CINtec® PLUS CYTOLOGY

Instructions for Use

CINtec® *PLUS* Kit

The CINtec® *PLUS* Kit is an immunocytochemistry assay for the simultaneous qualitative detection of the $p16^{INK4a}$ and Ki-67 proteins in cervical cytology preparations. It is intended to be used as an aid in the identification of women with high-grade cervical intraepithelial lesions in a screening population, and in the sub-groups of patients with a Pap cytology result of ASC-US (atypical squamous cells of undetermined significance) or LSIL (low grade squamous intraepithelial lesion), or in patients with positive high-risk HPV test results.

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I. Product Name

CINtec® *PLUS* Kit

II. Intended Use

For in vitro diagnostic use.

The CINtec® *PLUS* Kit is an immunocytochemistry assay for the simultaneous qualitative detection of the $p16^{INK4a}$ and Ki-67 proteins in cervical cytology preparations.

It is intended to be used as an aid in the identification of women with high-grade cervical intraepithelial lesions in a screening population, and in the sub-groups of patients with a Pap cytology result of ASC-US (atypical squamous cells of undetermined significance) or LSIL (low grade squamous intraepithelial lesion), or in patients with positive high-risk HPV test results.

The kit is intended to be used in medical cytology laboratories. Interpretation of the test results may only be made by a certified professional in conjunction with the patient´s clinical history and additional diagnostic tests that have been performed.

The test is intended for manual use, or for use on Autostainer instruments.

III. 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. Thus, $p16^{INK4a}$ provides an anti-proliferative effect when expressed during regular cell cycle progression [1]. In terminally differentiated epithelial cells, $p16^{INK4a}$ expression is down-regulated to levels typically not detectable by immunocytochemistry [2].

In cervical dysplasia, overexpression of p16 is regarded as a surrogate biomarker for transforming HPV infections, reflecting the activation of HPV E6/E7 oncoprotein-driven cell proliferation [2-5]. Detection of p16 in cervical cytology preparations has been proposed as a valuable adjunctive marker to triage women with abnormal Pap cytology results as well as with positive HPV test results [3-5]. However, because p16-specific staining may be observed in individual metaplastic or endocervical cells in which p16 may be expressed to exert its physiological normal, growth-suppressive cellular function, interpretation of p16 single-stained cervical cytology preparations requires identification of p16 immunoreactive cells and further classification of these cells regarding signs of morphologic abnormalities [2-4].

The combined simultaneous detection of p16 and the proliferation marker Ki-67 within the same cell by ICC has been shown to be a valuable tool to identify dysplastic cervical cells in cytology preparations without the need for morphologic interpretation [3,6,7]. Ki-67 is a nuclear and nucleolar protein strictly associated with cell proliferation and is undetectable by standard immunostaining methods in rest-

ing (G0) cells [8]. Under normal physiologic conditions, expression of the proliferation-associated protein Ki-67 is mutually exclusive of the anti-proliferative protein p16. In contrast, cells where the retinoblastoma protein (pRB)-mediated pathway controlling the cell-cycle progression is abrogated upstream of the tumor suppressor function of p16 (such as in epithelial cells expressing the high-risk HPV E6/E7 oncoproteins) may proliferate and thus may express Ki-67 in the presence of functional p16 [2,3].
Therefore, the detection of individual cells in cervical cytology preparations that simultaneously co-express p16 and Ki-67 may serve as a morphology-independent indicator of cells with cell cycle dysregulation and thus may be used as an indicator of the presence of transforming HPV infections and underlying cervical intraepithelial neoplasia [2,3]. In the recent past, numerous studies have been performed and published evaluating the potential value and clinical usefulness of p16/Ki-67 dual-stained cytology for the identification of women that may benefit from referral to colposcopy based on various primary cervical cancer screening results, including for the triage of women with Atypical Squamous Cells of Undetermined Significance (ASC-US) or Low grade Squamous Intraepithelial Lesion (LSIL) Pap cytology results, women who are high-risk HPV positive in primary HPV screening, or women who are Negative for Intraepithelial Lesion or Malignancy (NILM)/HPV positive in clinical settings where Pap cytology/HPV co-testing is used for primary screening [6,7;9-25].

Clinical Significance

The interpretation of cervical cytology slide preparations immuno-stained for the simultaneous detection of the expression of the cell cycle regulatory protein $p16^{INK4a}$ and the proliferation marker Ki-67 has been shown to identify women with high-grade cervical intraepithelial neoplasia (HGCIN) with high sensitivity and specificity when used

- in a screening population as an adjunct to routine Pap cytology testing,
- in the subgroup of patients with Pap cytology results of ASC-US or LSIL, or
- in the subgroup of patients with negative Pap cytology, but positive HPV test results.

It is a major public health challenge to efficiently identify women in a routine cervical cancer screening population that have HGCIN. Due to the low prevalence of high-grade pre-cancerous lesions (typically less than 1% of women in an asymptomatic screening population) the availability of screening tests and procedures that identify women with established HGCIN with both high sensitivity and high specificity is of high importance [26].

Over the last decades, Pap cytology testing for morphological abnormalities was shown to be successful in reducing the cervical cancer associated morbidity and mortality in those countries that have established cervical cancer screening protocols. However, despite its overall success, there is an unsatisfactorily low sensitivity for a single Pap cytology test (typically in the range of 50-70% [27], which requires short screening intervals to compensate for the rather low sensitivity of a single cytology test result. At the same time, the specificity of Pap cytology testing was shown to be relatively high, although it may vary substantially based on the characteristics of the individual screening population, including age, HPV prevalence, differences in Pap methodologies (conventional smears vs. liquid-based cytology), technologies used (i.e. manual interpretation vs. computer-assisted cytology/imaging), and dependent on the experience of the individual reviewers.

HPV testing has been widely accepted as an alternative approach to Pap cytology-

based screening for cervical cancer and its precursor lesions [28-30]. HPV testing is currently being used either as co-testing together with Pap cytology in women aged 30 years and older, or as a primary screening test in women aged 25 years and older [28-30].

Besides defining the optimal approach to implement effective primary screening technologies and algorithms there are additional areas of interest in cervical cancer screening where existing technologies may be improved to optimize the accuracy of identifying HGCIN, or where there are currently no tools available at all. The triage of equivocal (ASC-US, atypical squamous cells of undetermined significance) or mildly abnormal (LSIL, low-grade squamous intraepithelial lesion) Pap cytology results belong to this category, as well as the management of women aged 30 years and older with screening results from Pap/HPV co-testing approaches that were categorized as Negative for Intraepithelial Lesions or Malignancy (NILM) on Pap, but positively tested for high-risk HPV.

For the triage of ASC-US cytological abnormalities a triage strategy that incorporates HPV detection within the ASC-US population was demonstrated to be sensitive for detecting underlying HGCIN. However, specificity of HPV-based ASC-US triage is relatively low and shown to be age-dependent [31,32]. Furthermore, as the vast majority of LSIL cases have been demonstrated to be positive for high-risk HPV types [33], triaging women with LSIL by HPV testing is not a very effective approach for managing women with these Pap cytology screening results.

As colposcopy services are limited and the cost of these services and the subsequent treatment of low-grade lesions are considerably high, there is a continuous interest in the availability of triage tests for women with ASC-US and LSIL with improved performance characteristics, especially with regard to the specificity level provided. A biomarker test with high sensitivity and specificity that is capable of identifying patients with established high-grade cervical disease is beneficial in the management of women with equivocal and low-grade cytological abnormalities to 1) better stratify patients with ASC-US Pap cytology results, and 2) to open the opportunity to efficiently triage LSIL Pap cytology results.

Similar to the situation for the triage of LSIL Pap cytology results there are currently only limited options available to further triage screening results that are negative on Pap cytology, but tested positive for high-risk HPV. With about 5-7% of the Pap/HPV co-testing results, this represents a growing group of cases that would require a complete colposcopy follow-up to take advantage of the higher detection rate potential of the HPV test over Pap cytology testing. However, as the rate of underlying high-grade disease within this group of screening test results is still relatively low, the availability of an efficient triage tool is highly desirable to effectively manage women with positive HPV test results that are not matched by abnormal Pap cytology results. Current triage options include HPV16/18 genotyping and repeat testing for 12 Other High-risk HPV-positive women, or Pap cytology triage of all HPV-positive women [30,34].

Principle of Procedure

The CINtec® *PLUS* Kit contains a set of reagents for the immunocytochemical detection of the $p16^{INK4a}$ and Ki-67 antigens. The kit is designed to perform a two-step immunocytochemical staining procedure for cytological specimens obtained from the cervix uteri. For the detection of the antigens, a primary monoclonal mouse antibody clone E6H4™directed to human $p16^{INK4a}$ protein and a primary monoclonal rabbit antibody clone 274-11 AC3V1 directed against human Ki-67 protein are used.

Ready-to-use visualization reagents comprising (i) a polymer reagent conjugated to horseradish peroxidase and goat anti-mouse Fab' antibody fragments, and (ii) a polymer reagent conjugated to alkaline phosphatase and goat anti-rabbit Fab' antibody fragments are used. The visualization reagents have been subjected to solid-phase absorption to eliminate cross reactivity with human immunoglobulins.

The chromogen reactions are based on horseradish peroxidase-mediated conversion of a DAB chromogen, and alkaline phosphatase-mediated conversion of Fast Red chromogen to visible reaction products at the respective antigen sites. After counterstaining, a two step mounting protocol must be applied: in a first step, an aqueous mounting of the specimens using an aqueous mounting medium provided with the kit must be used. Subsequently, the slides may be cover-slipped using e.g. a permanent mounting medium. The results can be evaluated by light microscopy inspection.

IV. Reagents

Materials Provided

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

1 Peroxidase Blocking Reagent

Peroxidase Blocking Reagent
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 Primary Antibodies Solution p16/Ki-67

Primary Antibodies Solution p16/Ki-67
11.5 mL, ready-to-use
Monoclonal mouse anti-Human $p16^{INK4a}$ antibody (< 5 µg/mL), Clone E6H4™, and monoclonal rabbit anti-Human Ki-67 antibody Clone 274-11 AC3V1 (< 1 µg/mL), supplied in 50 mM Tris buffer pH 7.2, containing 15 mmol/L sodium azide (NaN_3) and stabilizing protein.

3 Visualization Reagent HRP

Visualization Reagent HRP
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.

4 Visualization Reagent AP

Visualization Reagent AP
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 work-place.
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

Polymer reagent conjugated with alkaline phosphatase and affinity purified goat anti-rabbit Fab' antibody fragments, supplied in stabilizing solution comprising preservatives and stabilizing protein.

5 DAB Buffered Sub-strate

DAB Buffered Substrate
16.0 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 Naphthol Phosphate Substrate

Naphthol Phosphate Substrate
25.0 mL,
Substrate buffer solution, pH 9.2 containing Naphthol AS-TR Phosphate Substrate, stabilizers and enhancers.

8 Fast Red Chromogen

Fast Red Chromogen
1.33 mL, Fast Red chromogen solution.

Danger

H314 Causes severe skin burns and eye damage.

P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.
P301 + P330 + P331 IF SWALLOWED: Rinse mouth. Do NOT induce vomiting.
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.
P501 Dispose of contents/ container to an approved waste disposal plant.

9 Epitope Retrieval Solution 10X

Epitope Retrieval Solution 10X
500 mL, 100 mM Tris, 10 mM EDTA, pH 9.0, containing 15 mmol/L sodium azide (NaN_3).

10 CINtec® *PLUS* Mount

CINtec® *PLUS* Mount
18.0 mL, aqueous based permanent mounting medium for the permanent preservation of slide preparations stained with peroxidase and alkaline phosphatase based visualization systems. Contains 7.7 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.

The CINtec® *PLUS* Mount vial (Vial 10) should be removed from the kit box after the kit has been opened for the first time and may be further stored at ambient temperature (2 – 30°C) to reduce viscosity and to facilitate usage.

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® *PLUS* Kit is available under catalog number 10215364001 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 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)

Alcohol-free hematoxylin for counterstaining;

Distilled or deionized water;

PreservCyt® Solution; may be required to prepare ThinPrep® slides from residual sample volume remaining in the original ThinPrep® vial. Sample volume must be at least 17 ml to prepare an-other slide according to the ThinPrep® 2000 System Operator's Manual, Instrument Troubleshooting, pg. 6.27;

SurePath™ Preservative Fluid; is required for preparing SurePath slides intended for CINtec® *PLUS* Cytology stain-ing. See section VI. Procedures, Cytological Specimen Preparations for details;

Ethanol, 99%;

Xylene-based mounting medium for glass coverslipping;

Slides: SuperFrost® PLUS, ThinPrep® Microscopy Slides; BD SurePath™ PreCoat slides

Xylene;

Glass or film coverslips;

Process controls e.g. ThinPrep® slides produced from the residual specimen vial:

- Positive control: ThinPrep® slide from (pooled) specimen(s) with Pap cytology result confirmed as High-grade squamous intraepithelial lesion (HSIL);
- Negative control: ThinPrep® slide from (pooled) specimen(s) with Pap cytology result confirmed as Negative for intraepithelial lesion or malignancy (NILM).

Equipment Required

Light microscope (10 - 40x objective magnification);

Heat resistant staining jars (plastic);

Measuring cylinders;

Squirt bottle (to be filled with wash buffer);

Timer (capable of 30 seconds - 60 minutes intervals);

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

Thermometer;

Optional: Drying oven capable of maintaining a temperature of 37 or 60°C;

Optional: Dako or LabVision Autostainer instrument*;*

Optional: Shandon Coverplate™ system.

V. 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, 9 and 10 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. Safety Data Sheet for the kit is available upon request.
5. When handling and disposing of cytological 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 reagents, DAB Chromogen and Fast Red 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® *PLUS* Kit. Refer to the Safety Data Sheet 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® *PLUS* 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.

10. 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.

VI. Procedures

Cytological Specimen Preparations

Cytological specimens must be adequately handled to preserve the specimens for immunocytochemical procedures. All specimens should be subjected to standard methods of cell processing.

ThinPrep® (Hologic™ Inc.) slides prepared on a ThinPrep® 2000 Processor (Hologic™ Inc.) according to the manufacturer's protocol, BD SurePath™ (BD Diagnostics) slides prepared according to the manufacturer´s protocol, as well as manually prepared slides (conventional smears) are suitable for use.

Notes:

1. ThinPrep® sample preparation:

   Cytologic samples in PreservCyt Solution (PC) intended for immunocytochemistry staining using CINtec® *PLUS* can be stored at 15-30°C for 6 weeks followed by 12 additional weeks refrigerated at 2-8°C.

   Dried slides can be stored at room temperature protected from light and must be stained with the CINtec *PLUS*® Kit within 7 days of preparation.

   Please note that the use of the ThinPrep® 3000 Processor is not recommended, as the spray fixation procedure provided by the instrument may lead to substantial cell loss.

2. BD SurePath™ sample preparation:

   Cytologic samples in SurePath™ Preservative Fluid intended for immunocytochemistry staining using CINtec® *PLUS* can be stored for up to 4 weeks at 15-30°C, or for 6 months at 2-8°C.

   Dried slides can be stored at room temperature protected from light and must be stained with the CINtec *PLUS*® Kit within 7 days of preparation.

   As it has been occasionally reported that storing the residual cell material after the enrichment process in water may have a negative impact on the intensity of the immunocytochemical signal, we strongly recommend to follow the instructions outlined below when preparing slides for CINtec® *PLUS* testing to avoid any risk of signal loss:

   a. Slide preparation **directly** after processing the Pap slide
      i. A second slide for each case can be prepared immediately after the Pap staining of the corresponding SurePath™ slide is completed.
      ii. Place a second set of labeled slides into slide racks.

iii. On the PrepStain™ using the GYN version 1.1 or 1.2, Slide Preparation, select program „Transfer only".

b. Slide preparation **from the enriched cell pellet** after preparation of Pap slide at a later time

iv. Remove the tube racks from the PrepStain™ System and add approximately 2 mL of SurePath™ Preservative Fluid to each tube.

v. Cap each tube and store at room temperature for 4 weeks or refrigerated (2 – 8°C) for up to 6 months.

vi. If a slide for CINtec® *PLUS* is to be made on a stored sample, allow the sample to adapt to room temperature for 1 hour. Start with the second centrifugation step of the GYN Enrichment Process and carry through the remainder of the Prep procedure.

c. Slide preparation **from left-over sample material** remaining in the original sample vial (approximately 2 mL)

vii. Add 8mL of SurePath™ Preservative Fluid to the residual sample in the SurePath™ vial (approx. 2 mL).

viii. The diluted sample should be processed on the PrepMate™ using the standard technique and on the PrepStain™ using the GYN version 1.1 or 1.2, Slide Preparation, program „Transfer only".

Immediately after preparation, ThinPrep® or BD SurePath™ slides should be fixed in 99% ethanol for 10 minutes to 1 hour and air-dried for 20 minutes to 16 hours (over night). **ThinPrep® or BD SurePath™ cytology preparation must not be fixed with cytological spray fixation reagent containing polyethylene glycol (e.g. Merckofix®, Merck).**

Conventional smears should be fixed with cytological spray fixation reagent containing polyethylene glycol (e.g. Merckofix®, Merck) immediately after sample collection. Spray-fixed conventional smear slides can be stored at room temperature protected from light and must be stained with CINtec® *PLUS* within 7 days of preparation.

Before beginning the immunostaining procedure, all specimens must be rehydrated following specific protocols described in Section 2.1.

NOTE: **Ethanol with minimal impurities is needed for fixation of ThinPrep® liquid based cytology slides to prevent background staining. During the preparation of ThinPrep® slides that are intended to be used for immunostaining renew the ethanol bath after fixation of every 25 ThinPrep® slides.**

Heat-induced epitope retrieval treatment of specimens prior to staining

Heat-induced epitope retrieval (HIER) using the Epitope Retrieval Solution included in the kit is necessary for optimal assay performance. Deviation from the described procedure may affect results!

For heat-induced epitope retrieval, the cytological specimen 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 devia-

tion from the procedure described herein.

After heat-induced epitope retrieval, the cytological specimen must be kept at ambient temperature for 20 minutes or longer until the temperature has cooled down to 50°C or below before further processing. Thereafter, staining of the cytological specimen must be performed without delay.

Slide Staining Procedures

The kit contains reagent volume which is sufficient to perform 50 tests. The number of tests is based on the use of 200 µL of the reagents per slide.

200 µL per slide is recommended to process ThinPrep® or BD SurePath™ cytological preparations on Dako or LabVision Autostainer instruments (two drop zones per slide with 100 µL each) or by using the Shandon Coverplate™ system (Thermo Fisher Scientific Inc.).

200 µL per slide is also recommended to process conventional smears by using the Shandon Coverplate™ system. However, to ensure complete coverage of conventional smears on Dako or LabVision Autostainer instruments the use of 300 µL per slide (100 µL for all three available drop zones) is recommended.

1. Reagent Preparation

It is recommended to prepare the following reagents, except the Fast Red working solution, before starting with the staining procedure. All reagents should be equilibrated to room temperature (20 – 25°C) prior to immunostaining.

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 9 (Epitope Retrieval Solution 10X) 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 distilled or 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 CINtec® Wash Buffer 10X, Catalog number 10215364001, provided by Roche in combination with the CINtec® *PLUS* 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 10X 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 Solutions (DAB and Fast Red)

Ensure that both Chromogenes and Substrates are equilibrated to room tempera-

ture (20 – 25°C). Addition of excess Chromogen to the Substrate will result in deterioration of the positive signal.

A) Preparation of the DAB working solution prior to the staining run

The DAB working solution is stable for 8 hours after preparation.

Prepare the DAB working solution as follows:

i) Transfer 1 mL of the DAB Substrate Solution (Vial 5) into a clean reaction tube;

ii) Add one drop (25 – 30 µL) of the DAB Chromogen (Vial 6) and mix gently by inverting the tube (do not vortex);

iii) When using the Autostainer, transfer the DAB working solution into an Autostainer Reagent Vial before starting the staining run and load it on the appropriate Autostainer rack position.

B) Preparation of the Fast Red working solution directly before use

Prepare the Fast Red working solution directly before use, as otherwise decreased staining intensity and thus loss of sensitivity may result. Do not vortex the Fast Red working solution as this may result in formation of precipitates.

Prepare the Fast Red working solution as follows:

i) Transfer 1 mL of the Naphthol Phosphate Substrate Solution (Vial 7) into a clean reaction tube;

ii) Add one drop (40 – 45 µL) of the Fast Red Chromogen (Vial 8) and mix gently by inverting the tube (do not vortex);

iii) When using the Autostainer, prepare the Fast Red working solution when requested by the Autostainer software. Then transfer the working solution into an Autostainer Reagent Vial and load it on the appropriate Autostainer rack position. Avoid delay of the “substrate batch” step during the Autostainer run to minimize the risk of drying artefacts.

1.4 Counterstain

The DAB and Fast Red staining reactions result in water insoluble coloured end products (DAB: brown; Fast Red: red).

NOTE: Alcohol-free hematoxylin must be used for counterstaining, as the use of alcohol may negatively impact the intensity of the signal generated by the Fast Red, or remove it completely. 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 two-step mounting procedure is needed as described below.

First CINtec® *PLUS* Mount (Vial 10), an aqueous-based permanent mounting medium, is applied manually in a thin coat and allowed to dry (“liquid coverslipping”). In a second step a glass coverslip is applied onto the dried surface of the aqueous mounting media using xylene-based mounting medium.

Equilibrate CINtec® *PLUS* Mount at room temperature before use and store it at ambient temperature for further use.

2. Staining Procedure

The CINtec® *PLUS* Kit has been validated for use on Autostainer instruments (Lab-Vision Autostainer 480 or Dako Autostainer PLUS) according to the template outlined below (see Section 2.3.1), as well as for the Shandon Coverplate™ system (see Section 2.3.2). It may be possible to use other instruments or systems with comparable function after appropriate validation by the user.

Prior to staining, the specimens and reagents should be prepared as stated in Sections 1.1-1.3 and 2.1.

All reagents should be equilibrated to ambient temperature (20 – 25°C) prior to performing the immuno-staining procedure.

Likewise, all steps should be performed at ambient temperature. Do not allow specimens to dry during the staining procedure. Dried specimens may display increased non-specific staining. If prolonged incubations are used, place specimens in a humid environment.

2.1 Specimen rehydration

For all cytological specimens a rehydration step is necessary prior to staining. This step should be performed at ambient temperature (20 - 25°C).

A) ThinPrep® liquid based cytology slides & conventional smears

- Place slides in distilled or deionized water and incubate for 10 (±3) minutes;
- Commence staining procedure as outlined in Section 2.2, Step 1: Epitope retrieval.

B) BD SurePath™ cytology slides

Alcohol fixed BD SurePath™ cytology slides have to undergo a special rehydration procedure to make specially coated glass slides compatible with liquid coverslip medium.

- Make sure that the slides have been completely air-dried;
- Place slides in a fresh bath of 100% xylene (use xylene-resistant jars); dip the slides a couple times and incubate for 2 minutes;
- Transfer the slides into a fresh bath of 99% ethanol and incubate for 2 minutes;
- Transfer the slides into a fresh bath of 70% ethanol and incubate for 2 minutes;
- Transfer the slides in distilled or deionized water and incubate for 2 minutes

Note: Replace the baths used for the dehydration after processing of 50 slides or once a week if a smaller quantity of slides is processed.

2.2 Epitope Retrieval

A) ThinPrep® liquid based cytology slides & conventional smears:

- Fill heat resistant staining jars (plastic) 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. The temperature has to be measured inside the staining jars. It is important to adjust the level of the water in the water bath to make sure that the jars are im-

mersed in the water to a level of 80%. To stabilize the temperature and avoid evaporation, cover jars with lids;

- When the temperature of 95 – 99°C has been reached immerse the re-hydrated cytology slides 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. Keep the temperature probe in the jars and close the lid of the staining jars as good as possible.
- 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 counting 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;
- Remove the lid off the staining jars and allow the slides to cool in the Epitope Retrieval Solution for 20 (±1) minutes at ambient temperature until it has reached 50°C or below;
- Transfer the slides into a staining jar filled with Wash Buffer (see Procedure, Section 1.2) and incubate for 5 (±1) minutes prior to loading the slides onto the programmed Autostainer instrument. When using the Shandon Coverplate™ System, assemble the slides and coverplates according to the Protocol REF 2010-953-009EN (available from Roche mtm laboratories AG).

B) BD SurePath™ cytology slides

- Fill heat resistant staining jars (plastic) with the diluted Epitope Retrieval Solution (see Procedure, Section 1.1).Please note that when using jars that are not made of heat-resistant plastic, but e.g. metal or heat-resistant glass the time for the epitope retrieval must be modified on an individual basis;
- Immerse slides into the cold Epitope Retrieval Solution; close the lid of the staining jar;
- Place the staining jar in a cold water bath and bring the temperature of the water bath and the Epitope Retrieval Solution to 95 – 99°C and incubate for 15 minutes as soon as the temperature has been reached; please note that the time period for heating up the solution to 95 – 99°C should be no longer than 40 minutes at maximum;
- Remove the entire jar with slides from the water bath;
- Remove the lid off the staining jars and allow the slides to cool in the Epitope Retrieval Solution for 20 (±1) minutes at ambient temperature until it has reached 50°C or below;
- Transfer the slides into a staining jar filled with Wash Buffer (see Procedure, Section 1.2) and incubate for 5 (±1) minutes prior to loading the slides onto the programmed Autostainer instrument. When using the Shandon Coverplate™ System, assemble the slides and coverplates according to the Protocol REF 2010-953-009EN (available from Roche mtm laboratories AG).

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

2.3 Staining Protocols

2.3.1 Staining Protocol for Autostainer Instruments (Dako, LabVision)

Prior to the first application of the CINtec® *PLUS* Kit on an Autostainer Instrument, a new template needs to be set up and the CINtec® *PLUS* Kit reagents need to be added to the Autostainer "Reagent List". Please refer to the Operator's Manual for the dedicated Autostainer Instrument.

The use of 200 µL per slide is recommended to process ThinPrep® or BD SurePath™ cytological preparations. For conventional smears on Dako or LabVision Autostainer instruments the use of 300 µL of the reagents per slide may be required.

The following is an outline of the program run:

Program Step	**ThinPrep® cytological preparations & Conventional Smears**	**BD Surepath™ cytological preparations**
1	rinse*	rinse*
2	Peroxidase-Blocking Reagent 5 minutes	Peroxidase-Blocking Reagent 5 minutes
3	rinse*	rinse*
4	Primary Antibodies Solution p16/Ki-67 30 minutes	Primary Antibodies Solution p16/Ki-67 30 minutes
5	rinse*	rinse*
6	Visualization Reagent HRP 15 minutes	Visualization Reagent HRP 15 minutes
7	rinse*	rinse*
8	rinse*	rinse*
9	rinse*	rinse*
10	Visualization Reagent AP 15 minutes	Visualization Reagent AP 15 minutes
11	rinse*	rinse*
12	rinse*	rinse*
13	rinse*	rinse*
14	switch	switch
15	“Substrate” step: DAB 10 minutes	“Substrate” step: DAB 10 minutes
16	rinse with distilled or deionized water	rinse with distilled or deionized water
17	rinse*	rinse*
18	“Substrate-batch” step: Fast Red 15 minutes	“Substrate-batch” step: Fast Red 15 minutes
19	rinse*	rinse*
20	---	**For LabVision Autostainer**: “Substrate-batch” step: Fast Red 15 minutes **For Dako Autostainer:** “Substrate” step: Fast Red 15 minutes
21	----	rinse*
22	rinse with distilled or deionized water	rinse with distilled or deionized water
23	switch	switch

*use Wash Buffer for the respective rinsing steps.

NOTE: If the Autostainer Instrument that is used for the staining procedure rinses slides with buffer, the slides must be rinsed with distilled or deionized water after they have been removed from the Autostainer.

Subsequent to programming proceed as follows:

- Transfer the reagents from the kit bottles into Autostainer Reagent Vials. Use the Autostainer-generated map for program times and reagent volumes;
- Place the Autostainer reagent vials into the Autostainer Reagent Rack according to the “Reagent Layout Map”;
- Load the slides onto the Autostainer according to the “Slide Layout Map” and rinse the slides with Wash Buffer to prevent specimen from drying out.

2.3.2 Staining Protocol for Shandon Coverplate™ System

Assemble the slides one after another with coverplates according to the Protocol REF 2010-953-009EN (available from Roche mtm laboratories AG) and place them in an upright position into the slide rack.

Check correct assembly of Shandon Coverplate™ System by filling reagent reservoir with distilled or deionized water (2 mL) and incubate for 5 minutes to allow the water to run completely through the gap. The assembled slide-coverplate system is leak-proof when the water runs slowly through the gap between the slide and coverplate.

1. Equilibration: Fill reagent reservoir with Wash Buffer (2 mL) and incubate for 5 minutes to allow the wash buffer completely run through the gap; **repeat this step once**;

2. Apply 200 µL Peroxidase Blocking Reagent. Incubate for 5 minutes;

3. Fill reagent reservoir with wash buffer (2 mL) and incubate for 5 minutes to allow the Wash Buffer to run completely through the gap;

4. Apply 200 µL Primary Antibodies Solution p16/Ki-67 ($p16^{INK4a}$/Ki-67). Incubate for 30 minutes;

5. Fill reagent reservoir with Wash Buffer (2 mL) and incubate for 5 minutes to allow the Wash Buffer to run completely through the gap;

6. Apply 200 µL Visualization Reagent HRP. Incubate for 15 minutes;

7. Fill reagent reservoir with Wash Buffer (2 mL) and incubate for 5 minutes to allow the Wash Buffer to run completely through the gap;

   Repeat this step twice;

8. Apply 200 µL Visualization Reagent AP. Incubate for 15 minutes;

9. Fill reagent reservoir with Wash Buffer (2 mL) and incubate for 5 minutes to allow the wash buffer to run completely through the gap;

   Repeat this step twice;

10. Apply 200 µL **DAB** Substrate-Chromogen Working Solution that has been prepared according to the procedure described in Section 1.3 above. Incubate for 10 minutes;

11. Fill reagent reservoir with distilled or deionized water (2 mL) and incubate for 5 minutes to allow the water to run completely through the gap;

12. Fill reagent reservoir with Wash Buffer (2 mL) and incubate for 5 minutes to allow the wash buffer to run completely through the gap;

13. Apply 200 µL **Fast Red** Substrate-Chromogen Solution that has been prepared according to the procedure described in Section 1.3 above. Incubate for 15 minutes;

    Note: For BD SurePath™ slide preparations this step has to be repeated once.

14. Fill reagent reservoir with distilled or deionized water (2 mL) and incubate for 5 minutes to allow the water to run completely through the gap;

    Repeat this step once.

To retrieve slides gently pull the whole slide/coverplate assembly out of the slide rack by using the thumb and second finger at the rear and then lift the slide gently off the coverplate. This process ideally is performed submerse in water.

Place the slides in distilled or deionized water and proceed further with counterstaining.

2.4 Counterstaining with Hematoxylin

- Immerse slides in a bath of **alcohol-free hematoxylin**. Incubate for 2 - 10 minutes, depending on the potency of the alcohol-free hematoxylin used;

NOTE: The use of alcohol-free hematoxylin is absolutely mandatory.

- For bluing place slides in a tap water bath or alkaline solution such as a weak ammonia solution (NH_4OH, 0.08% in deionized water) and incubate for 30 seconds - 2 minutes;
- To ensure that all residual hematoxylin has been cleared, subsequently replace the tap water in the jar several times (3 – 5x) with fresh tap water until no stain residue can be observed any more;
- Briefly incubate slides 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 coloration of the nuclei of the cells. Excessive or incomplete counterstaining may interfere with proper interpretation of results.

2.5 Mounting

To maintain optimal sensitivity and to prevent fading of Fast Red Chromogen a two-step mounting procedure is needed.

After counterstaining & bluing the slides will be mounted following a two-step protocol. Steps A and B have to be performed subsequently:

A) Liquid coverslipping

- Incubate slides in distilled or deionized water for at least 1 min;
- Remove slides from distilled or deionized water without further drying;
- Tap off excess liquid without allowing drying of specimens; carefully wipe the bottom side of the slides with a paper towel to remove water;
- Apply 4 drops of CINtec® *PLUS* Mount (1 drop corresponds to 35 – 40 µL of this aqueous mounting medium) per LBC slide and 8 drops per conventional smear, respectively. Avoid the generation of air bubbles. To prevent formation of tiny bubbles the first drop can be discarded onto a paper towel before applying CINtec® *PLUS* Mount on the specimen;
- Gently tilt and rotate the glass slide to generate a thin layer of mounting medium and to fully cover the specimen (Do NOT yet apply a glass or film coverslip!); check the distribution of the mounting medium on the slide by visual inspection;
- For drying place slides in a horizontal position:
  a. Incubate ThinPrep® or BD SurePath™ samples at 37-60°C for 1 hour, or alternatively overnight at ambient temperature;
  b. Incubate Conventional smears at 37°C for 4 hours, or at 60°C for 1 hour, or alternatively overnight at ambient temperature;

B) Glass or film coverslipping

- After complete drying of the aqueous mount, incubate slides in xylene for a minimum of 1 minute and up to a maximum of 20 minutes. Then, coverslip the slides using a xylene-based mounting medium.

NOTE: Sildes **must not** be dehydrated by ascending series of alcohol before being glass or film coverslipped.

- Let the xylene-based mounting medium dry at room-temperature;

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

VII. Quality Control

Deviations from the recommended procedures for fixation and further processing of the cervical cytological specimens may produce substantial variability in results, necessitating regular performance of in-house controls.

Positive Control

Specimens processed in the same manner as the patient sample(s) should be used as positive controls. Positive controls are indicative of correctly prepared specimens and proper staining techniques. One positive control should be included in each staining run.

Known positive controls should only be utilized for monitoring the correct performance of processed specimens and test reagents, rather than as an aid in formulating a specific diagnosis of patient samples. If the positive controls fail to demonstrate appropriate positive staining, results with the test specimens should be considered invalid.

Negative Control

Use a negative control processed in a manner identical to the patient sample(s) with each staining run to verify the specificity of the staining procedure and to provide an indication of background staining. A variety of different cell types present in representative cervical cytology specimens and that are known to be negative for the expression of the $p16^{INK4a}$ and Ki-67 antigens (such as e.g. superficial cells) may serve as an additional internal negative control to assess any background staining (this should be verified by the user).

Assay Verification

Prior to initial use of an antibody or staining system in a diagnostic procedure, the user should verify the antibody's specificity by testing it on a series of in-house specimens with known immunocytochemical performance characteristics representing known positive and negative specimens. Refer to the quality control procedures previously outlined in this section of the product insert and to the quality control requirements of the CAP Certification Program for Immunocytochemistry and/or CLSI (NCCLS) Quality Assurance for Immunocytochemistry, Approved Guideline.

VIII. Interpretation of Results

The CINtec® *PLUS* procedure causes two distinct coloured reaction products: a brown precipitate at the $p16^{INK4a}$ antigen sites, and a red precipitate at the Ki-67 antigen sites. Brown staining of cells (cytoplasm and/or nuclei) indicates $p16^{INK4a}$ over-expression. Red staining of cells (nuclei) indicates expression of Ki-67. Cells stained for both antigens exhibit brown cytoplasmic staining with typically pronounced red nuclei. A qualified pathologist/cytologist experienced in immunocytochemical procedures and trained on the interpretation of CINtec® *PLUS* stained slides must evaluate positive and negative controls before interpreting results. Interpretation of results must be made within the context of the patient's history and other diagnostic tests by a certified professional.

For the interpretation of cervical cytology slides stained with the CINtec® *PLUS* Kit the slides should be evaluated with regard to the presence of cervical epithelial cells showing both cytoplasmic brown and nuclear red staining indicative of simultaneous p16 and Ki-67expression.

The presence of one or more cervical epithelial cells with co-localization of brown cytoplasmic immuno-staining AND red nuclear immuno-staining within the same

cell is regarded as a positive CINtec® *PLUS* test result.

In case there is no cervical epithelial cell showing simultaneous brown cytoplasmic immuno-staining AND red nuclear immuno-staining the CINtec® *PLUS* test result is considered negative.

Please note that the presence of cervical epithelial cells that do show a single immuno-reactivity only for one of the two markers (such as e.g. brown staining for p16 only, or red staining for Ki-67 only) is not considered as a positive result for the CINtec® *PLUS* test, even when both potential types of cervical cells showing single immuno-reactivity only are present in the same cytology slide.

In case of the presence of cells showing morphological signs of severe dyskaryosis, but no dual staining for p16 and Ki-67, morphological interpretation criteria should not be disregarded.

IX. Limitations

- For professional use only. Special training is required for the performance of immunocytochemical procedures.
- The clinical interpretation of any positive or negative staining should be evaluated within the context of clinical presentation and cytological criteria. The clinical interpretation of any positive or negative staining should be complemented by morphological studies using proper positive and negative controls as well as other diagnostic tests. It is the responsibility of a qualified pathologist/cytologist who is familiar with the proper use of antibodies, reagents, and methods to interpret all of the steps used to prepare and interpret the final immunocytochemistry preparation.
- The staining results in immunocytochemistry are strongly influenced by the quality of the cells stained. Accordingly, the appropriate handling of the protocol steps for fixation, washing, drying, or heating of the slides, as well as the avoidance of the use of reagents contaminated with bacteria will significantly contribute to the overall result of the staining procedure. Deviations from the protocol may lead to artefacts, antibody trapping, or false-negative results. Inconsistent results may be due to variations in fixation or non-adequate sampling.
- 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 immunocytochemistry testing on prepared liquid-based cytology (LBC) slides or conventional smears. 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 (e.g. cytochrome C).
- The presence of leukocytes, mucus or vaginal deodorant in the sample may affect the cellularity of the slide preparation and thus may have a negative

effect on the overall performance of CINtec® *PLUS*.

- Cervical specimens may show visibly detectable levels of blood. If a sample has excessive amounts of blood, the specimen should be lysed with glacial acetic acid (GAA) according to the ThinPrep protocol prior to slide preparation.
- Do not replace kit reagents with reagents carrying different lot numbers or with reagents from other manufacturers.

X. Performance Characteristics

Clinical performance

The clinical performance of the CINtec® *PLUS* Kit has been evaluated in three separate clinical studies:

a. The PALMS Trial – Primary ASC-US LSIL Marker Study
b. The EEMAPS Trial – European Equivocal or Mildly Abnormal Pap Cytology Study
c. The Wolfsburg Trial – A nested substudy to the Wolfsburg Pap/HPV cotesting project

***Study descriptions*:**

a. PALMS – Primary ASC-US LSIL Marker Study

The PALMS trial was conducted as a prospective, multi-national, multi-center, diagnostic clinical study. The study was designed to demonstrate the appropriateness of the CINtec® *PLUS* Kit as an aid to identify women with established high-grade cervical intraepithelial neoplasia (i.e. CIN2 and higher, CIN2+) (a) within women attending routine cervical cancer screening, (b) within the subgroup of patients with a Pap cytology result of ASC-US (atypical squamous cells of undetermined significance), or (c) within the subgroup of patients with a Pap cytology result of LSIL (low-grade squamous intraepithelial lesion).

The main study objectives have been

1. To evaluate the sensitivity and specificity of the CINtec® *PLUS* Kit for identifying high-grade cervical intraepithelial neoplasia (CIN2+)
   - in a primary cervical cancer screening setting;
   - in women with a Pap cytology result of ASC-US;
   - in women with a Pap cytology result of LSIL.
2. To compare the sensitivity and specificity of the CINtec® *PLUS* Kit for identifying high-grade cervical intraepithelial neoplasia (CIN2+) to Pap cytology testing (screening) and HPV testing (ASC-US and LSIL triage; screening).

The study was performed on a total of 27,349 women attending routine cervical cancer screening visits in five European countries (Belgium, France, Germany, Italy, and Spain), and who were prospectively enrolled to the trial after giving their written informed consent. During the screening visit two cervical samples were collected from all participating women:

- A first cervical sample was collected using a broom-type sampling device and used for performing a Pap cytology test, either by preparing a smear on a glass slide for conventional Pap smear analysis, or by transferring the cervical material into a liquid-based cytology collection vial (ThinPrep® Pap Test, Hologic™, Marlborough, MA; or SurePath™, BD Diagnostics, Burlington, NC) for subsequent slide preparation for Pap testing. A second slide

was prepared from all specimens, either from residual material on the sampling device that had been used for the initial preparation of a conventional Pap smear ("split sample" technique), or from residual material out of the respective liquid-based cytology vial, and subsequently was used for p16/Ki-67 dual-stained cytology testing.

- A second cervical sample was collected from each study participant using the DNAPap Cervical Sampler ™ (Qiagen, Hilden, Germany), a brush type sampling device combined with a collection medium, specifically designed for the collection of specimens for HPV testing. These specimens were subsequently used for determining the presence of HR-HPV infections using the digene HC2 High-Risk HPV DNA Test® (Qiagen).

Pap testing was performed locally in a total of 16 European cytology laboratories and using the Bethesda classification system [35].

Dual-stained cytology testing was performed centrally following the protocols in the Instructions for Use of the CINtec® *PLUS* Kit (Roche mtm laboratories AG). For interpretation, slides were screened for the presence of dual-stained cervical cells by individual members of an independent team of 8 cytotechnologists contracted for slide interpretation in the course of the clinical trials. All positive dual-stained cytology results were reviewed and confirmed by members of a team of 5 European pathologists.

HPV testing was performed by a total of 6 independent clinical laboratories in France, Germany, and Italy.

Any positive test result, i.e. either a Pap cytology result of ASC-US or higher (ASC-US+), and/or a positive CINtec® *PLUS* test result, and/or a positive HR-HPV test result (in women aged 30 years or older), triggered a referral to colposcopy. During colposcopy, biopsies were collected as clinically indicated. Histology diagnoses were established on H&E stained, formalin-fixed, paraffin-embedded tissue sections. The local pathology result for each case was compared to the result of an independent European QC review pathologist. Any discrepancy between local and first QC review results as well as any CIN2 or higher grade (CIN2+) diagnosis resulted in a full QC review by a panel of European pathologists considered experts in gynaecological pathology. The two out of three majority consensus diagnosis, or adjudicated diagnoses for cases where no majority had been achieved before was used as Gold standard for study purposes. Histology results were confirmed by an independent evaluation of the p16 IHC staining pattern on consecutive sections prepared from the tissue blocks and immunostained using the CINtec® Histology Kit (Roche mtm laboratories AG, REF 9511). Only for those cases where the p16 staining pattern was not supporting the histological diagnosis established on H&E slides, a separate adjudication by 3 QC review pathologists was performed on both H&E and p16 stained tissue sections.

All data from PALMS trial are reported after statistical correction for verification bias due to some disparity in colposcopic follow-up procedures and thus disease ascertainment for positive results of the three individual tests, i.e. Pap cytology, CINtec® *PLUS*, or HPV testing.

b. EEMAPS – European Equivocal or Mildly Abnormal Pap Cytology Study

The EEMAPS trial was a multi-national, multi-center, case-control clinical study using retrospectively collected liquid-based cervical cytology preparations (ThinPrep®, Hologic™). The study was designed to prove the utility of the CINtec® *PLUS* test as an aid to identify women with underlying CIN2+ within the subgroup of pa-

tients with a Pap cytology result categorized as either ASC-US or LSIL according to the Bethesda classification system [35] and where residual material was available in the ThinPrep® liquid-based cytology vial. Cases were selected for which corresponding histology tissue specimens were available that had been obtained during diagnostic follow-up procedures performed within a 6 months period following the referral Pap cytology. Histologic tissue samples comprised a distribution of cervical punch biopsies, cone biopsies, and endocervical curretage specimens.

The main study objectives were

1. To evaluate the sensitivity and specificity of the CINtec® *PLUS* Kit for identifying CIN2+ in women with a Pap cytology result of ASC-US or LSIL;
2. To compare the sensitivity and specificity of detecting CIN2+ by using the CINtec® *PLUS* Kit with the sensitivity and specificity by using the digene HC2 High-Risk HPV DNA Test® (Qiagen) for women with a Pap cytology result of ASC-US or LSIL.

HPV testing was performed centralized in a qualified clinical laboratory accredited in Germany and according to the manufacturer´s instruction.

For p16/Ki-67 dual-stained cytology, residual material out of ThinPrep® liquid-based cytology vials was used to prepare fresh slides using a T2000 ThinPrep® Processor (Hologic™). Slides were subsequently immuno-stained following the protocol outlined in the Instructions for Use for the CINtec® *PLUS* Kit.

Slides were screened by a single cytotechnologist for the presence of dual-stained cervical cells. All cases identified by the cytotechnologist review as showing one or more dual-stained cell(s) were subject to confirmation by a pathologist review for final dual-stained cytology results.

The histological diagnosis established on re-cut tissue block specimens was used as the gold standard (i.e. diagnostic accuracy criterion) for the study to which the results of the dual-stained cytology and the HPV tests were compared.

A majority consensus diagnosis was established for each case by two or more QC review pathologists out of a team of five pathologists. The reviewers were blinded to the original histopathology result of the local study center from which the specimens had been retrieved, and to all other test results. A combined review of H&E-stained slides and consecutive slides stained for p16 with the CINtec® Histology Kit (Roche mtm laboratories AG) was performed by the QC review pathologists in this particular study.

A total of 776 cases, thereof 361 cases of ASC-US and 415 cases of LSIL were available for the analysis.

c. The Wolfsburg Study – A nested substudy to the Wolfsburg Pap/HPV co-testing project

The overall aim of the study was to investigate the clinical utility of the CINtec® *PLUS* Kit as a screening test as well as a reflex test to Pap negative, HPV positive test results in women aged 30 years and older. The evaluation of the performance characteristics of the CINtec® *PLUS* Kit for the identification of women with established high-grade cervical intra-epithelial lesions (HGCIN) was done as a nested substudy to the Wolfsburg Pap/HPV co-testing project. The study objectives were

1. To evaluate the sensitivity and specificity of dual-stained cytology for identifying HGCIN in a cervical cancer screening setting;

2. To compare the sensitivity and specificity of dual-stained cytology for identifying HGCIN in a cervical cancer screening setting to the sensitivity and specificity of liquid-based Pap cytology testing and HPV testing, respectively;
3. To evaluate the diagnostic performance of dual-stained cytology in the triage of Pap negative, HPV positive screening test results.

The study was performed as a multicenter, retrospective diagnostic study on prospectively collected study specimens. Women aged 30 and older with signed written informed consent were enrolled into the Wolfsburg screening project.

At the screening visit, a first cervical sample was collected using either spatula/brush or a broom-type sampling device and directly transferred into a ThinPrep® Pap test (Hologic™) sampling vial. Subsequently, a second cervical sample was collected using the DNAPap Cervical Sampler™ (Qiagen) and the respective sampling vial for subsequent HPV testing using the digene HC2 High-Risk HPV DNA Test® (Qiagen).

Pap cytology (ThinPrep® Pap test) was performed centralized in an independent laboratory directly after the enrollment visit. The first slide specimen prepared from the liquid-based cytology vial was used for Pap testing. HPV testing was performed in a centralized clinical laboratory in Wolfsburg.

Women were referred to colposcopy based on their Pap cytology and HPV test results only, following the requirements of the Wolfsburg Pap/HPV-co-testing study algorithm:

- Women positive for both Pap cytology (Pap IIw;using Munich nomenclature), almost equivalent to ASC-US), used as the threshold) and HPV testing were referred to immediate colposcopy;
- Women with either a positive Pap, but negative HPV result, or a negative Pap, but positive HPV result were referred to colposcopy only when either Pap or HPV test results were persistently positive in the course of one or more repeat testing procedures over the following 6-12 months, or longer;
- Women with negative test results for both Pap cytology and HPV testing were not subject to repeat cervical cancer screening testing for a 5 years period.

The p16/Ki-67 dual-stained cytology testing was performed retrospectively after 1-2 years of storage of the ThinPrep® vials. Slide preparation and immunostaining was performed centrally at the sponsor´s laboratory. Slides were screened for the presence of dual-stained cervical cells by individual members of a team of 8 independent cytotechnologists contracted for slide interpretation during PALMS and Wolfsburg trials. All positive dual-stained cytology results were reviewed and confirmed by a single independent pathologist.

Histology diagnoses on biopsy materials served as Gold standard. All local histology results were verified by members of an independent QC review board of 6 European pathologists, masked to the clinical center interpretation and all other test results. The QC review process was performed in analogy to the process established for the PALMS Trial (see above).

For a cross-sectional analysis of the screening population in women aged 30 years and older, 4,246 cases were randomly selected out of a total of 7,976 women enrolled in the Wolfsburg Project during 2007 and 2008 and with sufficient residual cellular material left in the liquid-based cytology vial. To assess the utility of CINtec® *PLUS* testing in the triage of women tested Pap negative but HPV positive, all cas-

es with such test results (n=425) out of the group of 7,976 women were included in the analysis.

Results:

ALL TABLES AND FIGURE 1 ARE SHOWN IN ANNEX 2!

CLINICAL PERFORMANCE OF CINtec® *PLUS* TEST IN SCREENING FOR CERVICAL CANCER

Table 1 shows that positivity rates for CINtec® *PLUS* were found at comparable levels as Pap abnormality rates (threshold of ASC-US or higher; ASC-US+), and at approx. half of the positivity rates for HR-HPV testing, irrespective of age groups.

In Table 2 sensitivity, specificity, as well as other diagnostic performance characteristics such as Positive (PPV) and Negative Predictive Values (NPV) and positive (DLR+) and negative Diagnostic Likelihood Ratios (DLR–) for CINtec® *PLUS* testing in a screening population as confirmed in the PALMS trial are reported. Sensitivity of CINtec® *PLUS* testing for CIN2+ over all ages was 90.1%, significantly higher than sensitivity of Pap cytology testing (66.4%; Increase by 36%, p<0.0005). HPV testing sensitivity for the detection of CIN2+ was found at 96.4%. Specificity of CINtec® *PLUS* testing (95.3) was found at the same high level as specificity of Pap cytology testing (95.4%), whereas specificity of HPV testing was 90.2% over all ages. The differences in specificity rates between CINtec® *PLUS* or Pap cytology vs. HPV testing were statistically highly significant (p< 0.0005), with rates of false-positive results double as high for HPV testing over all ages as well as within the <30 vs. ≥30 years age groups.

The results for the evaluation of sensitivity, specificity, PPV, NPV, DLR+ and DLR– for CINtec® *PLUS* vs. Pap cytology and HPV testing, respectively, in the Wolfsburg Study are shown in Table 3. The high levels of sensitivity and specificity of CINtec® *PLUS* for the detection of high-grade CIN as found in the PALMS trial (see Table 2) were independently confirmed by the results from the Wolfsburg study in women aged 30 years and older.

CLINICAL PERFORMANCE OF CINtec® *PLUS* TEST IN THE TRIAGE OF WOMEN WITH ASC-US PAP CYTOLOGY TO DETERMINE THE NEED FOR REFERRAL TO COLPOSCOPY

Sensitivity and specificity of CINtec® *PLUS* for detecting biopsy-confirmed CIN2+ at time of colposcopy in the triage of ASC-US cytology cases in women of all ages were found in PALMS at 94.6% and 77.5%, respectively (Table 4), and in EEMAPS at 92.2% and 80.6%, respectively (Table 5).

HPV testing provided comparable sensitivity levels (100% in PALMS), and 90.9% in EEMAPS), but at significantly lower specificity levels (60.7% in PALMS, and 36.3% in EEMAPS). This improvement in specificity was even higher in the younger age group (i.e. women aged less than 30 years old) as illustrated in Table 5, however, also in the older age group (≥30 years) specificity of CINtec® *PLUS* was double as high compared to HPV testing (85.5% vs. 43.6%).

As positivity rates for CINtec® *PLUS* in the ASC-US category in the prospective PALMS trial were found at 25.6% vs. 41.9% for HPV testing, testing with CINtec® *PLUS* may allow for a more effective ASC-US triage, providing comparable sensitivity for underlying high-grade CIN despite a lower rate of women referred to colposcopy.

CLINICAL PERFORMANCE OF CINtec® *PLUS* TEST IN THE TRIAGE OF WOMEN WITH LSIL PAP CYTOLOGY TO DETERMINE THE NEED FOR REFERRAL TO COLPOSCOPY

Positivity rates of CINtec® *PLUS* testing in cytology cases categorized as LSIL in the *PALMS* and *EEMAPS* trials are shown in Table 6 and Table 7 and compared to HPV test positivity rates. The positivity rate of 52.4% (*PALMS) and* 52.3% (*EEMAPS*) for CINtec® *PLUS* testing in LSIL was substantially lower than the rate of 83.9% (*PALMS*) and 86.2% (*EEMAPS*) positive HR-HPV test results, showing the potential of CINtec® *PLUS* testing as an efficient triage tool for LSIL Pap cytology results.

Sensitivity and specificity of CINtec® *PLUS* for detecting biopsy-confirmed CIN2+ at time of colposcopy in the triage of LSIL cytology cases in women of all ages were found in PALMS at 85.4% and 53.9%, respectively (Table 6), and in EEMAPS at 94.2% and 68.0%, respectively (Table 7).

CLINICAL PERFORMANCE OF CINtec® *PLUS* TEST IN THE TRIAGE OF WOMEN WITH NEGATIVE PAP CYTOLOGY AND POSITIVE HPV TEST RESULTS TO DETERMINE THE NEED FOR IMMEDIATE REFERRAL TO COLPOSCOPY

Figure 1 shows the positivity rate of CINtec® *PLUS* in the triage of cases tested negative by Pap cytology, but positive for HR-HPV. Approximately one quarter (n=108; 25.4%) of the Pap negative, HPV positive cases displayed cervical epithelial cell(s) dual-stained for p16 and Ki-67, whereas 317 out of 425 cases (74.6%) tested negative.

Over 90% of underlying CIN2+ was found in the group of CINtec® *PLUS* positive cases, i.e. 34 out of 37 biopsy confirmed cases of CIN2+ that were identified during follow-up (mean follow-up time of 12.5 months). Sensitivity, specificity, PPV, NPV, and positive and negative diagnostic likelihood ratios (DLR+ and DLR–) for CINtec® *PLUS* for the detection of CIN2+ and CIN3+ are shown in Table 8.

Analytical performance:

Analytical Sensitivity:

One sample pool each of ThinPrep® and of SurePath™ specimens categorized negative for intraepithelial lesion and malignancy (NILM), respectively, were initially divided in 5 aliquots. The first NILM Pool was spiked with 100, the second with 50, the third with 20 and the fourth with 10 C4.I cells per mL respectively. The remaining NILM pool aliquot was used as negative control and the C4.I ThinPrep stock as a positive control. The dilution with 10 double-immunoreactive C4.I cells per mL reflected the “limit of detection”.

Both for ThinPrep® and SurePath™, at least 1 cell was found on 1 of 2 samples with each lot in dilution with 10 double-immunoreactive C4.I cells per mL, and at least one single double-immunoreactive C4.I cell was detected in all samples containing 20 or more C4.I cells per mL.

It was therefore verified that the CINtec® *PLUS* Kit is able to detect double-immunoreactive cells which show over-expression of $p16^{INK4a}$ and Ki-67 within the same cell.

Analytical Specificity:

The specificity of $p16^{INK4a}$ antibody clone E6H4 has been verified in the course of verification studies comprising immuno-histochemistry (IHC), immuno-

cytochemistry (ICC), and Western-Blot analyses. The specificity of the Ki-67 antibody has been demonstrated by IHC and ICC, as well as Western blot analyses.

In order to demonstrate that double-immunoreactive cells are detected by this assay, two ThinPrep® and two SurePath™ pools consisting of patient material mainly categorized as HSIL were used as a positive control (staining performed with three different lots of CINtec® *PLUS*). In addition, ThinPrep® and SurePath™ cytological preparations of double-immunoreactive C4.I cells and p16 negative/Ki-67 positive Jurkat cells were included as controls. For HSIL pool controls co-localization of p16 and Ki-67 expression was restricted to dysplastic cells only. For staining of one formalin-fixed paraffin-embedded (FFPE) cervical biopsy (categorized as CIN2/3; histological tissue sections of one tissue) dysplastic, proliferating cells (simultaneous immunoreactivity of p16 and Ki-67) were detected within the lesion.

In addition, it was verified that normal epithelial cells of cytological specimen do not show double-immunoreactivity for both p16 and Ki-67. No double-immunoreactive cells were detected for all cytological samples categorized as NILM with any of the three lots.

Precision inter-lot and intra-run:

The objective of the intra-run and inter-lot study (performed within the same study) was to verify that the CINtec® *PLUS* Kit provides comparable staining results within the acceptable range. The study was conducted by staining of ThinPrep® and SurePath™ liquid based cytology (LBC) preparations consisting of pooled samples categorized as HSIL with 3 consecutive CINtec® *PLUS* lots.

The staining intensity for C4.I control cells prepared like ThinPrep® or SurePath™ samples did not differ significantly (±0.5 on a scale of 0 to 3) between the individual ThinPrep® and SurePath™ slides among all lots. All acceptance criteria were fulfilled, demonstrating that the CINtec® *PLUS* Kit provides comparable staining results within the same staining run and between different lots.

Precision inter-run:

The objective of the inter-run study was to verify that the CINtec® *PLUS* Kit provides comparable staining results within the acceptable range when one operator processes identical samples (3 slides prepared from the same sample vials) on one Autostainer instrument on three different days. The study was conducted by staining of ThinPrep® and SurePath™ LBC preparations consisting of pooled samples categorized as HSIL with one CINtec® *PLUS* lot.

The intensity of p16 staining did not vary significantly (±0.5 on a scale of 0 to 3) and neither did the score for Ki-67 staining, demonstrating the reproducibility in that regard. All acceptance criteria were fulfilled, demonstrating that the CINtec® *PLUS* Kit provides comparable staining results between several runs on several days, when all other variables (operator, instrument, etc.) are kept the same.

Precision inter-operator:

The inter-operator reproducibility was tested using the manual staining procedure and the LabVision Autostainer procedure. Both methods were tested with three operators each.

The staining intensity among all staining runs did not differ significantly (±0.5 on a scale of 0 to 3) for both specimen types (ThinPrep®, Sure Path™) used. All acceptance criteria were fulfilled, demonstrating a good reproducibility of the CINtec® *PLUS* Kit when used by different operators.

Precision inter-method:

Inter-method reproducibility was verified by comparing the staining results of one

CINtec® *PLUS* lot on the Lab Vision Autostainer with the Coverplate™ system. The tests have been performed using both ThinPrep® and SurePath™ samples. Especially for SurePath™ preparations where two Fast Red substrate steps must be applied, the “Dako® Autostainer software setting” (two Fast Red steps, but working solution can be prepared only once per run) was compared with the Lab Vision Autostainer (two Fast Red steps with freshly prepared working solution) and the manual (Shandon Coverplate) results.

The staining intensity for C4.I control cells among all staining runs did not differ significantly (±0.5 on a scale of 0 to 3) for each specimen type (ThinPrep®, Sure Path™) used. All acceptance criteria were fulfilled, demonstrating sufficient inter-method reproducibility.

Precision inter-instrument:

The objective of the inter-instrument study was to verify that the CINtec® *PLUS* Kit provides comparable staining results within the acceptable range when one operator processes identical samples (2 slides prepared from the same sample vials) on two different Autostainer instruments. The study was conducted by staining of ThinPrep® and SurePath™ LBC preparations consisting of pooled samples categorized as HSIL with one CINtec® *PLUS* lot.

The staining intensity between staining runs on both instruments did not differ significantly (±0.5 on a scale of 0 to 3) for both specimen types (ThinPrep®, Sure Path™) used. All acceptance criteria were fulfilled, demonstrating a good reproducibility of the CINtec® *PLUS* Kit, when used on different instruments.

Note that the staining intensity scoring method with a scale of 0 to 3 was solely used for analytical performance evaluation purposes and shall not be used for the interpretation of the staining in clinical practice. Instead, the qualitative interpretation of stained slides as described in Section VIII shall be used for the routine interpretation.

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;
	1b. Reagent vials were not loaded in the correct locations in the reagent racks;	**1b.** Check the Reagent Map to verify the proper location of the reagent vials;
	1c. Insufficient reagent in vial;	**1c.** Ensure that enough reagent is loaded into the vials prior to commencing the run. Refer to Reagent Map for volumes required;
	1d. Missing Wash Buffer for Dako or LabVision Autostainers;	**1d.** Check that there is sufficient buffer. If not, then fill the container with buffer and prime the pump; Check if the clear plastic tubing around the syringe (placed on the left side of the moving arm) contains air bubbles - if so removal of the bubbles may solve the problem;
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 down for - additional 20 minutes; Ensure that no water slops over into the heat resistant staining jars during epitope retrieval procedure. Review 2.2 for recommendations;
	2b. Inadequate reagent incubation times;	**2b.** Review 2.3.1 / 2.3.2 Staining protocol recommendations;
	2c. Inappropriate fixation method;	**2c.** Ensure that cytology preparations are fixed as outlined in Section VII 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 the ion exchange column for producing deionized water has been checked by routine maintenance;
	2e. Missing Wash Buffer for Dako or LabVision Autostainers;	**2e.** Refer to troubleshooting Section 1d;
	2f. Dilution of kit components with washing buffer due to inadequate waiting time after washing steps for the Shandon Coverplate System;	**2f.** Wait 5 minutes after adding the Wash buffer into the funnel of the Shandon Coverplate System and check, that no residual Wash buffer remains in the funnel which may cause a dilution of the following reagent;

3. No or weak Ki-67 staining (red) on positive control slides	**3a.** Use of alcohol containing Hematoxylin (e.g. Harris');	**3a.** Fast Red is soluble in EtOH therefore, the use of alcohol-free Hematoxylin is mandatory to prevent fading of Fast Red dye (Ki-67 signal);
	3b. Slides were dehydrated using ascending series of alcohol before being glass or film coverslipped;	**3b.** Follow the 2-step mounting protocol using CINtec® *PLUS* Mount followed by permanent glass or film coverslipping; Review 2.5 for recommendations;
	3c. Fast Red working solution was not prepared according to the recommendations;	**3c.** Prepare Fast Red working solution immediately before use; apply the solution to the slides within 15 minutes of preparation, as otherwise a decrease in signal intensity and test sensitivity may occur;
4. Excessive background staining of slides	**4a.** Incomplete removal of spray fixation reagent (polyethylene glycol) when using conventional smears;	**4a.** Follow procedure as outlined in Section 2.1.;
	4b. Insufficient rinsing of slides;	**4b.** Use fresh solution in buffer baths and wash bottles; For Autostainer Instrument, ensure that before running the instrument is primed properly and that sufficient amounts of buffer are available for the whole run; use fresh solutions of buffers and reagents;
	4c. Drying of specimen during staining procedure;	**4c.** When using the Autostainer Instrument, ensure that the amount of reagent supplied is sufficient and that the hood of the instrument is closed during the run; Do not expose slides to elevated temperatures or to dry environment; Consider whether the programmed reagent volume and dispense location (drop zone) were adequate to cover all areas on a slide which contain cell materials; Check that there is sufficient buffer. If not, then fill the container with buffer and prime the pump; Check if the clear plastic tubing around the syringe (placed on the left side of the moving arm) contains air bubbles - if so removal of the bubbles may solve the problem; Avoid delay of the Fast Red working solution incubation step during autostainer run to minimize the risk of drying artefacts;

	4d. Inappropriate fixation method;	**4d.** Use only fixatives as recommended herein. Aberrantly fixed cells may exhibit excessive background staining;
	4e. Non-specific binding of reagents to cells;	**4e.** Check fixation method used for the specimen;
	4f. Drying of specimens during set up of Autostainer Instrument;	**4f.** Keep specimens covered with reagent (buffer) while initiating the run;
	4g. Drying of specimens during assembly of Shandon Coverplate system;	**4g.** Fill a plain bowl with distilled or deionized water. Take one slide out of the Epitope Retrieval Solution and assemble the slide and the coverplate submerged in the bowl (underwater);
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 2.3.1 / 2.3.2 above;
	5c. Inappropriate wash solution;	**5c.** Use the Wash Buffer (catalog number 10215364001);
6. Evaluation of double-immuno-reactive cells not possible	**6a.** Hematoxylin counterstain too strong;	**6a.** Reduce incubation time for hematoxylin and/or bluing in tap water or ammonia water;
7. Glass or film coverslipping is not adequate	**7a.** Glass or film coverslip cannot be applied correctly;	**7a.** Prevent formation of air-bubbles when applying CINtec® *PLUS* Mount (aqueous coverslip); Adhere to recommended drying time for liquid coverslip;
8. Patchy or sporadic staining pattern after using the Shandon Coverplate System	**8a.** Air bubbles embedded between slide and coverplate;	**8a.** Assembly of slide and coverplate has to be performed according to Protocol REF 2010-953-009EN (available from Roche mtm laboratories AG); Air bubbles on the slide need to be removed before assembling;
9. Crack formation after mounting the slides with xylene-based mounting medium	**9a.** Insufficient drying of CINtec® *PLUS* Mount before mounting the slides with xylene-based mounting medium;	**9a.** Dry slides which have been covered with CINtec® *PLUS* Mount over night at room temperature or at least for 4h at 37°C. CINtec® *PLUS* Mount has to be dried completely.

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
	Contact for technical support (Telephone)
	Contains materials of animal origin
CONTENT	Content

XIII. Manufacturer

Manufactured by:

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany

https://navifyportal.roche.com

Contact for technical support (Telephone): +800 5505 6606

The summary of safety and performance can be found here:

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

XIV. Revision Status

The current Instructions for Use represent Version 1.0 released June 2024.

Changes to previous version for product 9531 / 06595367001 (2.7, released December 2022):

- Manufacturer changed from Roche mtm laboratories AG to Roche Diagnostics GmbH, and thus new product number, 10215348001, issued
- Editorial changes

XV. Intellectual Property

CINtec and E6H4 are trademarks of Roche.

All other trademarks are the property of their respective owners.

Annex 1
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Annex 2
Tables

Glossary for Tables and Figure 1

Woman aged xx–xx years

Pap cytology

ASC-US or higher

Sensitivity

Specificity

PPV:

Positive predictive value

NPV:

Negative predictive value

DLR+:

Positive diagnostic likelihood ratio

DLR–:

Negative diagnostic likelihood ratio

CI:
Confidence Interval

Referral to colposcopy

HGCIN:
High-grade cervical intraepithelial neoplasia

Table 1:

PALMS trial: CINtec® *PLUS* test positivity rates in screening population, in comparsion to results from Pap cytology (i.e. ASC-US or higher result), and HC2 High-Risk HPV DNA testing, respectively.

	Pap cytology, ASC-US or higher		**CINtec® *PLUS* Positive**		**HPV Positive**	
	n	**%**	n	**%**	n	**%**
Women aged 18-65 years n= 27,248	1,407	**5.2%**	1,462	**5.4%**	2,918	**10.7%**
Women aged 18-29 years n= 6,798	563	**8.3%**	605	**8.9%**	1,376	**20.2%**
Women aged 30-65 years n= 20,450	844	**4.1%**	857	**4.2%**	1,542	**7.5%**

Table 2:

PALMS trial: Sensitivity, specificity, PPV, NPV, and positive and negative diagnostic likelihood ratios (DLR+, DLR–) for CINtec® *PLUS* in Screening population for the detection of CIN2+. Results for Pap cytology and HC2 High-Risk HPV DNA testing are shown for comparison.

	CINtec® *PLUS* (95% CI)	**Pap cytology** (95% CI)	**HPV** (95% CI)
Women aged 18-65 years (n=25,577; 205 CIN2+, prevalence = 0.80%)			
Sensitivity	**90.1%** (85.3, 93.5)	**66.4%** (59.5, 72.6)	**96.4%** (93.1, 98.3)
Specificity	**95.3%** (95.0, 95.6)	**95.5%** (95.2, 95.7)	**90.2%** (88.1, 92.4)
PPV	**18.4%**	**14.6%**	**10.2%**
NPV	**99.9%**	**99.6%**	**99.9%**
DLR+	**19.219**	**14.573**	**9.850**
DLR–	**0.104**	**0.352**	**0.040**
Women aged 18-29 years (n=6,372; 82 CIN2+, prevalence = 1.29%)			
Sensitivity	**93.3%** (85.7, 97.0)	**67.7%** (56.3, 77.4)	**97.4%** (91.3, 99.5)
Specificity	**92.3%** (91.6, 93.0)	**92.8%** (92.1, 93.4)	**81.4%** (77.3, 85.7)
PPV	**19.7%**	**16.0%**	**9.7%**
NPV	**99.9%**	**99.3%**	**99.9%**
DLR+	**12.092**	**9.359**	**5.234**
DLR–	**0.073**	**0.348**	**0.032**
Women aged 30-65 years (n=19,205; 123 CIN2+, prevalence = 0.64%)			
Sensitivity	**87.8%** (80.8, 92.5)	**64.9%** (56.0, 72.9)	**95.6%** (89.0, 98.3)
Specificity	**96.3%** (96.0, 96.6)	**96.3%** (96.0, 96.6)	**93.1%** (92.7, 93.5)
PPV	**17.6%**	**13.7%**	**10.5%**
NPV	**99.9%**	**99.7%**	**99.9%**
DLR+	**23.765**	**17.676**	**13.851**
DLR–	**0.127**	**0.364**	**0.047**

Table 3:

Wolfsburg Study: Sensitivity, specificity, PPV, NPV, and positive and negative diagnostic likelihood ratios (DLR+, DLR–) for CINtec® *PLUS* in Screening of women aged 30 years and older for the detection of CIN2+. Results for Pap cytology and HC2 High-Risk HPV DNA testing are shown for comparison.

	CINtec® *PLUS* (95% CI)	**Pap cytology** (95% CI)	**HPV** (95% CI)
Women aged 30-65 years (n=4,246; 40 CIN2+, prevalence = 0.94%)			
Sensitivity	**92.5%** (79.6, 98.4)	**65.0%** (48.3, 79.4)	**100%** (91.2, 100)
Specificity	**97.5%** (97.0, 97.9)	**98.7%** (98.3, 99.0)	**94.1%** (93.3, 94.8)
PPV	**25.9%** (18.9, 33.9)	**32.1%** (22.2, 43.4)	**13.8%** (10.1, 18.4)
NPV	**99.9%** (99.8, 99.9)	**99.7%** (99.4, 99.8)	**100%** (99.9, 100)
DLR+	**36.703** (29.821, 45.173)	**49.707** (35.122, 70.350)	**16.892** (14.974, 19.054)
DLR–	**0.077** (0.026, 0.228)	**0.355** (0.232, 0.541)	**0.0** (NA)

Table 4:

PALMS trial: Test positivity (colposcopy referral rate), sensitivity, specificity, PPV, NPV, and positive and negative diagnostic likelihood ratios (DLR+, DLR–) for CINtec® *PLUS* in the triage of ASC-US Pap cytology results for the detection of CIN2+. Results for HC2 High-Risk HPV DNA testing are shown for comparison.

	CINtec® *PLUS* (95% CI)	**HPV** (95% CI)
Women aged 18-65 years, ASC-US (n=575; CIN2+ prevalence = 4.3%)		
Referral to colposcopy	**25.6%**	**41.9%**
Sensitivity	**94.6%** (70.2, 99.2)	**100%** (81.5, 100)
Specificity	**77.5%** (73.7, 80.9)	**60.7%** (56.5, 64.7)
PPV	**15.8%**	**10.2%**
NPV	**99.7%**	**100%**
DLR+	**4.207**	**2.543**
DLR–	**0.069**	**0.000**
Women aged 18-29 years, ASC-US (n=219; CIN2+ prevalence = 6.8%)		
Referral to colposcopy	**32.0%**	**57.7%**
Sensitivity	**100%** (69.2, 100)	**100%** (69.2, 100)
Specificity	**73.0%** (66.2, 78.8)	**45.5%** (38.8, 52.5)
PPV	**21.1%**	**11.7%**
NPV	**100%**	**100%**
DLR+	**3.698**	**1.836**
DLR–	**0.000**	**0.000**
Women aged 30-65 years, ASC-US (n=356; CIN2+ prevalence = 2.8%)		
Referral to colposcopy	**21.6%**	**32.3%**
Sensitivity	**87.2%** (45.7, 98.2)	**100%** (63.1, 100)
Specificity	**80.3%** (75.6, 84.2)	**69.7%** (64.6, 74.3)
PPV	**11.3%**	**8.7%**
NPV	**99.5%**	**100%**
DLR+	**4.417**	**3.295**
DLR–	**0.160**	**0.000**

Table 5:

EEMAPS trial: Test positivity (colposcopy referral rate), sensitivity, specificity, PPV, NPV, and positive and negative diagnostic likelihood ratios (DLR+, DLR–) for CINtec® *PLUS* in the triage of ASC-US Pap cytology results for the detection of CIN2+. Results for HC2 High-Risk HPV DNA testing are shown for comparison.

	CINtec® *PLUS* (95% CI)	**HPV** (95% CI)
Women aged 18-65 years, ASC-US (n=361; CIN2+ prevalence = 21.3%)		
Referral to colposcopy	**34.8%**	**69.6%**
Sensitivity	**92.2%** (83.8, 97.1)	**90.9%** (82.2, 96.3)
Specificity	**80.6%** (75.6, 85.1)	**36.3%** (30.7, 42.2)
PPV	**56.4%** (47.2, 65.2)	**27.9%** (22.4, 33.9)
NPV	**97.5%** (94.5, 99.1)	**93.6%** (87.3, 97.4)
DLR+	**4.761** (3.723, 6.089)	**1.426** (1.274, 1.596)
DLR–	**0.097** (0.045, 0.209)	**0.251** (0.122, 0.516)
Women aged 18-29 years, ASC-US (n=136; CIN2+ prevalence = 22.8%)		
Referral to colposcopy	**43.1%**	**81.8%**
Sensitivity	**96.8%** (83.3, 99.9)	**100%** (88.8, 100)
Specificity	**72.4%** (62.8, 80.7)	**23.8%** (16.0, 33.1)
PPV	**50.9%** (37.5, 64.1)	**27.9%** (19.8, 37.2)
NPV	**98.7%** (93.0, 99.97)	**100%** (86.3, 100)
DLR+	**3.504** (2.554, 4.807)	**1.313** (1.179, 1.461)
DLR–	**0.045** (0.006, 0.308)	**0.0** (N/A)
Women aged 30-65 years, ASC-US (n=225; CIN2+ prevalence = 20.4%)		
Referral to colposcopy	**29.8%**	**62.2%**
Sensitivity	**89.1%** (76.4, 96.4)	**84.8%** (71.1, 93.7)
Specificity	**85.5%** (79.5, 90.3)	**43.6%** (36.2, 51.2)
PPV	**61.2%** (48.5, 72.9)	**27.9%** (20.6, 36.1)
NPV	**96.8%** (92.8, 99.0)	**91.8%** (83.8, 96.6)
DLR+	**6.136** (4.241, 8.879)	**1.503** (1.258, 1.795)
DLR–	**0.127** (0.055, 0.292)	**0.349** (0.173, 0.705)

Table 6:

PALMS trial: Test positivity (colposcopy referral rate), sensitivity, specificity, PPV, NPV, and positive and negative diagnostic likelihood ratios (DLR+, DLR–) for CINtec® *PLUS* in the triage of LSIL Pap cytology results for the detection of CIN2+. Results for HC2 High-Risk HPV DNA testing are shown for comparison.

	CINtec® PLUS (95% CI)	**HPV** (95% CI)
Women aged 18-65 years, LSIL (n=529; CIN2+ prevalence = 15.9%)		
Referral to colposcopy	**52.4%**	**83.9%**
Sensitivity	**85.4%** (74.5, 92.2)	**98.1%** (87.9, 99.7)
Specificity	**53.9%** (49.1, 58.6)	**18.8%** (15.4, 22.7)
PPV	**26.0%**	**18.6%**
NPV	**95.1%**	**98.1%**
DLR+	**1.853**	**1.208**
DLR–	**0.271**	**0.101**
Women aged 18-29 years, LSIL (n=250; CIN2+ prevalence = 15.2%)		
Referral to colposcopy	**54.8%**	**88.0%**
Sensitivity	**84.2%** (65.1, 93.9)	**95.3%** (73.7, 99.3)
Specificity	**50.5%** (43.6, 57.3)	**13.3%** (9.3, 18.7)
PPV	**23.3%**	**16.4%**
NPV	**94.7%**	**94.1%**
DLR+	**1.700**	**1.099**
DLR–	**0.313**	**0.354**
Women aged 30-65 years, LSIL (n=279; CIN2+ prevalence = 16.4%)		
Referral to colposcopy	**50.2%**	**80.3%**
Sensitivity	**86.0%** (70.7, 94.0)	**100%** (90.5, 100)
Specificity	**56.9%** (50.2, 63.3)	**23.6%** (18.5, 29.5)
PPV	**28.2%**	**20.5%**
NPV	**95.4%**	**100%**
DLR+	**1.994**	**1.309**
DLR–	**0.246**	**0.000**

Table 7:

EEMAPS trial: Test positivity (colposcopy referral rate), sensitivity, specificity, PPV, NPV, and positive and negative diagnostic likelihood ratios (DLR+, DLR–) for CINtec® *PLUS* in the triage of LSIL Pap cytology results for the detection of CIN2+. Results for HC2 High-Risk HPV DNA testing are shown for comparison.

	CINtec® *PLUS* (95% CI)	**HPV** (95% CI)
Women aged 18-65 years, LSIL (n=415; CIN2+ prevalence = 33.0%)		
Referral to colposcopy	**52.3%**	**86.2%**
Sensitivity	**94.2%** (88.8, 97.5)	**96.4%** (91.7, 98.8)
Specificity	**68.0%** (62.2, 73.4)	**19.1%** (14.6, 24.2)
PPV	**59.2%** (52.3, 65.8)	**37.0%** (21.0, 42.2)
NPV	**95.9%** (92.2, 98,2)	**91.4%** (81.0, 97.1)
DLR+	**2.941** (2.466, 3.508)	**1.190** (1.115, 1.271)
DLR–	**0.086** (0.044, 0.169)	**0.191** (0.078, 0.468)
Women aged 18-29 years, LSIL (n=142; CIN2+ prevalence = 38.7%)		
Referral to colposcopy	**60.6%**	**87.3%**
Sensitivity	**96.4%** (87.5, 99.6)	**94.6%** (84.9, 98.9)
Specificity	**62.1%** (51.0, 72.3)	**17.2%** (10.0, 26.8)
PPV	**61.6%** (50.5, 71.9)	**41.9%** (33.1, 51.1)
NPV	**96.4%** (87.7, 99.6)	**83.3%** (58.6, 96.4)
DLR+	**2.540** (1.932, 3.340)	**1.142** (1.018, 1.282)
DLR–	**0.059** (0.015, 0.231)	**0.316** (0.096, 1.043)
Women aged 30-65 years, LSIL (n=273; CIN2+ prevalence = 30.0%)		
Referral to colposcopy	**48.4%**	**85.3%**
Sensitivity	**92.7%** (84.8, 97.3)	**97.6%** (91.5, 99.7)
Specificity	**70.7%** (63.7, 77.0)	**19.9%** (14.5, 26.3)
PPV	**57.6%** (48.7, 66.1)	**34.3%** (28.3, 40.8)
NPV	**95.7%** (91.0, 98.4)	**95.0%** (83.1, 99.4)
DLR+	**3.161** (2.516, 3.972)	**1.218** (1.126, 1.317)
DLR–	**0.104** (0.048, 0.225)	**0.123** (0.030, 0.496)

Figure 1:

Wolfsburg Study: Flow chart showing the distribution of cases with biopsy- confirmed CIN2+ in women aged 30 and higher and tested Pap negative, HPV positive, within the CINtec® *PLUS* positive vs. negative groups, respectively.

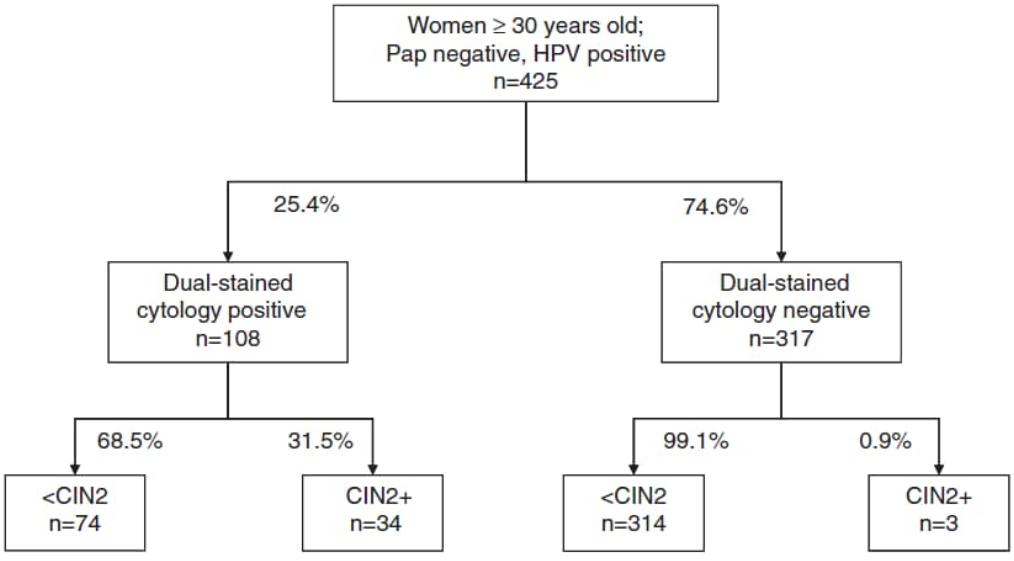

Table 8:

Wolfsburg Study: Sensitivity, specificity, PPV, NPV, and positive and negative diagnostic likelihood ratios (DLR+, DLR–) of CINtec® *PLUS* in the triage of Pap negative, HPV positive test results for the detection of CIN2+ and CIN3+. Numbers are based on the evaluation of 132 cases with colposcopic biopsies collected during a mean follow-up period of 12.5 months.

CINtec® *PLUS*	**CIN2+** (95% CI)	**CIN3+** (95% CI)
Women aged 30-65 years, HPV pos/Pap neg, with biopsy results (n=132)		
Referral to colposcopy	**34.8%**	**69.6%**
Sensitivity	**91.9%** (78.1, 98.3)	**96.4%** (81.7, 99.9)
Specificity	**82.1%** (72.9, 89.2)	**76.9%** (67.6, 84.6)
PPV	**66.7%** (52.1, 79.2)	**52.9%** (38.5, 67.1)
NPV	**96.3%** (89.6, 99.2)	**98.8%** (93.3, 99.9)
DLR+	**5.135** (3.303, 7.983)	**4.179** (2.921, 5.978)
DLR–	**0.099** (0.033, 0.293)	**0.046** (0.007, 0.319)