# The Abbott RealTime High Risk HPV test: comparative evaluation of analytical specificity and clinical sensitivity for cervical carcinoma and CIN 3 lesions with the Hybrid Capture 2 HPV DNA test

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## ABSTRACT

**Background:** The Abbott RealTi*m*e High Risk HPV test (RealTi*m*e) is a novel assay designed to detect 14 high-risk human papillomavirus genotypes (hr-HPV) and concurrently distinguish HPV-16 and HPV-18 from other hr-HPV within a single test.

**Objective:** To evaluate analytical specificity and clinical sensitivity for cervical carcinoma and cervical intraepithelial neoplasia grade 3 (CIN3) of the RealTi*m*e test in comparison with the Digene Hybrid Capture II Test (hc2).

**Materials and methods:** Analytical specificity of the RealTi*m*e assay was evaluated on 37 samples with previously determined hc2 false-positive results due to cross-reactivity of the hc2 high-risk probe cocktail with untargeted low-risk HPV genotypes. All 37 samples were negative for 14 hr-HPV using the RealTi*m*e test. Clinical sensitivity of RealTime was evaluated in comparison to hc2 on 95 and 267 archived routine cervical specimens collected from women with histologically confirmed cervical carcinoma and CIN3 lesions, respectively. Archived specimens were selected for the present study after linkage with the Slovenian national registry of CIN3 and cervical cancer to obtain histology data.

**Results:** Concordant results between RealTi*m*e and hc2 were obtained in 90/95 cervical cancer samples (94.7% agreement) and in 250/267 CIN3 samples (93.6% agreement). Clinical sensitivity of RealTi*m*e and hc2 for cervical cancer in the total study cohort was 88.4% (95% confidence interval (CI): 80.3–93.6%) and 87.3% (95% CI: 79.0–92.8%), respectively, and analytical sensitivity for samples containing at least one targeted hr-HPV was 98.8% (95% CI: 93.0–100.0%) and 95.3% (95% CI: 88.2–98.5%), respectively. Clinical sensitivity of RealTi*m*e and hc2 for CIN3 lesions of the total study cohort was 91.8% (95% CI: 87.8–94.5%) and 89.1% (95% CI: 84.8–92.3%), respectively, and analytical sensitivity for samples containing at least one targeted hr-HPV was 96.4% (95% CI: 93.3–98.2%) and 92.5% (95% CI: 88.5–95.2%), respectively.

**Conclusion:** The RealTi*m*e test showed excellent analytical specificity and no cross-reactivity with low risk HPV genotypes that tested positive with hc2. Clinical sensitivity of the RealTi*m*e assay using archived routine cervical specimens was comparable to hc2. The RealTi*m*e test is an important new method applicable to cervical carcinoma screening and management of cervical precancerous lesions.



KEY WORDS

**HPV, Abbott, Hybrid Capture, real-time PCR, CIN 3, cervical cancer**

# Introduction

Human papillomaviruses (HPVs) are remarkably diverse DNA viruses that are causally involved in the etiology of various benign and malignant neoplastic lesions of mucosal and skin epithelium (1). More than 200 HPV genotypes have currently been described, of which 115 have been officially designated and taxonomically defined (2, 3). Forty different HPV genotypes from the alpha genus are known to infect the mucosal epithelium, with a subset of at least 15 genotypes being associated with lesions that can progress to cancer (2, 4). These cancer-associated HPV genotypes are designated as high-risk HPV (hr-HPV) genotypes and are the etiological agents of virtually all cervical carcinomas (1, 5). The clinically most important hr-HPV genotypes are HPV-16 and HPV-18, found in 50 to 65% and 7 to 20% of cases of cervical cancer, respectively (6). In addition to cervical carcinoma, hr-HPV genotypes, the most frequent being HPV-16, play the leading etiological role in the development of anal cancer and substantial proportion of vaginal, penile, vulvar, and oropharyngeal (mainly tonsillar) cancers (7–10).

Detecting hr-HPV has recently become an important part of cervical carcinoma screening and detection algorithms (11, 12). Five years ago the US Food and Drug Administration (FDA) approved concurrent HPV and Pap smear screening of women 30 years and older. In addition to primary screening, several consensus guidelines recommend HPV testing for (i) triage in cases of equivocal cytology results showing the presence of atypical squamous cells of undetermined significance (ASC-US) to determine which patients should be referred for colposcopy and (ii) for follow-up after treatment of precancerous cervical lesions (11–14). For these screening purposes, a range of in-house and commercial molecular tests have been developed in order to distinguish hr-HPV infections from no HPV infection (11). The Hybrid Capture II Test (hc2) originally developed by the Digene Corporation (Gaithersburg, MD) and currently marketed by Qiagen (Hilden, Germany), is the most frequently used HPV screening test at present. hc2 is a solution-based hybridization assay designed to detect 13 hr-HPV genotypes (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, and HPV-68) using a mixture of unlabeled full genomic length RNA probes (15). Although several studies have clearly demonstrated that hc2 is a sensitive, specific, and reliable test for detecting clinically relevant HPV infections, some recently conducted studies showed a significant analytical inaccuracy of hc2, mainly due to the cross-reactivity of its probe cocktail outside the spectrum of 13 targeted hr-HPV genotypes (16–18).

Abbott RealTi*m*e High Risk HPV test (Abbott Molecular Inc., Des Plaines, IL) based on real-time polymerase chain reaction (PCR) was recently introduced in Europe. The Abbott RealTi*m*e High Risk HPV test (RealTi*m*e) is performed on the *m*2000rt real-time PCR instrument (Abbott Molecular) and is designed to identify 14 hr-HPV genotypes (HPV-16, HPV-18, HPV-31, HPV-33, HPV-35, HPV-39, HPV-45, HPV-51, HPV-52, HPV-56, HPV-58, HPV-59, HPV-66 and HPV-68) using a modified GP5+/6+ primer mix consisting of three forward and two reverse primers (19). The assay uses four channels for detecting fluorescent probes; one for detecting the internal control (human beta-globin), a second one for detecting HPV-16, a third one for detecting HPV-18, and a fourth one for detecting the remaining 12 hr-HPV genotypes. The assay turnaround time is 6 to 8 hours for 96 samples and depends on the method used for DNA extraction (19). The fully automated high throughput *m*2000*sp* instrument (Abbott Molecular Inc.) or smaller *m24sp* instrument (Abbott Molecular Inc.) can be used for DNA extraction, or alternatively DNA can be prepared manually. The RealTi*m*e assay is currently validated for use with cervical specimens collected in PreservCyt solution (Hologic, Marlborough, MA) only (19).

To the best of our knowledge, three studies evaluating various aspects of RealTi*m*e have been published in peer-reviewed journals to date by manufacturer's researchers (19–21). The first study described design principles and evaluated analytical performance of RealTi*m*e (19). Probit analysis showed that the analytical sensitivity of RealTi*m*e was between 500 and 5,000 copies of HPV DNA per assay, depending on the genotype (19). An analytical specificity study on a panel of 41 bacteria, viruses, and fungi, including 15 low-risk HPV genotypes and other microorganisms that can be found in the female anogenital tract, revealed no cross-reactivity with any of the organisms tested (19). Interference or PCR-inhibition was not observed with any of the tested substances that may be present in cervical specimens (19). In the second study, clinical sensitivity of the RealTi*m*e test was evaluated on archived cervical specimens from Amsterdam and the presence of hr-HPV was detected in 97.2% (246/253) and 98.5% (335/340) of cervical intraepithelial neoplasia grade 3 (CIN3) and cancer specimens, respectively (20). In specimens from women 30 years or older with

normal cytology in a screening population, the HPV positivity rate was 6.5% (49/757) (20). In the third study, RealTi*m*e demonstrated similar clinical performance detecting CIN2+ and CIN3+ when compared to hc2 on 702 patients with abnormal cytology referred to colposcopy (21). The clinical sensitivity for detecting CIN2+ was shown to be 97.8% with RealTi*m*e and 95.6% with hc2, and for detecting CIN3+ 100% with RealTi*m*e and 97% with hc2 (21).

This study evaluated the analytical specificity and clinical sensitivity of RealTi*m*e for cervical carcinoma and CIN3 lesions in comparison with the FDA-approved hc2 test in the settings of a routine virological laboratory. Due to the fact that the analytical performance of RealTi*m*e has been evaluated in detail in a previous study (19), our analytical evaluation of RealTi*me* was performed only on a subset of 37 samples with previously determined false-positive hc2 results. The clinical sensitivity of RealTi*m*e was evaluated in comparison to hc2 on a total of 362 archived routine cervical specimens collected from women with histologically confirmed cervical carcinoma or CIN3 lesions.

# Materials and Methods

## Analytical specificity

Thirty-seven archived routine cervical specimens collected in 1 ml of Digene Specimen Transport Medium (STM; Qiagen, Gaithersburg, MD) with previously determined hc2 hr-HPV false-positive results were included in the study. All 37 samples tested hc2 hr-HPV–positive using a high-risk probe cocktail with relative light unit per cutoff (RLU/CO) values between 1.1 and 4.0, but were negative for 14 hr-HPV genotypes (13 hr-HPV genotypes targeted in the hc2 plus HPV-66) using three commercial assays: the Amplicor HPV Test (Roche Diagnostics, Branchburg, NJ), the INNO-LiPA HPV Genotyping *Extra* Test (Innogenetics, Gent, Belgium), and the Linear Array HPV Genotyping Test (Roche Diagnostics). Because the presence of only low-risk HPV genotypes was detected in all 37 specimens after extensive testing using the INNO-LiPA HPV Genotyping *Extra* Test, the Linear Array HPV Genotyping Test, HPV-6/-11/-42/-43/-44/-55 in-house real-time PCR assays (22, 23), and sequencing of GP5+/GP6+ and PGMY09/11 PCR-products (24), all 37 samples were considered hc2 hr-HPV false-positive due to the cross-reactivity of the hc2 high-risk probe cocktail with untargeted low-risk HPV genotypes (16, 17). For this study, 200 µl aliquots of the specimens collected in STM were removed immediately upon arrival of specimens in the laboratory and prior to the addition of the hc2 denaturing reagent solution (the first step of hc2 testing). Specimens were stored at −70 °C. Prior to sample extraction, 20 µl of the specimens was spiked to mLysis Buffer (Abbott Molecular Inc.) and processed on the *m*2000*sp* and *m*2000*rt* instruments, following the manufacturer's instructions (19).

## Clinical sensitivity for cervical carcinoma and CIN3 lesions

The clinical sensitivity of RealTi*m*e for cervical carcinoma and CIN3 lesions was evaluated in comparison to hc2 on a total of 362 archived routine cervical specimens. No samples were collected solely for the purpose of this study. The samples included in the study were selected from a total of 8,967 samples, which were routinely tested in our laboratory between 2005 and 2008 for the presence of hr-HPV using hc2. Sample inclusion was based on the ability to link with the Slovenian national registry of CIN3 and cervical cancer to obtain histology data. Only samples taken within 1 year before entering the national registry of CIN3 and cervical cancer were included in the study. In total, 95 and 267 samples collected in women with histologically confirmed cervical carcinoma and CIN3 lesion, respectively, were included in the study. Samples had originally been collected in 1 ml of STM by approximately 70 different public or private practices for various reasons (clinician-based decision). For this study, 500 µl aliquots of STM were removed immediately upon arrival of the specimens in the laboratory, out of which 20 µl was used for RealTime test as described above, and the remaining aliquots were tested by hc2 after addition of hc2 denaturing reagent solution, following the manufacturer's instructions (16). The two aliquots of one clinical sample kept at −70 °C were tested in parallel within 1 week using the two comparator assays.

The INNO-LiPA HPV Genotyping *Extra* Test was used as the main test for resolving discordance between RealTi*m*e and hc2, as described previously (25). The INNO-LIPA test is capable of recognizing 27 different alpha-HPV genotypes. It specifically identifies all hr-HPV genotypes covered by RealTi*m*e and hc2, two additional hr-HPV genotypes (HPV-73 and HPV-82), and two probable hr-HPV genotypes (HPV-26 and HPV-53).

All samples negative for both tests and some samples with discordant results were further tested

*Table 1. Results of comparative evaluation of RealTime and hc2 on 95 archived routine cervical specimens collected from the same number of women with histologically confirmed cervical carcinoma.*

| | hc2 hr-HPV–positive | hc2 hr-HPV–negative | Total |
|---|---|---|---|
| RealTime hr-HPV–positive | 81 | 3* | 84 |
| RealTime hr-HPV–negative | 2** | 9*** | 11 |
| Total | 83 | 12 | 95 |

**All three samples tested hr-HPV–positive by the INNO-LiPA HPV Genotyping Extra Test method.*
***Both samples tested hr-HPV–negative using six additional PCR-based HPV methods.*
****8 out of 9 samples tested hr-HPV–negative using six additional PCR-based HPV methods. The presence of HPV-16 in very low quantity was confirmed in one sample.*

using three commercial assays: the Amplicor HPV Test, INNO-LiPA HPV Genotyping *Extra* Test, and Linear Array HPV Genotyping Test, as well as three in-house PCRs amplifying a 188-bp fragment of the HPV E1 gene, 450-bp fragment of the HPV L1 gene, and 150-bp fragment the HPV L1 gene using CPI/CPIIg, PGMY09/PGMY11, and GP5+/GP6+ primers, respectively, as described in detail previously (24, 26, 27).

# Results

## Analytical specificity

All 37 samples with previously determined hc2 hr-HPV false-positive results due to cross-reactivity of the hc2 high-risk probe cocktail with untargeted low-risk HPV genotypes tested as hr-HPV–negative using RealTime. The results obtained with RealTime were in full concordance with previously obtained hr-HPV–negative results using the Amplicor HPV Test, INNO-LiPA HPV Genotyping *Extra* Test, and Linear Array HPV Genotyping Test in the same collection of samples.

## Clinical sensitivity for cervical carcinoma

The results of comparative evaluation of clinical sensitivity of RealTime and hc2 for histologically confirmed cervical carcinoma performed on 95 archived routine cervical specimens are summarized in Table 1. As shown in Table 1, concordant results were obtained from 90 out of 95 samples (94.7% agreement). Nine samples with concordant RealTime and hc2 HPV-negative results were additionally tested using three commercial HPV assays and three in-house PCRs, and 8 out of 9 samples tested HPV-negative using all six additionally applied PCR-based HPV methods. The presence of HPV-16 in very low quantity was confirmed in one sample with concordant HPV-negative results using the INNO-LiPA HPV Genotyping *Extra* Test and Linear Array HPV Genotyping Test. As shown in Table 1, three samples tested RealTime-positive and hc2-negative. In all three RealTime-positive/hc2-negative samples, the presence of hr-HPV genotypes was confirmed using the INNO-LiPA HPV Genotyping *Extra* Test: two samples contained HPV-16 (both RealTime-positive for HPV-16 only) and one sample contained HPV-51 (RealTime-positive for other hr-HPVs). Thus, all three RealTime-positive/hc2-negative samples were considered analytically and clinically hc2 hr-HPV false-negative. As shown in Table 1, two samples tested RealTime-negative and hc2-positive. In both RealTime-negative/hc2-positive samples, the presence of any hr-HPV genotypes could not be confirmed by six additionally applied PCR-based HPV methods. According to our interpretation criteria, both RealTime-negative/hc2-positive samples were considered analytically true-negative samples; however, according to the clinical diagnosis, clinically false-negative. Taking all results together, the clinical sensitivity of RealTime and hc2 for cervical cancer in the total study cohort was 88.4% (95% confidence interval (CI): 80.3–93.6%) and 87.4% (95% CI: 79.0–92.8%), respectively, and analytical sensitivity of RealTime and hc2 for samples containing at least one targeted hr-HPV (after exclusion of 10 samples in which the presence of hr-HPV DNA could not be detected using any of six additionally applied PCR-based HPV methods) was 98.8% (95% CI: 93.0–100.0%) and 95.3% (95% CI: 88.2–98.5%), respectively.

*Table 2. Results of comparative evaluation of RealTi*m*e and hc2 on 267 archived routine cervical specimens collected from women with histologically confirmed CIN3 lesions.*

| | hc2 hr-HPV–positive | hc2 hr-HPV–negative | Total |
|---|---|---|---|
| RealTi*m*e hr-HPV–positive | 233 | 12* | 245 |
| RealTi*m*e hr-HPV–negative | 5** | 17*** | 22 |
| Total | 238 | 29 | 267 |

**All 12 samples tested positive by the INNO-LiPA HPV Genotyping Extra Test method.*
***2 samples hr-HPV–positive and 3 samples hr-HPV–negative in discordant testing.*
****10 out of 17 samples tested HPV-negative using six additional PCR-based HPV methods and the presence of at least one targeted hr-HPV genotype was detected in 7 samples.*

## *Clinical sensitivity for CIN3 lesions*

The results of comparative evaluation of the clinical sensitivity of RealTi*m*e and hc2 for histologically confirmed CIN3 lesions performed on 267 archived routine cervical specimens are summarized in Table 2. As shown in Table 2, concordant results were obtained in 250 out of 267 samples (93.6% agreement). Similar to the cervical cancer group, 17 samples with concordant RealTi*m*e and hc2 HPV-negative results were additionally tested using three commercial HPV assays and three in-house PCR methods. Ten out of 17 samples tested HPV-negative using all six additional PCR-based HPV methods. The presence of at least one targeted hr-HPV genotype was determined in the remaining 7 samples: HPV-51 and HPV-33 in two samples each and HPV-16, HPV-35, and HPV-39+HPV-6 in one sample each. Interestingly, 4 out of 7 samples containing at least one targeted hr-HPV genotype, which tested negative using RealTi*m*e, showed HPV-specific amplification in late PCR cycles but were determined as RealTi*m*e HPV "not detected" due to the manufacturer's cut-off criteria. As shown in Table 2, 12 samples tested RealTi*m*e-positive and hc2-negative. In all 12 RealTi*m*e-positive/hc2-negative samples, the presence of hr-HPV genotypes was confirmed using the INNO-LiPA HPV Genotyping *Extra* Test: seven samples contained HPV-16, two samples contained HPV-51, and one sample each contained HPV-18, HPV-52, and HPV-31+HPV-33. The RealTi*m*e assay accurately grouped HPV genotypes into HPV-16, HPV-18, and other hr-HPV groups in all 12 samples. According to our interpretation criteria, all 12 RealTi*m*e-positive/hc2-negative samples were considered analytically and clinically hc2 hr-HPV false-negative. As shown in Table 2, five samples tested RealTi*m*e-negative and hc2-positive. Further testing of discordant samples revealed the presence of targeted hr-HPV in two samples containing HPV-31 and HPV-58 each and the presence of untargeted low-risk HPV-67 in two samples, and in one sample HPV was not detected by any of the six additional PCR-based HPV methods. According to our interpretation criteria, two out of five RealTi*m*e-negative/hc2-positive samples were considered analytically and clinically RealTi*m*e false-negative, two samples were interpreted to be hc2 analytically false-positive but clinically true-positive, and one sample was considered analytically RealTi*m*e true-negative – but according to the clinical diagnosis as clinically RealTi*m*e false-negative. Taking all of the results together, the clinical sensitivity of RealTi*m*e and hc2 for CIN3 lesions in the total study cohort was 91.8% (95% CI: 87.8–94.5%) and 89.1% (95% CI: 84.8–92.3%), respectively, and analytical sensitivity of RealTi*m*e and hc2 for samples containing at least one targeted hr-HPV (after exclusion of 13 samples in which the presence of hr-HPV DNA could not be detected with any of six additionally applied PCR-based HPV methods) was 96.4% (95% CI: 93.3–98.2%) and 92.5% (95% CI: 88.5–95.2%), respectively.

## *Discussion*

Detection of hr-HPV has recently become an important part of cervical carcinoma screening and detection algorithms (11, 12). Most hr-HPV infections are spontaneously cleared by the host immune system and only about 3 to 10% of hr-HPV–infected women eventually develop cervical cancer or other carcinomas in the anogenital region (4). As a result, a clinically useful HPV test needs to be designed with optimal clinical sensitivity and specificity in order to effectively

detect high-grade cervical disease and at the same time minimize the number of positives associated with highly prevalent transient HPV infections (19, 28). The recently developed RealTi*m*e test is the first commercially available test that utilizes real-time amplification technology to detect 14 hr-HPV types and concurrently type HPV-16 and HPV-18. It was designed to meet emerging needs in cervical carcinoma screening and management of cervical precancerous lesions. Namely, although for almost two decades we have mainly relied on HPV DNA-based screening tests capable of detecting hr-HPV infection (hc2 being a prototype of such tests), recent studies have clearly shown that HPV-16 and HPV-18 are remarkably powerful human carcinogens that are different from other hr-HPV genotypes in a way that merits separate laboratory and clinical consideration. HPV-16 and HPV-18 showed significantly lower clearance rates in comparison with other hr-HPV (29) and remarkable enrichment in high-grade cervical lesions and cancer (30). Thus, infection with HPV-16 and HPV-18 together can be identified in 2 to 4% of women with normal cytology, 6 to 27% of ASC-US lesions, 16 to 32% of low-grade squamous intraepithelial lesions (LSIL), 41 to 67% of high-grade squamous intraepithelial lesions (HSIL), and 65 to 75% of cervical carcinoma (30). A recent study of 1,213 cases of carcinoma in situ and 808 invasive cervical cancers diagnosed from 1980 to 1999 in New Mexico (USA) showed that the age at diagnosis of HPV-16 and HPV-18–related cancers was 5 years earlier than that of cancers caused by other hr-HPV genotypes (48.1 years [46.6–49.6 years] vs. 52.3 years [50.0–54.6 years]) (31). However, the most striking evidence for the remarkably powerful carcinogenic potential of HPV-16 and HPV-18 comes from three prospective studies measuring cumulative incidence of CIN2+ lesions, which showed the significantly higher progression risks associated with HPV-16 and HPV-18 in comparison to other hr-HPV in women with normal cytology in a general screening population (32–34). Based on these data, the American Society for Colposcopy and Cervical Pathology updated their 2006 Consensus Guidelines in March 2009, recommending that for cytology-negative women 30 years and older that are hr-HPV DNA-positive (for any of the hr-HPV genotypes) molecular genotyping assays that detect HPV-16 and HPV-18 would be clinically useful for determining which women should be referred for immediate colposcopy (HPV-16 or HPV-18–positive), and which could be followed-up with repeat cytology and hr-HPV testing in 12 months (hr-HPV–positive but HPV-16 or HPV-18–negative).

Although hc2 is the most widely used HPV test and many studies have demonstrated that hc2 is a sensitive and reliable test for detecting clinically important HPV infections, some recent studies have shown a significant analytical inaccuracy of hc2, mainly due to the cross-reactivity of its probe cocktails. Thus, in 2002, our research group determined the specificity of the hc2 high-risk probe cocktail by genotyping of HPV isolates obtained from 325 women recognized as HPV-positive using the high-risk probe cocktail (35). Genotyping of Slovenian samples showed that, in addition to the genotypes included in the high-risk probe cocktail, the hc2 high-risk probe cocktail detects several other HPV genotypes. These results were also confirmed by other research groups and, currently, it has been established that, in addition to 13 HPV genotypes included in the high-risk probe cocktail, the hc2 high-risk probe cocktail detects at least 28 other HPV genotypes, many of them classified as low-risk HPV genotypes. A recent genotyping study on 3,179 specimens from women participating in the ALTS trial (a clinical trial to evaluate management strategies for women with ASC-US or LSIL) found that 7.8% of all hc2-positive results in this population were false-positive due to the cross-reactivity of hc2 with untargeted, noncarcinogenic HPV genotypes (18). Our research group recently showed that the probability of hc2 false-positive results increased with the proximity to the hc2 cut-off value (17). According to these results, we recommended the introduction of an hc2 gray zone and retesting of samples with repeatedly borderline hc2 results (samples with RLU/CO values between 1.0 and 4.0) by an alternate detection method (17). At present, in Slovenia we routinely test all cervical samples for HPV using the hc2 high-risk cocktail. The samples with hc2 RLU/CO values less than 1.0 are considered hr-HPV DNA-negative and samples with hc2 RLU/CO values greater than 4.0 are considered hr-HPV DNA-positive, without any additional testing. In contrast to the manufacturer's recommendations, but based on our findings, all samples in the hc2 RLU/CO ranging between 1.1 and 4.0 (approximately 5% of all samples) are additionally tested using the Amplicor HPV Test (27), and final hr-HPV results are released according to the results of the Amplicor HPV Test (17). Our unique routine testing strategy and the fact that analytical inaccuracy of hc2 is currently accepted as the main disadvantage of the hc2 test were the main reasons why we analytically evaluated RealTi*m*e only on the subset of 37 samples with previously determined hc2 false-positive results. As expected, all 37 samples with previously determined hc2 hr-

HPV false-positive results due to cross-reactivity of the hc2 high-risk probe cocktail with untargeted low-risk HPV genotypes tested negative for 14 hr-HPV using RealTi*m*e, confirming the excellent analytical specificity of this test established in a previous study (19).

High clinical sensitivity for cervical carcinoma and CIN3 lesions is one of the main comparative advantages of HPV tests over cytology. Two studies have been published in peer-reviewed journals to date evaluating the clinical sensitivity of RealTi*m*e for cervical carcinoma or CIN3 lesions (20, 21), both of which showed higher clinical sensitivity than what was observed here. In one of the studies, the clinical sensitivity of RealTi*m*e was evaluated on archived cervical specimens from Amsterdam and the presence of hr-HPV was detected in 97.2% (246/253) and 98.5% (335/340) of histologically confirmed CIN3 and cancer specimens, respectively (20). In the other study, clinical sensitivity for CIN3+ specimens from a colposcopy referral population due to abnormal cytology was 100% (21). Although virtually all cervical carcinomas and CIN3 lesions should be hr-HPV–positive (5, 35), such a high detection rate is rarely achieved in the "real world" situation due to several (mainly technical) problems, including specimen quality. Thus the most probable reason for lower sensitivity in our study is suboptimal quality of some of our specimens. The specimens included in our study were not collected in strictly controlled clinical conditions (as is usually done in clinical trials), but were archived routine cervical specimens selected for this study after linkage with the Slovenian national registry of CIN3 and cervical cancer to obtain histology data. Samples were collected by approximately 70 different public or private practices for various reasons. Similar to the majority of other countries, no organized quality control program of sample collection exists in Slovenia. Because 18 out of 26 (69.2%) specimens that were hr-HPV–negative using both RealTi*m*e and hc2 were also HPV-negative using six additionally applied PCR-based HPV methods, we believe that the suboptimal quality of these specimens was the most probable reason for clinically false-negative results. Another possible reason for the lack of detection for CIN3 and cancer specimens is that some of the specimens had very low levels of HPV DNA despite having high-grade disease. Among 26 specimens negative by both tests, 6 specimens had HPV specific amplification in the RealTi*m*e test with a cycle number beyond the assay cut-off cycle, indicating the presence of very low levels of HPV target that may not be reliably detected. In addition histological misclassification is also a possible reason for the discrepancy of histology and hr-HPV results in 26 samples. Although histology is performed exclusively by certified specialists in clinical pathology in Slovenia and there is an organized national quality control program for cervical pathology and cytology, a certain degree of inter-observer variability is always present even in the best pathology departments (36, 37). An additional histological review of tissue slides in patients that tested HPV DNA-negative could resolve the dillema, but due to strict standards of patients' medical records protection in our country we were not allowed to perform such an additional review.

Several previously published studies comparatively evaluated clinical and analytical sensitivity of the FDA-approved hc2 test with other non-FDA-approved PCR-based HPV assays introduced to the diagnostic market in the past few years (28). As shown in Tables 1 and 2, concordance between hc2 and RealTi*m*e was similar to leveles obtained between hc2 and the majority of other non-FDA-approved PCR-based assays evaluated to date (28). Testing of samples with discordant results showed that all 15 RealTi*m*e-positive/hc2-negative samples (Tables 1 and 2) represent hc2 false-negative results. By additional testing of seven samples which tested RealTi*m*e negative and hc2 positive, targeted hr-HPV was detected in two samples, untargeted low-risk HPV-67 was found in two samples, and no HPV of any genotype could be identified in three samples. Our study confirms that, paradoxically, some level of analytical inaccuracy of the HPV test (in this case of hc2) can be clinically beneficial. Namely, although detecting low-risk HPV-67 in two samples using the hc2 high-risk cocktail should be considered an analytically inaccurate result, cross-reactivity of the hc2 high-risk probe cocktail with untargeted low-risk HPV genotypes (described in detail above) helped hc2 identify two women with CIN3 lesion.

In conclusion, in our hands the RealTi*m*e assay showed excellent analytical specificity and the absence of cross-reactivity with untargeted HPV genotypes that had resulted in hc2 false-positive results. The clinical sensitivity of RealTi*m*e evaluated on 362 archived routine cervical specimens collected from women with histologically confirmed cervical carcinoma or CIN3 lesions was comparable to hc2. The RealTime assay, designed to detect 14 hr-HPV genotypes and concurrently type HPV-16 and HPV-18 within a single test, is an important new diagnostic tool in cervical carcinoma screening and management of cervical precancerous lesions.

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## REFERENCES

1. zur Hausen H, de Villiers EM, Gissmann L. Papillomavirus infections and human genital cancer. Gynecol Oncol. 1981;12:S124–8.

2. de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H. Classification of papillomaviruses. Virology. 2004;324:17–27.

3. Bernard HU. The clinical importance of the nomenclature, evolution and taxonomy of human papillomaviruses. J Clin Virol. 2005;32(suppl 1):S1–6.

4. Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med. 2003;348:518–27.

5. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Muñoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol. 1999;189:12–9.

6. Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, Clifford GM. Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. Int J Cancer. 2007;121:621–32.

7. Hobbs CG, Sterne JA, Bailey M, Heyderman RS, Birchall MA, Thomas SJ. Human papillomavirus and head and neck cancer: a systematic review and meta-analysis. Clin Otolaryngol. 2006;31:259–66.

8. Backes DM, Kurman RJ, Pimenta JM, Smith JS. Systematic review of human papillomavirus prevalence in invasive penile cancer. Cancer Causes Control. 2009;20:449–57.

9. De Vuyst H, Clifford GM, Nascimento MC, Madeleine MM, Franceschi S. Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis. Int J Cancer. 2009;124:1626–36.

10. Smith JS, Backes DM, Hoots BE, Kurman RJ, Pimenta JM. Human papillomavirus type-distribution in vulvar and vaginal cancers and their associated precursors. Obstet Gynecol. 2009;113:917–24.

11. Cuschieri KS, Cubie HA. The role of human papillomavirus testing in cervical screening. J Clin Virol. 2005;32(suppl 1):S34–42.

12. Cuzick J, Arbyn M, Sankaranarayanan R, Tsu V, Ronco G, Mayrand MH, Dillner J, Meijer CJ. Overview of human papillomavirus-based and other novel options for cervical cancer screening in developed and developing countries. Vaccine. 2008;26(suppl 10):K29–41.

13. Smith RA, Cokkinides V, Eyre HJ, American Cancer Society. American Cancer Society guidelines for the early detection of cancer, 2003. CA Cancer J Clin. 2003;53:27–43.

14. Wright TC Jr, Cox JT, Massad LS, Twiggs LB, Wilkinson EJ, and ASCCP-Sponsored Consensus Conference. 2001 Consensus guidelines for the management of women with cervical cytological abnormalities. JAMA. 2002;287:2120–9.

15. Poljak M, Brenčič A, Seme K, Vince A, Marin IJ. Comparative evaluation of first- and second-generation digene hybrid capture assays for detection of human papillomaviruses associated with high or intermediate risk for cervical cancer. J Clin Microbiol. 1999;37:796–7.

16. Poljak M, Marin IJ, Seme K, Vince A. Hybrid capture II HPV test detects at least 15 human papillomavirus genotypes not included in its current high-risk probe cocktail. J Clin Virol. 2002;25(suppl 3):S89–97.

17. Seme K, Fujs K, Kocjan BJ, Poljak M. Resolving repeatedly borderline results of Hybrid Capture 2 HPV DNA Test using polymerase chain reaction and genotyping. J Virol Methods. 2006;134:252–6.

18. Castle PE, Solomon D, Wheeler CM, Gravitt PE, Wacholder S, Schiffman M. Human papillomavirus genotype specificity of hybrid capture 2. J Clin Microbiol. 2008;46:2595–604.

19. Huang S, Tang N, Mak W-B, Erickson B, Salituro J, Li Y, Krumpe E, Schneider G, Yu H, Robinson J, Abravaya K. Principles and analytical performance of Abbott RealTime HR HPV test. J Clin Virol. 2009;45(suppl 1):S13–7.

20. Tang N, Huang S, Erickson B, Mak W-B, Salituro J, Robinson J, Abravaya K. High-risk HPV detection and concurrent HPV 16 and 18 typing with Abbott RealTime High Risk HPV test. J Clin Virol. 2009;45(suppl 1):S25–9.

21. Huang S, Erickson B, Tang N, Mak W-B, Salituro J, Robinson J, Abravaya K. Clinical performance of Abbott RealTime High Risk HPV test for detection of high-grade cervical intraepithelial neoplasia in women with abnormal cytology. J Clin Virol. 2009;45(suppl 1):S19–23.

22. Kocjan BJ, Seme K, Poljak M. Detection and differentiation of human papillomavirus genotypes HPV-6 and HPV-11 by FRET-based real-time PCR. J Virol Methods. 2008;153:245–9.

23. Poljak M, Kocjan BJ, Kovanda A, Lunar MM, Židovec Lepej S, Planinić A, Seme K, Vince A. Human papillomavirus genotype specificity of Hybrid Capture 2 low-risk probe cocktail. J Clin Microbiol. 2009; 47:2611–5.

24. Jančar N, Kocjan BJ, Poljak M, Lunar MM, Vrtačnik-Bokal E. Distribution of human papillomavirus genotypes in women with cervical cancer in Slovenia. Eur J Obstet Gynecol Reprod Biol. 2009;145:184–8.

25. Kleter B, van Doorn LJ, Schrauwen L, Molijn A, Sastrowijoto S, ter Schegget J, Lindeman J, ter Harmsel B, Burger M, Quint W. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. J Clin Microbiol. 1999;37:2508–17.

26. Kovanda A, Juvan U, Šterbenc A, Kocjan BJ, Seme K, Jančar N, Vrtačnik Bokal E, Poljak M. Pre-vaccination distribution of human papillomavirus (HPV) genotypes in women with grade 3 cervical intraepithelial neoplasia (CIN 3) lesions in Slovenia. Acta Dermatovenerol Alp Pannonica Adriat. 2009; 18:47–52.

27. Poljak M, Fujs K, Seme K, Kocjan BJ, Vrtačnik-Bokal E. Retrospective and prospective evaluation of the Amplicor HPV test for detection of 13 high-risk human papillomavirus genotypes on 862 clinical samples. Acta Dermatovenerol Alp Pannonica Adriat. 2005;14:147–52.

28. Meijer CJ, Berkhof J, Castle PE, Hesselink AT, Franco EL, Ronco G, Arbyn M, Bosch FX, Cuzick J, Dillner J, Heideman DA, Snijders PJ. Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older. Int J Cancer. 2009;124:516–20.

29. Bulkmans NW, Berkhof J, Bulk S, Bleeker MC, van Kemenade FJ, Rozendaal L, Snijders PJ, Meijer CJ; POBASCAM Study Group. High-risk HPV type-specific clearance rates in cervical screening. Br J Cancer. 2007;96:1419–24.

30. Bosch FX, Burchell AN, Schiffman M, Giuliano AR, de Sanjose S, Bruni L, Tortolero-Luna G, Kjaer SK, Muñoz N. Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. Vaccine. 2008;26(suppl 10):K1–16.

31. Wheeler CM, Hunt WC, Joste NE, Key CR, Quint WG, Castle PE. Human papillomavirus genotype distributions: implications for vaccination and cancer screening in the United States. J Natl Cancer Inst. 2009;101:475–87.

32. Khan MJ, Castle PE, Lorincz AT, Wacholder S, Sherman M, Scott DR, Rush BB, Glass AG, Schiffman M. The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. J Natl Cancer Inst. 2005;97:1072–9.

33. Bulk S, Bulkmans NW, Berkhof J, Rozendaal L, Boeke AJP, Verheijen RH, Snijders PJ, Meijer CJ. Risk of high-grade cervical intra-epithelial neoplasia based on cytology and high-risk HPV testing at baseline and at 6 months. Int J Cancer. 2007;121:361–7.

34. Winer RL, Kiviat NB, Hughes JP, Adam DE, Lee SK, Kuypers JM, Koutsky LA. Development and duration of human papillomavirus lesions, after initial infection. J Infect Dis. 2005;191:731–8.

35. Bohmer G, van den Brule AJ, Brummer O, Meijer CL, Petry KU. No confirmed case of human papillomavirus DNA-negative cervical intraepithelial neoplasia grade 3 or invasive primary cancer of the uterine cervix among 511 patients. Am J Obstet Gynecol. 2003;189: 118–20.

36. Castle PE, Stoler MH, Solomon D, Schiffman M. The relationship of community biopsy-diagnosed cervical intraepithelial neoplasia grade 2 to the quality control pathology-reviewed diagnoses: an ALTS report. Am J Clin Pathol. 2007;127:805–15.

37. Stoler MH, Schiffman M. Atypical Squamous Cells of Undetermined Significance-Low-Grade Squamous Intraepithelial Lesion Triage Study (ALTS) Group. Interobserver reproducibility of cervical cytologic and histologic interpretations: realistic estimates from the ASCUS-LSIL Triage Study. JAMA. 2001;285:1500–5.

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