-negative staining. These recommendations are for guidance only. Laboratory managers should determine their own procedures and quality policies.
2. Take reasonable precautions when handling reagents. Use protective equipment such as disposable gloves and lab coats when handling suspected carcinogens or toxic materials. Read Safety Data Sheets (SDS) prior to use.
   a. Thimerosal is used as a preservative in this solution and the substance is classified as toxic substance. Inhalation causes respiratory and CNS effects and severe delayed neurotoxicity.
   b. WARNING! Prop 65: DAB product contains a chemical(s) known to the state of California to cause genetic effects and/or cancer. If exposed or concerned, seek medical attention. See DAB SDS for more information.
3. Avoid contact of reagents with eyes and mucous membranes. If reagents come in contact with sensitive areas, wash with copious amounts of water.
4. Patient specimens and all materials that come into contact with patient specimens should be handled as bio-hazardous materials and disposed of appropriately.
5. Consult local or state authorities with regard to recommended method of disposal of bio-hazardous and hazardous chemical waste materials.
6. Incubation time and temperature other than those specified may give erroneous results. The user must validate any such changes.
7. Use lab grade quality chemicals such as acetone, ethanol and water when preparing fixatives and buffers. Users should validate performance including stability for laboratory prepared reagents (at 1X).
8. Avoid microbial contamination of reagents.

Staining Procedures:

General Operating Notes:

1. Equilibrate all reagents to room temperature prior to use. **Gently** swirl or shake the pHRP-labeled antibody solution before use. **Do not vortex.** Calculate the amount of DAB working solution needed (100 µl per tissue) and **freshly** prepare DAB working solution by adding the ratio of 30 µl of ihc DAB to 1.0 ml of ihc DAB Diluent into an Eppendorf Tube.
2. Drying can result in damage the tissue structure. It is best to prevent slides from dry out at any time in process. Store in PBS-T rather than leave exposed.
3. Gently wash tissues during manual wash steps. Avoid direct high velocity streams of that might tend to damage or cut delicate tissues.
4. Following each manual assay step, remove excess fluids on tissue slides with tissue paper. Excessive residual solution may dilute subsequent reagents, causing negative or uneven staining.
5. For the tissues with high level of oxidase activity, e.g. gastrointestinal or renal tissues, an additional quench with H_2O_2 is recommended.
6. The following protocol has been validated at temperatures between 21°-30°C (70°- 86°F) for incubating ihc Blocker, Mart-1 pHRP and DAB working solution. If room temperature is less than 21°C, incubate labeled antibody for a longer period of time (≥4 minutes depending upon temperature). Consistent results have been obtained using a slide warmer set to 30°C.

Cryostat Tissues:

1. Place cryostat tissue sections into fixation solution immediately after sectioning. **Prolonged exposure to the air at room temperature may damage targeted antigens.**
2. The DAB working solution incubation step is a range from 1-3 minutes. Users should determine the optimal incubation time for their lab environment and observe the brown color formation via visual inspection during incubation.

Test Timing Est. (10-minute IHC protocol for frozen tissue sections):

Procedure **Frozen**	**Time** **in minutes**
Fixation, use ihc Fixative or Acetone	0:30
- Wash with ihc Wash	0:15
Block with ihc Blocker	1
- Remove excess blocker	- - -
Mart-1 pHRP	3
ihc Wash	0:15
DAB working solution	1-3
ihc Wash	0:15
Hematoxylin counterstaining	0:20
ihc Wash	0:15
Mounting media and coverslip	0:45
Total	**10**

Paraffin Tissues:

1. Deparaffinization: Soak slides in Xylene 3 times for 5 minutes each. Next, 3 minutes each in 100%, 95% and 75% ethanol. Then wash slides with tap water in slide tank for two times, 2 minutes each time.
2. Antigen retrieval: Using a water bath, incubate slides in antigen retrieval buffer in a slide tank at 96°C for 30 minutes, then cool the slides down to room temperature for 30 minutes. Rinse the slides twice with tap water, 2 minutes each time.
3. (Optional) Quench with H_2O_2: Soak the slides in 3% H_2O_2 in slide tank, stand for 10 minutes. Rinse the slides with tap water twice and then wash once with PBS-T in slide tank for 2 minutes.
4. Dispense 100 µl of ihc Blocker covering the entire tissue and incubate at room temperature for 15 minutes. Remove ihc Blocker as much as possible but do not rinse the slides with PBS-T or water.
5. Dispense 100µl of pHRP labeled anti-human Mart-1 antibody on slides covering the entire tissue and incubate for 15-30 minutes at room temperature. Rinse the slides three times with PBS-T in slide tank, 2 minutes each time. Note: Places slides in a wet chamber to prevent evaporation if longer incubation times are used.
6. Dispense 100µl of DAB working solution covering the entire tissue, incubate for 3-10 minutes at room temperature. Rinse the slides twice with tap water in slide tank, 2 minutes each time.
7. Counterstaining: Add hematoxylin and incubate for 1 minute at room temperature. Rinse twice with tap water for 2 minutes, each time.

8. Dehydration: Soak slides in the following order: 75% ethanol for 3 minutes, 95% ethanol for 3 minutes, 100% ethanol for 3 minutes and Xylene twice at 5 minutes each time.
9. Applying Coverslip: Add one drop of permanent mounting medium on both the slide and the coverslip, then apply coverslip.

Quality Control Procedures:

Positive Tissue Control: The recommended positive control tissues for this antibody are properly processed melanoma and skin. The staining is cytoplasmic for melanoma cells and melanocytes in skin. One positive tissue control for each set of test conditions should be included in each staining run. Previous tissue specimen and in some cases, an individual's own tissue may be used as a control.

The tissues used for the positive control should be selected from patient specimens with well-characterized low levels of the positive target activity that gives weak positive staining. Known positive tissue controls should only be utilized for monitoring the correct performance of processed tissues and test reagents, rather than as an aid in formulating a specific diagnosis of patient samples. If the positive tissue controls fail to demonstrate positive staining, results with the patient specimens should be considered invalid.

Negative Tissue Control: The same tissue used for the positive control may be used as the negative tissue control. The variety of cell types in most tissue sections offers internal negative control sites. But this should be verified by the user. The components that do not stain should demonstrate the absence of specific staining, and provide an indication of non-specific background staining. If specific staining occurs in the negative tissue control sites, results with the patient specimens must be considered invalid. Breast carcinoma and lung carcinoma tissues may be used as negative tissue control. If specific staining (false positive staining) occurs in the negative tissue control, results with the patient specimens should be considered invalid.

Troubleshooting:

If an unexpected staining pattern occurs on control tissues or patient sample, contact Novodiax Technical Service at 888-439-2716.

1. No staining: If no staining on positive control slide, please verify whether (1) chromogen was prepared freshly and correctly, (2) reagents were used in right order, (3) pHRP-labeled antibody was indeed added, and (4) for FFPE tissue, dewaxing and antigen retrieval were performed inadequately. Perform any corrective actions required and then repeat the procedure.
2. Weak staining: Please check whether (1) the reagents have expired, (2) room temperature was below 21°C if a 30°C slide warmer was not used, (3) chromogen was prepared freshly, (4) too much washing solution remained on slide and diluted next reagent, and (5) for FFPE tissue, dewaxing and antigen retrieval were performed inadequately. Perform any required corrective actions and repeat the procedure.
3. High background: Possible causes include (1) inadequate washes, (2) blocker not applied, (3) specimens dried out, (4) prolonged incubation with chromogen, (5) prolonged incubation with pHRP-labeled antibody and (6) specimens contain high level of endogenous peroxidase and need an additional blocking step (refer to the Quench with H_2O_2 step in "Staining Procedures Paraffin Tissues"). Perform any required corrective actions and repeat the procedure.
4. The ihc Chromogen volumes provided in the kit are matched to the size of the kit for a typical user. Occasionally, materials can stick to either the lid or the side of the vial. To gain access to all of the material, it may be necessary to centrifuge at a slow speed or tap down the bottle using caution prior to use.

Expected Results:

The antibody stains cytoplasm on positive cells, such as melanoma cells and melanocyte in skin tissue. Other types of cells in the same tissue are negative. Interpretation of the staining result is solely the responsibility of the user.

General Limitations:

Immunohistochemistry is a multistep diagnostic process that requires specialized training in the selection of the appropriate reagents; tissue selection, fixation, and processing; preparation of the IHC slide; and interpretation of the staining results. Improper fixation, freezing, thawing, washing, drying, heating, sectioning or contamination with other tissues or fluids may produce artifacts, antibody trapping, or false negative results. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue (Najd M, Morales AR. 1983).

The manufacturer provides these antibodies/reagents at optimal dilution for use following the provided instructions for IHC on prepared tissue sections. Any deviation from recommended test procedures may invalidate declared expected results; appropriate controls must be employed and documented. Users who deviate from recommended test procedures must accept responsibility for interpretation of patient results under these circumstances.

Performance Characteristics:

The ihcDirect Mart-1 pHRP test kit performance has been determined using both frozen and FFPE tissues. Novodiax has conducted studies to evaluate the performance of the antibody, accompanying kit reagents and ancillary supplies. The antibodies and systems have been found to be sensitive and show specific binding to the antigen of interest with minimal to no binding of non-specific tissues or cells. Novodiax antibodies and accompanying kit reagents have shown reproducible and consistent results when used within a single run, between runs and between lots. These products have been determined to be stable for the periods of time specified on the labels either by standard real-time and/or accelerated methods. Novodiax ensures product quality by testing each lot of material and by testing materials at regular intervals and via surveillance programs.

ihcDirect Mart-1 Key to Symbols

IVD	*In vitro* diagnostic medical device	Mart-1 pHRP	pHRP Mart-1 antibody conjugate
REF	Catalog Number	ihc Blocker	Blocking reagent
	Use by YYYY-MM-DD	ihc DAB	DAB Chromogen reagent
	Consult Instructions for Use	ihc DAB Diluent	DAB Diluent reagent
LOT	Batch Code	ihc Wash	Wash Buffer
Σ	Contains sufficient for < n > tests		Health Hazard
2°C 8°C	Temperature Limitation		Manufacturer

Bibliography:

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