Instructions for Use

CINtec® Histology Kit

For in vitro diagnostic use.

The CINtec® Histology Kit is an immunohistochemistry assay for the qualitative detection of the $p16^{INK4a}$ antigen on formalin-fixed, paraffin-embedded tissue sections prepared from cervical biopsies.

It is intended for use in medical pathology laboratories to provide adjunctive information after initial diagnosis has been made by established diagnostic methods. Interpretation of the test results may only be made by a certified professional in conjunction with the patient´s clinical history and any additional diagnostic tests that have been performed.

Manufactured by:
Roche mtm laboratories AG
Sandhofer Straße 116
68305 Mannheim
Germany
www.roche.com
https://dialog.roche.com

Distributed by:
Ventana Medical Systems, Inc.
1910 E. Innovation Park Drive
Tucson, Arizona 85755
USA
www.roche.com

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II. Product Name

CINtec® Histology Kit

III. Intended Use

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The CINtec® Histology Kit is an immunohistochemistry assay for the qualitative detection of the $p16^{INK4a}$ antigen on formalin-fixed, paraffin-embedded tissue sections prepared from cervical biopsies.

It is intended for use in medical pathology laboratories to provide adjunctive information after initial diagnosis has been made by established diagnostic methods. Interpretation of the test results may only be made by a certified professional in conjunction with the patient´s clinical history and any additional diagnostic tests that have been performed.

IV. Summary and Explanation of the Device

Background

In eukaryotic cells, control of progression of the cell division cycle is effected by a complex pattern of controlled expression and post-translational modifications (*e.g.*, phosphorylation) of cell-cycle regulating proteins. The $p16^{INK4a}$ protein plays a major role in this mechanism of regulation of the eukaryotic cell cycle. It is part of the retinoblastoma protein (pRB)-mediated control of the G_1-S-phase transition, and it triggers cell cycle arrest in the course of cellular differentiation processes. In terminally differentiated epithelial cells, $p16^{INK4a}$ expression is down-regulated to levels typically not detectable by immunohistochemistry.

In various tumor entities, the tumor suppressor gene $p16^{INK4a}$ has been found to be functionally inactivated by gene mutation or promoter hypermethylation. In replication competent cervical epithelial cells however, where high risk type human papillomavirus (HR-HPV) oncoproteins have initiated the cellular transformation process, $p16^{INK4a}$ has been shown to be highly up-regulated and thus strongly over-expressed.

Principle of Procedure

The CINtec® Histology Kit contains a set of reagents for the immunohistochemical detection of the $p16^{INK4a}$ antigen. The Kit is designed to perform a two-step immunohistochemical staining procedure for formalin-fixed, paraffin-embedded tissue specimens obtained from cervical biopsies. For the detection of the antigen, a primary monoclonal mouse antibody clone E6H4™ directed to human $p16^{INK4a}$ protein is used.

A ready-to-use visualization reagent comprising a polymer reagent conjugated with horseradish peroxidase and goat anti-mouse Fab' antibody fragments is used. The Visualization Reagent has been subjected to solid-phase absorption to eliminate cross reactivity with human immunoglobulins. The chromogen reaction is based on horseradish peroxidase-mediated conversion of a DAB chromogen to a visible reaction product at the antigen site. After counterstaining, the specimen may be cover-slipped and the results may be evaluated by light microscopy inspection.

V. Reagents

Materials Provided

The materials listed below are included in each kit and are sufficient to perform 50 tests and 50 negative control reactions. The number of tests is based on the use of 200 µL of the reagents per slide.

1 Peroxidase Blocking Reagent

Peroxidase Blocking Reagent
2x 11.5 mL, ready-to-use
3% hydrogen peroxide, containing 15 mmol/L sodium azide (NaN_3).
EUH210: Safety data sheet available on request.

2 Mouse anti-Human $p16^{INK4a}$ Antibody

Mouse anti-Human $p16^{INK4a}$ Antibody
11.5 mL, ready-to-use
Monoclonal mouse anti-Human $p16^{INK4a}$ antibody, Clone E6H4™, supplied in 50 mmol/L Tris buffer pH 7.2, containing 15 mmol/L sodium azide (NaN_3) and stabilizing protein.

3 Visualization Reagent

Visualization Reagent
2x 11.5 mL, ready-to-use

Warning

H317 May cause an allergic skin reaction.

P261 Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
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.

Contains:
26172-54-3 2-methyl-2H-isothiazol-3-one hydrochloride
55965-84-9 reaction mass of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC no. 247-500-7] and 2-methyl-2H -isothiazol-3-one [EC no. 220-239-6] (3:1)

Polymer reagent conjugated with horseradish peroxidase and affinity purified goat anti-Mouse Fab' antibody fragments, supplied in stabilizing solution comprising preservatives and stabilizing protein.

Contains 5-bromo-5-nitro-1,3-dioxane that may produce an allergic reaction.

4 Negative Reagent Control

Negative Reagent Control
11.5 mL, ready-to-use

Monoclonal mouse anti-Rat oxytocin-related neurophysin antibody, supplied in 50 mmol/L Tris buffer pH 7.2, containing 15 mmol/L sodium azide (NaN_3) and stabilizing protein. For verification of the specificity of the staining. Rat oxytocin-related neurophysin is not present in human tissues.

5 DAB Buffered Substrate

DAB Buffered Substrate
31 mL

Substrate buffer solution, pH 7.5, containing < 0.1 % hydrogen peroxide, stabilizers and enhancers.

6 DAB Chromogen

DAB Chromogen
0.85 mL, 3,3'-diaminobenzidine chromogen solution.

Danger

H314 Causes severe skin burns and eye damage.
H341 Suspected of causing genetic defects.
H350 May cause cancer.

P201 Obtain special instructions before use.
P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.
P303 + P361 + P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water.
P304 + P340 + P310 IF INHALED: Remove person to fresh air and keep comfortable for breathing. Immediately call a POISON CENTER/doctor.
P305 + P351 + P338 + P310 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER/doctor.
P308 + P313 IF exposed or concerned: Get medical advice/ attention.

Contains 868272-85-9 3,3′-Diaminobenzidine tetrahydrochloride hydrate

NOTE: Consult Federal, State, or local regulations for disposal.
Material Safety Data Sheet is available upon request.

7 Epitope Retrieval Solution 10 x

Epitope Retrieval Solution 10 x
500 mL, 100 mmol/L Tris buffer pH 9 containing 10 mmol/L EDTA and 15 mmol/L sodium azide (NaN_3).

Storage

Store at 2 – 8 °C. Do not use after the expiration date. No data has been generated respective to the storage of the reagents under any conditions other than those stated above.

After opening, kit components are stable for 6 months if stored at 2 – 8 °C. Solutions must be discarded if cloudy in appearance.

Diluted Wash Buffer and diluted Epitope Retrieval Solution are stable for up to one month if stored at 2 – 8 °C. Solutions must not be used if cloudy in appearance.

Materials and Reagents Required but not Provided

CINtec Wash Buffer 10X to be used with the CINtec® Histology Kit is available under catalog number 06595421001 (8557) from Roche but is not included in the kit. For order details please refer to the website www.roche.com.

500 mmol/L Tris buffer solution with 1.5 mol/L NaCl, pH 7.6, containing detergent and an antimicrobial agent.

Warning

H317 May cause an allergic skin reaction.

P261 Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
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.

Contains 55965-84-9 Mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-2H -isothiazol-3-one (3:1)

Absorbent wipes;

Hematoxylin counterstain;

Distilled or deionized water (Washing Water);

Ethanol, 95% and 70%;

Mounting medium;

Positive and Negative Tissues to use as process controls;

Slides (SuperFrost® Plus or equivalent);

Xylene;

Coverslips;

Equipment Required

Drying oven, capable of maintaining 60 °C or less;

Humid chamber (optional);

Light microscope (4 - 40x objective magnification);

Staining jars or baths;

Wash bottles;

Timer (capable of 2 - 60 minute intervals);

Water bath with lid (capable of maintaining Epitope Retrieval Solution at 95 – 99 °C);

VI. Warnings and Precautions

⚠ Warning

1. Caution! Some of the reagents comprised in this kit contain hazardous chemicals. When handling the components of this kit, adhere to safety precautions for handling hazardous laboratory reagents.
2. Components 1, 2, 4, and 7 of this product contain sodium azide (NaN_3), which is highly toxic in its pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
3. Components 2, 3 and 4 contain material of animal origin. Adhere to proper handling procedures as is applicable to any product derived from biological sources.
4. Material Safety Data Sheet for the kit is available upon request.
5. When handling and disposing of histology specimens, including all specimens before and after fixation, as well as all materials exposed to them, adhere to the safety precautions for handling potentially infectious material as well as applicable waste disposal requirements.
6. Never pipette reagents by mouth. Avoid contacting the skin and mucous membranes with reagents and specimens. In the event that reagents or specimens come in contact with skin or mucous membranes, wash with copious amounts of water.
7. The Visualization Reagent and DAB Chromogen may be affected adversely if exposed to excessive light levels. Do not store kit components or perform staining in strong light, such as direct sunlight.
8. Wear appropriate personal protective equipment to avoid contact with eyes and skin when handling any of the components included or to be used in conjunction with the CINtec® Histology Kit. Refer to the Safety Data Sheet (SDS) for additional information.
9. Product safety labeling primarily follows EU GHS guidance.

⚠ Caution

1. For in vitro diagnostic use.
2. For professional use only.
3. Minimize microbial contamination of reagents to avoid non-specific staining.
4. Incubation times, temperatures, or methods other than those specified may give erroneous results.

5. Do not use the kit if the packaging of any of its components is damaged. Should packaging be compromised or components damaged, please notify the manufacturer without delay.
6. Disposal of all waste materials must be in accordance with local guidelines and regulations.
7. All reagents are formulated specifically for use with this test. In order for the test to perform as specified, no substitutions should be made.
8. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the CINtec® Histology Kit is suspected, immediately refer to the contact information provided in section XIII. for further information on technical support.
9. Malfunction of the product due to handling problems or to instability does not result in obvious signs. Therefore, as a quality control measure, positive and negative controls should be run simultaneously with patient specimens.

VII. Procedure

Specimen Preparation

The CINtec® Histology Kit is designed to be used with tissue specimens preserved for immunohistochemistry procedures. Specimens should be prepared in accordance to standard methods of tissue processing.

Positively charged slides such as SuperFrost® Plus slides are recommended for optimal performance.

Paraffin-embedded Tissue Specimens

Neutral buffered formalin fixed, paraffin-embedded tissue specimens are appropriate for use with this kit. If specimens are prepared using another preservation method, the user must verify the appropriateness of the method.

Specimens from the biopsy should be fixed for 18 – 24 hours in neutral-buffered formalin and blocked into a thickness of 3 or 4 mm. The tissue blocks are then dehydrated in a series of alcohols and xylene, followed by infiltration by melted paraffin held at no more than 60 °C. Each tissue block will be sectioned at 4 – 5 µm and mounted on SuperFrost® Plus microscopy slides by a histopathology laboratory.

Heat-Induced Epitope Retrieval

For heat-induced epitope retrieval, the tissue sections mounted on slides must be heated by immersing into the Epitope Retrieval Solution in a calibrated water bath capable of maintaining the Epitope Retrieval Solution at a temperature of 95 - 99 °C. Laboratories located at higher elevations should determine the best method of maintaining the required water bath temperature. Manufacturer does not recommend any deviation from the procedure described herein.

After heat-induced epitope retrieval, tissue sections must be cooled at room temperature for 20 minutes before further processing. Thereafter staining of the tissue sections must be performed without delay.

Staining Procedure

1. Reagent Preparation

All reagents should be brought to ambient temperature (20 – 25 °C) before use in immunostaining. Accordingly all subsequent steps are to be performed at ambient temperature.

Care should be taken to avoid drying of the specimens during the immunostaining procedure as drying may lead to staining artefacts.

The following reagents should be prepared before starting with the staining procedure:

1.1 Epitope Retrieval Solution

Prepare the amount of Epitope Retrieval Solution sufficient for the staining procedure that is planned by dilution of a quantity of Vial 7 (Epitope Retrieval Solution 10 x) 1:10 using distilled or deionized water.

After dilution, the Epitope Retrieval Solution may be stored at 2 – 8 °C for up to one month. The diluted solution must be discarded if it is cloudy in appearance.

NOTE: Use of water with elevated levels of ions for dilution of the epitope retrieval solution may significantly reduce the staining performance of the test. Please make sure that the water used is properly deionized (i.e. ensure that your ion exchange column for producing deionized water has been checked by routine maintenance). Do not use Tap water!

1.2 Wash Buffer

Use Wash Buffer (10 x), Catalogue number 06595421001 (8557), provided by Roche mtm laboratories AG in combination with the CINtec® Histology Kit. For order details please refer to the website www.roche.com.

Prepare an amount of Wash Buffer sufficient for the wash steps of the staining procedure that is planned by diluting of a quantity of the Wash Buffer (10 x), Catalogue number 06595421001 (8557), 1:10 using distilled or deionized water.

After dilution the Wash Buffer may be stored at 2 – 8 °C for up to one month. The diluted solution must be discarded if it is cloudy in appearance.

1.3 Substrate-Chromogen Solution (DAB)

For preparation of Substrate-Chromogen Solution, one drop of DAB Chromogen must be added to 2 mL of DAB Buffered Substrate. Proceed as follows:

i) transfer 2 mL of DAB Buffered Substrate from Vial 5 to a test tube;

ii) add one drop (25-30 µL) of DAB Chromogen from Vial 6. Mix and apply to tissue sections with a pipette;

2 mL of the Substrate-Chromogen Solution (DAB) prepared according to the instruction above is usually sufficient for staining five tissue sections including the corresponding five control specimens.

NOTE: Use the prepared Substrate-Chromogen Solution (DAB) within the same day.

NOTE: Addition of excess DAB Chromogen to the DAB Buffered Substrate will result in deterioration of the positive signal.

1.4 Counterstain

The DAB staining reaction results in a water insoluble coloured end-product. Alcohol or water-based hematoxylin may be used for counterstaining. If used, adhere to the instructions provided by the supplier of the hematoxylin for performing the counterstaining.

1.5 Mounting Medium

For mounting slide specimens after staining, a non-aqueous, permanent mounting medium is recommended. However, aqueous mounting is also acceptable.

Eukitt Mounting Medium is recommended for non-aqueous mounting. Aquatex Merck is recommended for aqueous mounting.

2. Staining Procedure for Autostainer Instruments

The CINtec® Histology Kit has been adapted for use on the Autostainer Instruments (*e.g.*, the Lab Vision Autostainer 480 or Dako Autostainer Plus) according to the template outlined below. Prior to staining on the Autostainer Instrument, the specimens and reagents should be prepared as stated in sections 1.1-1.5 and 2.1

2.1 Deparaffinization and Rehydration

Prior to deparaffinization, place slides in a drying oven at a temperature of no more than 60 °C for at least 20 minutes but not more than one hour to quantitatively remove water thereby improving adherence of the tissue to the glass slide (“baking”) and to melt the paraffin. Tissue slides must be deparaffinized to remove embedding medium and must then be rehydrated before the staining procedure can be performed. It is crucial to avoid incomplete removal of paraffin as residual embedding medium will result in increased non-specific staining. Incubate the slides at ambient temperature (20 – 25 °C) according to the following steps.

- 5 (±1) minutes in a xylene bath;
- repeat this step once with a fresh bath;
- remove excess liquid;
- 3 (±1) minutes in 95% ethanol;

- repeat this step once with a fresh bath;
- remove excess liquid;
- 3 (±1) minutes in 70% ethanol;
- repeat this step once with a fresh bath;
- remove excess liquid;
- minimum of 30 seconds in distilled or deionized water;

Start staining procedure as described in Section 2.2, Step 1: Epitope retrieval.

Xylene and alcohol solutions should not be used for more than 40 slides.

NOTE: Users should note that variations in equipment temperature or exposure times during preanalytical sample preparation may lead to incomplete removal of paraffin from tissue slides. Residual paraffin can lead to incomplete staining with any histology stain, including the CINtec® Histology stain. Histopathology laboratories should include regular monitoring of the equipment to reduce variation in sample preparation before staining. Observation of sharply defined borders within immuno-reactive tissue areas or other staining inconsistencies within a slide may, with any immuno-histochemical stain, be an indicator of non-optimal or incomplete pre-analytical processing of the specimen. Users should consider checking the equipment and the preanalytical sample preparation methods if inconsistent staining is observed.

2.2 Staining Protocol for Autostainer Instruments

Step 1: Epitope Retrieval

- fill staining jars, *e.g.*, plastic Coplin jars, with the diluted Epitope Retrieval Solution (see Procedure, Section 1.1);
- place staining jars containing Epitope Retrieval Solution in water bath and heat water bath and the Epitope Retrieval Solution to 95 - 99 °C. It is important to adjust the level of the water in the water bath to make sure that the jars are immersed in the water to a level of 80%. To stabilize the temperature and avoid evaporation, cover jars with lids;
- immerse deparaffinized sections into the preheated Epitope Retrieval Solution in the staining jars; this step usually will lower the temperature in the jars to less than 90 °C;
- bring the temperature of the water bath **and** the Epitope Retrieval Solution in the jars back to 95 - 99 °C; check the temperature of the Epitope Retrieval Solution in the jars;
- incubate for 10 (±1) minutes at 95 - 99 °C; start count down only after the temperature of the Epitope Retrieval Solution in the jars has been verified to have reached a temperature of 95 – 99 °C;
- remove the entire jar with slides from the water bath;
- allow the slides to cool in the Epitope Retrieval Solution for 20 (±1) minutes at room temperature;
- decant the Epitope Retrieval Solution and rinse sections in the Wash Buffer (see Procedure, Section 1.2);
- for optimal performance, soak sections in Wash Buffer for 5 minutes after epitope retrieval and prior to staining;

NOTE: The Epitope Retrieval Solution is designed for single use application only. Do not re-use.

Step 2: Programming of the Instrument

Prior to the first application of the CINtec® Histology Kit on an Autostainer Instrument, a new template needs to be set up. Please refer to the Operator's Manual for the dedicated Autostainer Instrument.

Step 3: Autostainer Procedure

- transfer the reagents from the kit bottles into graduated Autostainer Reagent Vials. Use the Autostainer-generated map for program times and reagent volumes (see point 4 for specific times and volumes);
- place the Autostainer reagent vials in the Autostainer Reagent Rack according to the computer-generated Reagent Layout Map;
- load the slides onto the Autostainer according to the computer-generated Slide Layout Map;
- to prevent drying the specimens should be sprinkled with wash buffer after loading onto the Autostainer;
- the following is an outline of the program run:
    - rinse*;
    - 200 µL Peroxidase-Blocking Reagent - 5 minutes;
    - rinse*;
    - 200 µL Primary $p16^{INK4a}$ antibody or Negative Reagent Control - 30 minutes;
    - rinse*;
    - 200 µL Visualization Reagent - 30 minutes;
    - rinse*;
    - rinse*;
    - rinse*;
    - switch;
    - 200 µL Substrate-Chromogen Solution (DAB) - 10 minutes;
    - rinse*;
    - rinse slides in deionized water after the substrate-chromogen step;

    *use Wash Buffer for the respective rinsing steps.

NOTE: If the Autostainer Instrument used rinses slides in buffer, the slides must be rinsed with deionized water after they have been removed from the Autostainer.

Step 4: Counterstain (Instructions are for Hematoxylin)

- immerse slides in a bath of hematoxylin. Incubate for 2 - 5 minutes, depending on the strength of hematoxylin used;
- place slides in a tap water bath and rinse gently with running tap water. Ensure all residual hematoxylin has been cleared;
- briefly rinse slides gently in a bath of distilled or deionized water;
- Counterstain may be performed directly on the Autostainer Instrument;

NOTE: Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a pale to dark blue colouration of the cell nuclei. Excessive or incomplete counterstaining may interfere with proper interpretation of results.

Step 5: Mounting

Non-aqueous, permanent mounting medium is recommended. Otherwise, aqueous mounting medium is also acceptable. Adhere to the instructions for use of the supplier for mounting medium.

NOTE: To minimize fading, protect slides from light and store at ambient temperature (20 – 25 °C).

3. Staining Procedure for Manual Use

NOTE: Prevent drying of tissue sections during the staining procedure. Dried tissue sections may result in increased non-specific staining. For prolonged incubations keep tissues in a humid environment.

Adhere to standard procedures used in manual immunohistochemistry staining when using the CINtec® Histology Kit for manual staining.

3.1 Deparaffinization and Rehydration

Prior to deparaffinization place slides in a drying oven at a temperature of no more than 60 °C for at least 20 minutes but not more than one hour to quantitatively remove water thereby improving adherence of the tissue to the glass slide ("baking") and to melt the paraffin.
Tissue slides must be deparaffinized to remove embedding medium and must then be rehydrated before the staining procedure can be performed. It is crucial to avoid incomplete removal of paraffin as residual embedding medium will result in increased non-specific staining. Incubate the slides at ambient temperature (20 – 25 °C) according to the following steps.

- 5 (±1) minutes in a xylene bath;
- Repeat this step once with a fresh bath;
- Remove excess liquid;
- 3 (±1) minutes in 95% ethanol;
- Repeat this step once with a fresh bath;
- Remove excess liquid;
- 3 (±1) minutes in 70% ethanol;
- Repeat this step once with a fresh bath;
- Remove excess liquid;
- Minimum of 30 seconds in distilled or deionized water;

Start staining procedure as described in Section 3.2, Step 1: Epitope retrieval.

Xylene and alcohol solutions should not be used for more than 40 slides. In place of xylene, toluene and xylene substitutes, such as Histoclear or RotiHistol® may be used.

3.2 Staining Protocol for Manual Use

Step 1: Epitope Retrieval

- fill staining jars, *e.g.*, plastic Coplin jars, with the diluted Epitope Retrieval Solution (see Procedure, Section 1.1);
- place staining jars containing Epitope Retrieval Solution in water bath and heat water bath and the Epitope Retrieval Solution to 95 - 99 °C. At that step it is important to adjust the level of the water in the water bath to make sure that the jars are immersed in the water to a level of 80%. To stabilize the temperature and avoid evaporation, cover jars with lids;
- immerse deparaffinized sections into the preheated Epitope Retrieval Solution in the staining jars; this step usually will lower the temperature in the jars to less than 90°C;

- bring the temperature of the water bath **and** the Epitope Retrieval Solution in the jars back to 95 - 99 °C; check the temperature of the Epitope Retrieval Solution in the jars;
- incubate for 10 (±1) minutes at 95 - 99 °C; start count down only after the temperature of the Epitope Retrieval Solution in the jars has been verified to have reached a temperature of 95 – 99 °C;
- remove the entire jar with slides from the water bath;
- allow the slides to cool in the Epitope Retrieval Solution for 20 (±1) minutes at room temperature;
- decant the Epitope Retrieval Solution and rinse sections in the diluted Wash Buffer (see Procedure, Section 1.2);
- for optimal performance, soak sections in Wash Buffer for 5 (±1) minutes after epitope retrieval and prior to staining;

NOTE: The Epitope Retrieval Solution is designed for single use application only. Do not re-use.

Step 2: Peroxidase-Blocking Reagent

- apply 200 µL Peroxidase-Blocking Reagent to cover specimen;
- incubate for 5 (±1) minutes;
- tap off excess liquid and place slides in a fresh Wash Buffer bath for 5 (±1) minutes;

Step 3: Primary Antibody or Negative Reagent Control

- remove excess buffer;
- cover specimen with 200 µL of primary antibody (Mouse Anti-Human $p16^{INK4a}$ or Negative Reagent Control);
- incubate for 30 (±1) minutes;
- tap off excess liquid and place slides in a fresh Wash Buffer bath for 5 (±1) minutes;

Step 4: Visualization Reagent

- remove excess buffer;
- cover specimen with 200 µL of Visualization Reagent;
- incubate for 30 (±1) minutes;
- tap off excess liquid and place slides in a fresh buffer bath for 5 (±1) minutes;
- repeat this step twice with a fresh Wash Buffer bath;

Step 5: Substrate-Chromogen Solution (DAB)

- cover specimen with 200 µL of Substrate-Chromogen Solution (DAB) that has been prepared according to the procedure described in 1.3 above;
- incubate for 10 (±1) minutes;
- tap off excess liquid and rinse gently with distilled or deionized water;

Collect Substrate-Chromogen Solution (DAB) waste in a hazardous materials container for proper disposal.

Step 6: Counterstain (Instructions are for Hematoxylin)

- immerse slides in a bath of hematoxylin. Incubate for 2-5 minutes, depending on the strength of hematoxylin used.
- place slides in a tap water bath and rinse gently with running tap water. Ensure all residual hematoxylin has been cleared.
- briefly rinse slides gently in a bath of distilled or deionized water.

NOTE: Depending on the incubation length and potency of the hematoxylin used, counterstaining will result in a pale to dark blue colouration of the cell nuclei. Excessive or incomplete counterstaining may interfere with proper interpretation of results.

Step 7: Mounting

Non-aqueous, permanent mounting medium is recommended. For xylene-based permanent mounting media a dehydradation procedure is necessary, e.g.

- distilled or deionized water
- 3 min 70% Ethanol
- 3 min 70% Ethanol
- 3 min 96% Ethanol
- 3 min 99% Ethanol
- 5 min Xylene
- 5 min Xylene

Otherwise, aqueous mounting medium is also acceptable. Adhere to instructions of use of the supplier for mounting medium.

NOTE: To minimize fading, protect slides from light and store at ambient temperature (20 – 25 °C).

VIII. Quality Control

Deviations from the recommended procedures for specimen fixation and processing in the user's laboratory may produce significant variability in results, necessitating regular performance of in-house controls.

Positive Tissue Control

External Positive control materials should be fresh autopsy/biopsy/surgical specimens fixed, processed and embedded as soon as possible in the same manner as the patient sample(s). Positive tissue controls are indicative of correctly prepared tissues and proper staining techniques. One positive external tissue control for each set of test conditions should be included in each staining run. The tissues used for the external positive control materials should be selected from patient specimens with known positive staining for $p16^{INK4a}$. If the positive controls fail to demonstrate appropriate positive staining, results with the test specimens should be considered invalid.

Negative Tissue Control

Cell types known to be negative for p16^{INK4a} present in most tissue sections can be used by the laboratorian as internal negative control sites to verify the IHC's performance specifications. If specific staining (false positive staining) occurs in the negative tissue control, results with the patient specimens should be considered invalid.

Non-specific Negative Reagent Control

Use the non-specific Negative Reagent Control in place of the primary antibody with a section of each patient specimen to evaluate non-specific staining and allow better interpretation of specific staining at the antigen site.

If specific staining (false positive staining) occurs with the non-specific Negative Reagent Control, results with the patient specimens should be considered invalid.

IX. Interpretation of Results

Control Specimens stained with the Negative Reagent Control as a primary reagent must not show specific staining.

Positive staining using the monoclonal mouse anti-Human p16^{INK4a} antibody, Clone E6H4™, should be assessed within the context of any non-specific background staining of the Negative Reagent Control. As with any immunohistochemical test, a negative result means that the antigen was not detected, not that the antigen was absent in the cells/tissue assayed.

Interpretation of the results must take into consideration the fact that p16^{INK4a} is a cellular protein that may be expressed at detectable levels in cervical high-grade dysplastic lesions and cervical cancers as well as in some conditions not associated with cervical dysplasia, albeit at differing levels and with different patterns of expression.

The stained slide specimens are evaluated according to a binary rating system composed of the ratings "positive" and "negative".

The rating "positive" is assigned if the p16^{INK4a}-stained slide specimen shows a continuous staining of cells of the basal and parabasal cell layers of the squamous cervical epithelium, with or without staining of cells of superficial cell layers ("diffuse staining pattern"). An example for a slide rated as "positive" ("diffuse staining pattern") is shown in Annex 2, Figure 1.

The rating "negative" is assigned if the p16^{INK4a}-stained slide specimen shows either a negative staining reaction in the squamous epithelium ("negative staining pattern") or a staining of isolated cells or small cell clusters; *i.e.*, a non-continuous staining, particularly not of the basal and parabasal cells ("focal staining pattern"). An example for a slide rated as "negative" ("focal staining pattern") is shown in Annex 2, Figure 2.

The interpretation of slides stained for p16^{INK4a} using the CINtec® Histology V-Kit should be performed in conjunction with H&E-stained slides prepared from the same cervical tissue specimen. The additional information provided by the CINtec®-stained slides should be combined with the preliminary morphology-based diagnosis established on the H&E-stained slides in order to create a final diagnosis.

X. Limitations

- For professional use only. Special training is required for the performance of immunohistochemistry procedures.
- The clinical interpretation of any positive or negative staining should be evaluated within the context of clinical presentation, morphology, and other histopathological criteria. The clinical interpretation of any positive or negative staining should be complemented by morphological studies using proper positive and negative internal and external controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist who is familiar with the proper use of IHC antibodies, reagents, and methods to interpret all of the steps used to prepare and interpret the final IHC preparation.
- The staining results in immunohistochemistry are strongly influenced by the quality of the tissue stained. Accordingly, the steps of fixation, washing, drying, heating, sectioning, or contamination with other tissues significantly contribute to the overall result of the staining and may lead to artefacts, 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.
- Excessive or incomplete counterstaining may interfere with proper interpretation of results.
- The manufacturer provides these antibodies/reagents at optimal dilution for use according to the instructions provided herein, for IHC testing on prepared tissue sections. Any deviation from the recommended test procedures may invalidate declared expected results; appropriate controls must be employed and documented. Users who deviate from the recommended test procedures must accept responsibility for interpretation of patient results under these circumstances.
- False-positive results may be seen due to non-immunological binding of proteins or substrate reaction products. They may also be caused by pseudoperoxidase activity (erythrocytes) and endogenous peroxidase activity (cytochrome C).
- Reagents may demonstrate unexpected reactions in previously untested tissues. The possibility of unexpected reactions even in tested tissue groups cannot be completely eliminated due to biological variability of antigen expression in neoplasms, or other pathological tissues. Contact Roche mtm laboratories AG with documented unexpected reaction(s). For information on technical support please refer to the contact information provided in section XIII.
- Do not replace kit reagents with reagents carrying other lot numbers or with reagents from other manufacturers.

XI. Troubleshooting

Refer to section XIII. for contact details in case technical assistance is required.

Problem	Probable Cause	Suggested Action
1. No staining of slides	**1a.** Deviation from Instructions for Use;	**1a.** Carefully read Instructions for Use and adhere to the procedures outlined therein;
2. Weak staining of slides	**2a.** Inadequate epitope retrieval;	**2a.** Use freshly prepared Epitope Retrieval Solution and / or make sure that Epitope Retrieval Solution reaches 95 – 99 °C for a full 10 minutes and is allowed to cool for an additional 20 minutes;
	2b. Inadequate reagent incubation times;	**2b.** Review 2.2. / 3.2. Staining protocol recommendations;
	2c. Inappropriate fixation method;	**2c.** Ensure that patient tissue is not over-fixed or that no alternative fixative was used;
	2d. Water that has been used to dilute the epitope-retrieval solution contains an ion concentration that is too high;	**2d.** Ensure that your ion exchange column for producing deionized water has been checked by routine maintenance;

	2e. Inappropriate deparaffinization;	**2e.** Users should note that variations in equipment temperature or exposure times during preanalytical sample preparation may lead to incomplete removal of paraffin from tissue slides. Residual paraffin can lead to incomplete staining with any histology stain, including the CINtec® Histology stain. Histopathology laboratories should include regular monitoring of the equipment to reduce variation in sample preparation before staining. Observation of sharply defined borders within immuno-reactive tissue areas or other staining inconsistencies within a slide may, with any immuno-histochemical stain, be an indicator of non-optimal or incomplete pre-analytical processing of the specimen. Users should consider checking the equipment and the preanalytical sample preparation methods if inconsistent staining is observed;
3. Excessive background staining of slides	**3a.** Incomplete removal of paraffin;	**3a.** Use fresh xylene baths and follow procedure as outlined in Section 2.1. / 3.1.;
	3b. Mounting of sections to slides performed using starch additives;	**3b.** Starch additives used in mounting sections may exhibit immunoreactivity and should therefore be avoided;
	3c. Insufficient rinsing of slides;	**3c.** Use fresh solution in buffer baths and wash bottles;
	3d. Drying of sections during staining procedure;	**3d.** Use humidity chamber. Wipe only three to four slides at a time before applying reagent;
	3e. Inappropriate fixation method;	**3e.** Use only fixative as recommended herein. Aberrantly fixed tissue may exhibit excessive background staining;
	3f. Non-specific binding of reagents to tissue;	**3f.** Check fixation method of the specimen and presence of necrosis;
4. Tissue detaches from	**4a.** Use of inappropriate slides;	**4a.** Adhere to recommendation herein and use SuperFrost®

slides		Plus slides;
5. Excessively strong specific staining	**5a.** Inappropriate fixation method;	**5a.** Ensure proper fixative and fixation method;
	5b. Prolonged reagent incubation times;	**5b.** Review and adhere to staining protocol given in sections 2.2 / 3.2 above;
	5c. Inappropriate wash solution;	**5c.** Use the Wash Buffer (10 x) (catalog number 8557).

XII. Symbols

Symbol:	Explanation:
REF	Catalog number
LOT	Batch code
GTIN	Global Trade Item Number
UDI	Unique Device Identifier
IVD	In vitro diagnostic medical device
	Manufacturer
	Contains sufficient for <n> tests
	Consult instructions for use
	Use by
	Temperature limitation
	Date of manufacturing
	Do not re-use

XIII. Manufacturer

Manufactured by:	Roche mtm laboratories AG Sandhofer Straße 116 68305 Mannheim Germany www.roche.com https://dialog.roche.com
Contact for technical support (Telephone):	+800 5505 6606

XIV. Revision Status

The current Instructions for use represent Version 1.9 released May 2021.

Changes to previous version (1.8, released July 2020):

- Symbol “do not re-use” added to title page
- Reference to website for support added (title page and Section XIII.)
- Composition DAB Buffered Substrate updated (Section V.)
- Additional symbol explained (Section XII.)

XV. Intellectual Property

CINtec and E6H4 are trademarks of Roche.

All other trademarks are the property of their respective owners.

Annex 1

References

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Annex 2

Example for diffuse staining pattern

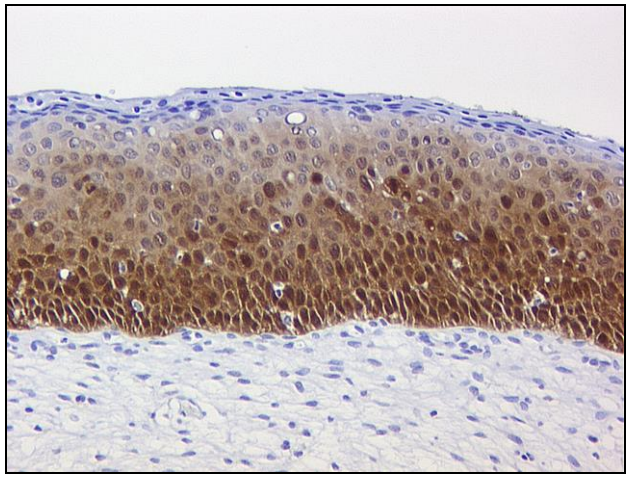

Fig. 1: CIN 3

Example for focal staining pattern

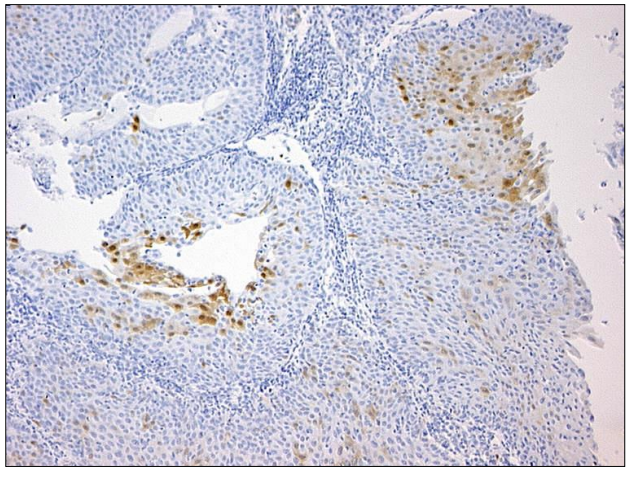

Fig. 2: Squamous metaplasia, mature