Diagnostic Microbiology and Infectious Disease 95 (2019) 46–54

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Principles and analytical performance of Papilloplex® HR-HPV, a new commercial CE-IVD molecular diagnostic test for the detection of high-risk HPV genotypes

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ARTICLE INFO

Article history:
Received 23 September 2018
Received in revised form 15 April 2019
Accepted 15 April 2019
Available online 25 April 2019

Keywords:
HPV
Multiplex real-time PCR
Cervical intraepithelial neoplasia
Molecular diagnostics

ABSTRACT

The accurate detection and genotyping of high-risk human papillomavirus (HR-HPV) are critical for cervical cancer screening and epidemiological investigations. GeneFirst Papilloplex® HR-HPV is a new CE-IVD–marked real-time PCR test based on patented multiplex probe amplification technology. Papilloplex® HR-HPV provides the simultaneous detection and differentiation of 14 HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68a/b) in a single closed-tube reaction ensuring rapid, cost-effective, and contamination-free results. In this study, the analytical performance characteristics in terms of the assay's sensitivity, specificity, range, reproducibility, and cross-reactivity were evaluated. Papilloplex® HR-HPV provided sensitive detection and differentiation of 14 HR-HPV types with highly reproducible results. The differential HR-HPV specificity and sensitivity were further confirmed through the participation in the WHO HPV Laboratory Network Proficiency Study (2014). Overall, GeneFirst Papilloplex® HR-HPV assay demonstrated a robust analytical performance with reproducible and reliable results in the detection of HR-HPV genotypes.

1. Introduction

Human papillomavirus (HPV) is a group of more than 250 related DNA viruses with varied oncogenic potential (Braaten and Laufer, 2008; Castle et al., 2005; Crosbie et al., 2013; de Villiers, 2013; Lindemann et al., 2012). HPV types are categorized into high-risk HPV (HR-HPV), associated with cervical neoplasia and a subset of anogenital and oropharyngeal cancers (Crosbie et al., 2013; Lindemann et al., 2012), and low-risk HPV (LR-HPV) genotypes, associated with benign lesions including genital warts (Braaten and Laufer, 2008; Munoz et al., 2003). HPV infection is the most common sexually transmitted infection worldwide, and most sexually active individuals of both sexes will acquire it at some point during their life (Dunne et al., 2007).

The prevalence of HPV includes a mix of incident and persistent infections that have accumulated over time due to lack of clearance (Castle et al., 2005; Munoz et al., 2004). Although >90% of HPV infections regress in 6–18 months (Castle et al., 2009), persistent infection with HR-HPV is a prerequisite for progression to cervical intraepithelial neoplasia (CIN) and cervical cancer. HR-HPV genotypes including 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 types have been detected in >99% of cervical cancers worldwide (Walboomers et al., 1999), and recent meta-analysis studies investigating the cross-sectional distribution of HR-HPV types across cytopathological and histopathological cervical diagnoses have reported that the most common HPV types in cervical cancer are types 16 (57%), 18 (16%), 58 (5%), 33 (5%), 45 (5%), 31 (4%), 52 (3%), and 35 (2%) (Guan et al., 2012; Li et al., 2011).

The implication of HR-HPV infections in human carcinogenesis underlies the clinical importance of sensitive diagnostic tests that offer differential detection of HR-HPV DNA genotypes. Application of these assays helps to determine the effectiveness of HPV vaccinations and improve risk stratification of patients with HPV in cervical screening programs. In addition, the clinical importance of highly sensitive and accurate HPV diagnostic tests is further highlighted by recent guidelines that encourage the replacement of cytology [*Papanicolaou* (Pap) smear test, the current gold standard] with HPV testing as the primary screening tool for cervical cancer screening (von Karsa et al., 2015). In fact, European countries including the Netherlands and Norway have recently implemented a cervical screening program which has fully transitioned from Pap smear test to primary HPV screening (Dijkstra et al., 2016).

Abbreviations: CIN, cervical intraepithelial neoplasia; CLSI, Clinical and Laboratory Standards Institute; Ct, cycle threshold; gDNA, genomic DNA; HPV, human papillomavirus; HR-HPV, high-risk HPV; IVD, *in vitro* diagnostic medical devices; LabNet, Laboratory Network; LR-HPV, low-risk HPV; LoD, limit of detection; T_m, melting temperature; MPA, multiplex probe amplification; NC, negative control; NTC, no template control; Pap, Papanicolaou; PC, positive control; THO, target-hybridizing oligonucleotide; % CV, % coefficient of variation.

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https://doi.org/10.1016/j.diagmicrobio.2019.04.005
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Although HPV tests are among the least clinically validated *in vitro* diagnostic medical devices (IVD) (Meijer et al., 2009), more than 125 HPV assays have been developed within the last 10 years, which utilize a variety of technologies including real-time PCR, Luminex, microarrays, and DNA hybridisation (Arbyn et al., 2015). Recently, a new HR-HPV genotyping assay based on multiplex probe amplification (MPA) technology, the Papilloplex® HR-HPV test developed by GeneFirst Ltd., has become commercially available and has achieved the European Community marking for *in vitro* diagnostic devices (CE-IVD). The Papilloplex® test is a real-time PCR test that provides the simultaneous detection and differentiation of all 14 HR-HPV genotypes in a single closed-tube reaction. The assay provides unambiguous results in short test time while reducing the risk of contamination and maintaining compatibility with widely available research and diagnostic real-time PCR platforms. The test also features an internal human DNA control reaction for specimen adequacy and quality of DNA extraction. In addition, external positive and negative controls are included in each run to assess run validity.

Preliminary clinical performance studies, using cervical liquid-based cytology samples with known clinical outcomes, provided compelling evidence that the Papilloplex® HR-HPV test results are comparable to well-established clinically validated HPV genotyping assays including the Hybrid Capture 2 HPV DNA Test™ (Qiagen), the RealTime High Risk HPV test (Abbott), the Linear Array® HPV test (Roche), and the Optiplex HPV genotyping assay (formerly the Multiplex HPV genotyping kit; DiaMex) (Bhatia et al., 2018).

In this study, we evaluate and describe the analytical performance characteristics of the Papilloplex® HR-HPV assay in terms of the assay's sensitivity, specificity, assay range, reproducibility, and the potential for interference with LR-HPV types or microorganisms/viruses that may be present in the female genital and urinary tract. In addition, the analytical sensitivity and differential HR-HPV specificity of the assay through the participation in the WHO HPV Laboratory Network (LabNet) Proficiency Study (2014) (Eklund et al., 2018) which covers all 14 HR-HPV types addressed by the Papilloplex® HR-HPV assay, are also described.

2. Methods and materials

2.1. Plasmid DNA and cell line genomic DNA

Linearized plasmid DNAs containing partial sequences of the L1 gene of the HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and LR-HPV types (6, 10, 11, 26, 30, 32, 34, 40, 53, 54, 57, 61, 67, 69, 70, 73, 82, 85, 90, and 97) were synthesized by GenScript (Piscataway, USA). Genomic DNA (gDNA) extracted from non-HPV organisms (*Candida albicans, Escherichia coli, Chlamydia trachomatis, Gardnerella vaginalis, Lactobacillus acidophilus, Mycoplasma hominis. Neisseria gonorrhoeae, Staphylococcus aureus, Trichomonas vaginalis*, and *Human herpesvirus 2*) was purchased from LGC (Middlesex, UK). Copy number calculation was performed using the ThermoFisher DNA Copy Number and Dilution Calculator (www.thermofisher.com). Linearized plasmids stocks' concentrations were calculated based on GenScript synthesis data and compared to commercially available HPV reference standards (ATCC, Manassas, USA) with known target copy numbers using quantitative PCR (qPCR). Serial 10-fold dilutions were undertaken to produce a titration series representing 10^6 to 10^2 copies/μL for plasmid DNA and 15,000 copies/μL for gDNA. To minimize changes in HPV plasmid concentrations or gDNA due to DNA adhesion to plastic tubes' walls, stock dilution and subsequent working dilutions were prepared in dH_2O alongside a constant background of a DNA sodium salt from *Escherichia coli* strain B (Sigma, UK) at 2.5 μg mL^{-1}.

2.2. Principles of MPA technology

The principles of MPA technology and the design of fluorescent probes with unique melting properties were recently described in detail by Fu et al. (2012). In brief, as in conventional real-time PCR, each amplification product is detected by a fluorescently labeled, target-hybridizing oligonucleotide. This allows differential identification of only 1 target per fluorescence channel. MPA technology allows differential identification of up to 6 targets per channel by examining the oligonucleotide dissociation curve after amplification. Each target hybridizing oligonucleotide (THO) has a partially complementary oligonucleotide, and each probe pair has a unique melting temperature that creates a characteristic peak on the dissociation curve (see Fig. 1). If a target is present, the respective THO is consumed during the reaction, and the characteristic peak disappears. Therefore, amplification of multiple targets within the same fluorescence channel can be differentially identified.

2.3. Papilloplex® HR-HPV probe design and oligonucleotide synthesis

The multiplex PCR system of the Papilloplex® HR-HPV assay includes probes designed to target specific genomic sequences of the L1 gene of HR-HPV DNA types (Table 1). The THOs of the HR-HPV probes were synthesized with a ROX, FAM, or HEX (JOE) label at the 5′ end and a BHQ1 or BHQ2 (Black Hole Quencher 1 or 2, LGC Biosearch, USA) quencher at the 3′ end. An additional probe designed to target a conserved internal human DNA control (IC) region was labeled with a CY5 fluorophore to provide a measure of sample adequacy and of the quality of DNA extraction and amplification.

2.4. Melting profiles of Papilloplex® HR-HPV probes

To distinguish all targets per fluorescent channel, each probe is designed to have a unique melting temperature (T_m) and shape which can be recognized in a melting curve analysis (Fig. 1). Probes labeled with a ROX fluorophore include: HPV 45 plus probe with a T_m of 33.5 ± 1 °C, HPV 51 plus probe with a T_m of 36.2 ± 1 °C, HPV 35 plus probe with a T_m of 42.8 ± 1 °C, HPV 66 plus probe with a T_m of 46.8 ± 1 °C, HPV 56 plus probe with a T_m of 50.3 ± 1 °C, and HPV 33 minus probe with a T_m of 55.8 ± 1 °C (Fig. 1A). Probes labeled with a FAM fluorophore include: HPV 59 plus probe with a T_m of 32.8 ± 1 °C, HPV 31 plus probe with a T_m of 39.5 ± 1 °C, HPV 16 plus probe with a T_m of 46.3 ± 1 °C, HPV 18 plus probe with a T_m of 52.1 ± 1 °C, and HPV 52 minus probe with a T_m of 57.6 ± 1 °C (Fig. 1B). Probes labeled with a HEX fluorophore include: HPV 39 plus probe with a T_m of 37.6 ± 1 °C, HPV 68a plus probe with a T_m of 45.1 ± 1 °C, HPV 68b plus probe with a T_m of 46 ± 1 °C, and HPV 58 minus probe with a T_m of 58.7 ± 1 °C (Fig. 1C). The melting profile obtained per channel is a combination of the melting data generated from all HR-HPV probes included in each given channel (Fig. 1). The distinct change in the characteristic melting profile(s) (red melting curve) in the sample compared to the negative control (NC) reference melting profile (green melting curve) indicates which HR-HPV DNA type(s) is/are present in the sample (Figs. 2-5). Each probe of the Papilloplex® HR-HPV assay detected only the corresponding HR-HPV specific genotype (Supplementary Table 1).

2.5. Assay procedure

Papilloplex® HR-HPV PCR mix consists of a buffer (dNTPs and Mg^{2+}), master mix (Taq polymerase, UNG enzyme, and dUTP), and working mix (primers and probes). Following preparation according to manufacturer's instructions, the PCR amplification reaction mix is aliquoted into a 96-well plate, followed by the addition of plasmid DNA samples (positive control, PC), gDNA, nuclease-free H_2O (no template control, NTC), or TE buffer (NC).

2.6. Real-time PCR instrument settings

Thermocycling, fluorescence detection, and melting profiles were performed on 2 ABI 7500 Fast Real-Time PCR Systems (Applied Biosystems, Warrington, UK). The thermal profile was set to: stage 1 (50 °C for 2 min followed by 95 °C for 3 min), stage 2 (9 cycles of

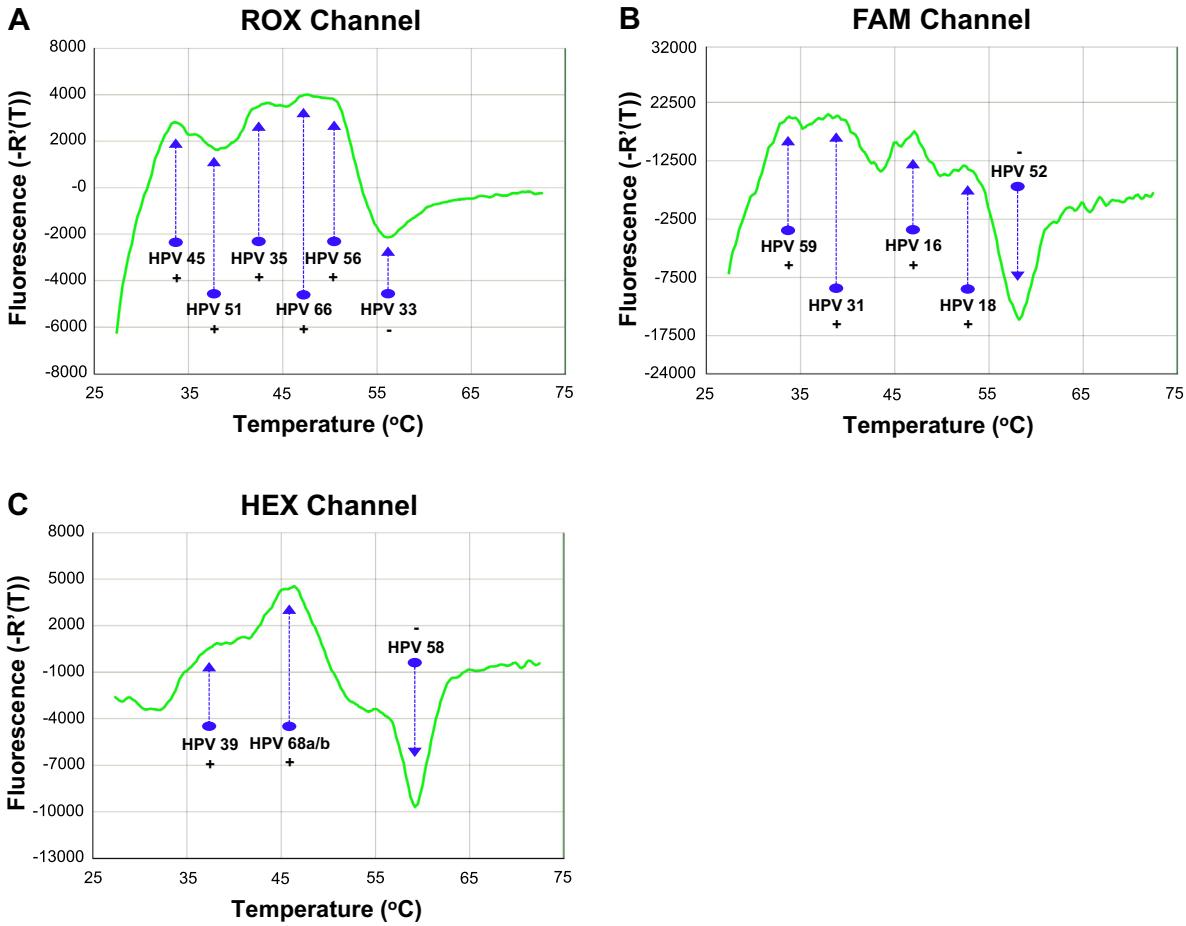

Fig. 1. Design of Papilloplex® HR-HPV specific probes. (**A**) Melting profile of the mix of HR-HPV 45 (+), 51 (+), 35 (+), 66 (+), 56 (+), and 33 (−) probes designed to target specific genomic regions (sequences) of the respective HR-HPV DNA types. Probes were labeled with a ROX fluorophore. (**B**) Melting profile of the mix of HR-HPV 59 (+), 31 (+), 16 (+), 18 (+), and 52 (−) probes designed to target specific DNA sequences of the respective HR-HPV types. Probes were labeled with a FAM fluorophore. (**C**) Melting profile of the mix of HR-HPV 39 (+), 68a (+), 68b (+), and 58 (−) probes designed to target specific sequences of the respective HR-HPV DNA types. Probes were labeled with a HEX fluorophore. All graphs plotted as the negative derivative of the emission reading versus temperature. Note the unique T_m of each probe. "(+)" indicates plus probes; "(−)" indicates minus probes.

95 °C for 6 s followed by 66 °C for 45 s), and stage 3 (42 cycles of 95 °C for 3 s followed by 60 °C for 33 s and 63 °C for 15 s). Fluorescence measurements in the ROX, FAM, HEX, and CY5 channels were recorded during step 2 of amplification stage 3 (60 °C for 33 s). A preset dissociation stage (stage 4) was included following the final PCR cycle of amplification stage 3. The postamplification melting profile protocol comprised of the following conditions: 95 °C for 15 s, followed by 25 °C for 1 min, followed by 75 °C for 15 s and 60 °C for 15 s. The fluorescence emission data were continually collected during the rising temperatures. The negative derivative of the emission reading, with respect to temperature, was plotted against the temperature to form melting curves (per fluorescent channel) generated during the dissociation stage of the reaction (from 25 °C to 75 °C).

2.7. Controls

An NC and a PC are required for each run to verify that the sample processing, amplification, HR-HPV type identification, and detection steps are performed correctly. Both the NC and PC are provided with the Papilloplex® HR-HPV assay.

Table 1
Fluorescent probe labeling.

Fluorescent channel	HR-HPV specific probes					
ROX	33	35	45	51	56	66
FAM	16	18	31	52	59	
HEX	39	58	68a	68b		
CY5	Internal control					

2.8. Amplification and melting profile analysis

The cycle threshold (Ct) values and melting curve profiles per detection channel are automatically reported by the Applied Biosystems® 7500 Fast System SDS Software. For the reaction result to be considered valid, amplification of human DNA in the CY5 channel must have a Ct value <38. In addition, the NC and NTC should not show amplification in any channels, the Ct value should be either undetermined or ≥39 for all detection channels. Samples are considered positive for HR-HPV DNA if the Ct value is <36 in any of the ROX, FAM, and HEX fluorescent channels. Samples which produce either an undetermined or a Ct value >36 in all three HPV channels will have an interpretation of "No HR-HPV Detected." Samples positive for HR-HPV DNA types are processed for genotypic analysis. The change in the characteristic melting profile of the sample compared to the NC reference melting profile indicates the sample to be positive for the respective HR-HPV type(s) (see Figs. 2–5).

2.9. Data analysis and evaluation of Papilloplex® HR-HPV performance characteristics

The guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) document EP05-A3 "Evaluation of Precision of

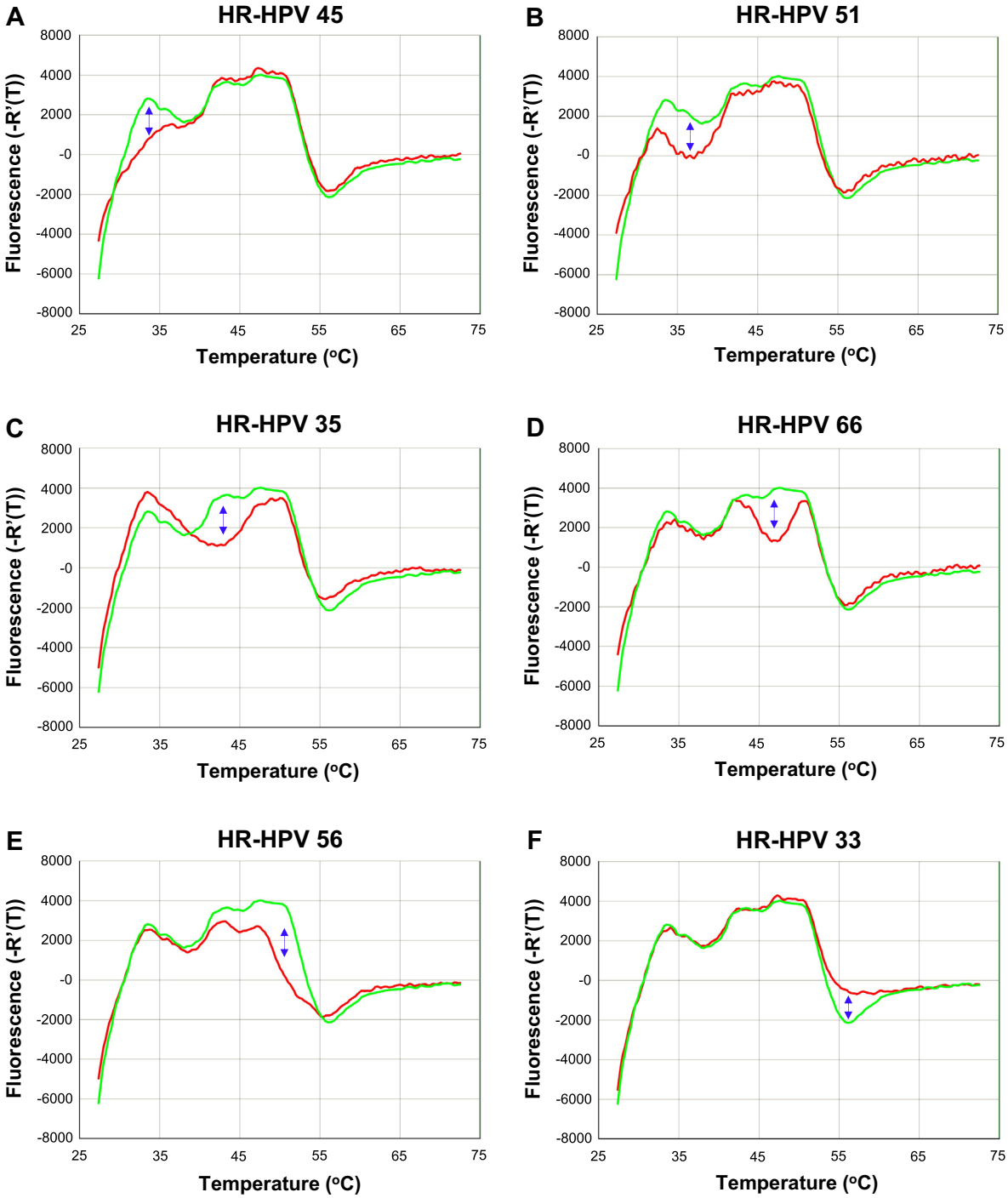

Fig. 2. Melting curve analysis (ROX detection channel) of the amplification reactions of HR-HPV targets. When an HR-HPV target is present in a reaction, its corresponding probe is consumed, and in comparison with the NC, the respective melting peak either is reduced or disappears from the melting curve. The change in the melting peak of a probe indicates which target has been amplified and hence present in a sample. The NC melting curve is marked in green, and the target melting curve (PC) is marked in red. (**A**) HR-HPV 45, (**B**) HR-HPV 51, (**C**) HR-HPV 35, (**D**) HR-HPV 66, (**E**) HR-HPV 56, and (**F**) HR-HPV 33. Note the double-headed arrows which depict the distinct change in the melting profile. HR-HPV DNA plasmids were used at a concentration of 3000 copies per PCR.

Quantitative Measurement Procedures; Approved Guideline-Third Edition. CLSI document EP05-A3. Wayne, PA, CLSI; 2014" were followed to evaluate the analytical performance of the Papilloplex® CE-IVD assay. CLSI document EP05-A3 included guidelines regarding the experimental design, materials, data analysis, summarization, and interpretation of laboratory measurement procedures. The Papilloplex® HR-HPV assay has been designed, optimized, and validated in strict compliance with the MIQE guidelines (Bustin et al., 2009).

2.10. Participation of Papilloplex® HR-HPV in the 2014 HPV LabNet international proficiency study

The differential HR-HPV specificity and analytical sensitivity of the Papilloplex® HR-HPV genotyping assay to concurrently detect and discriminate all 14 HR-HPV DNA types were also validated through the participation in the WHO HPV LabNet Proficiency Study (2014). The test was regarded as proficient in genotyping if it could detect a minimum of 50 international units (IU) of HR-HPV 16 and 18, and 500 genome equivalents

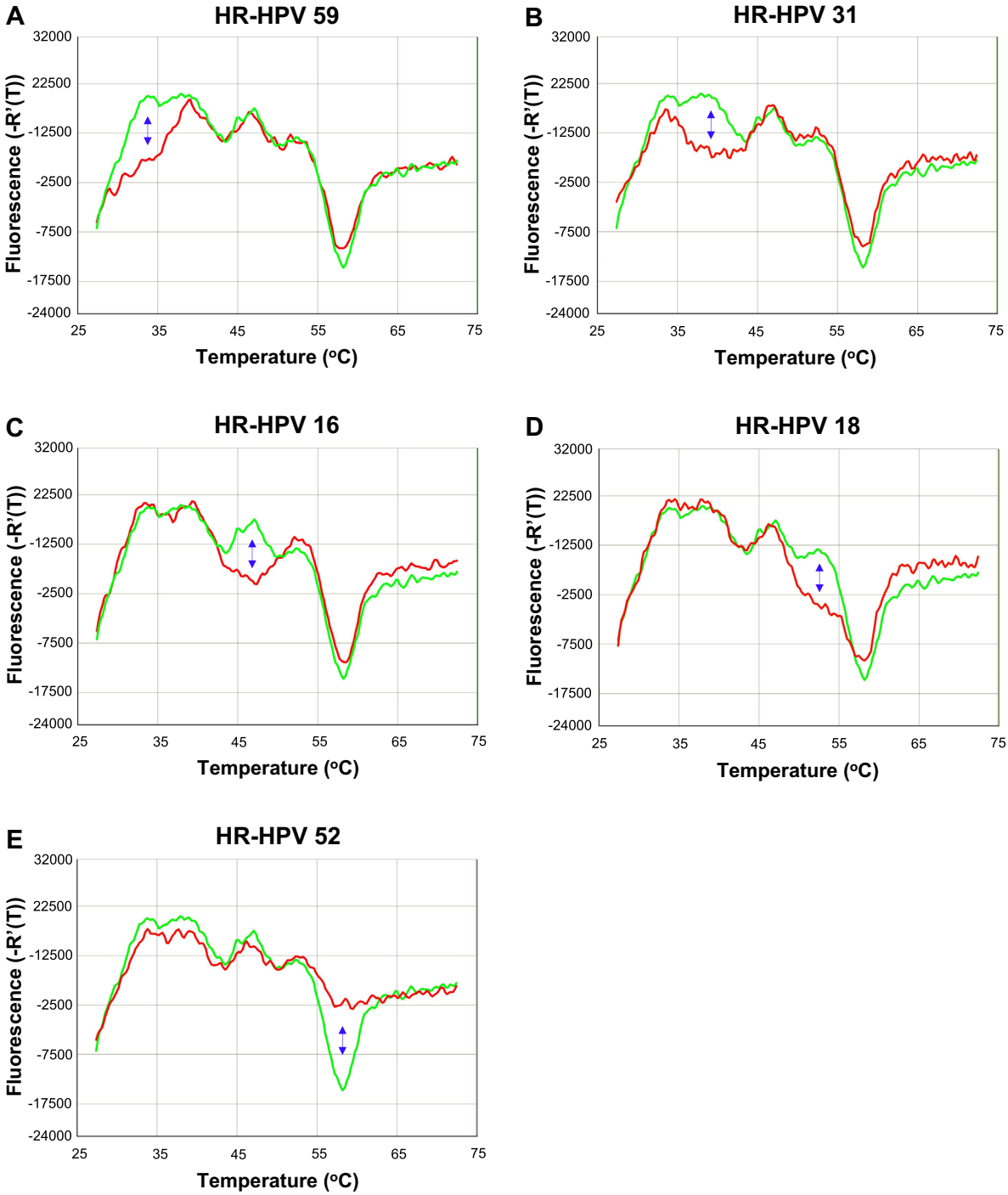

Fig. 3. Melting curve analysis (FAM detection channel) of the amplification reactions of HR-HPV targets. When an HR-HPV target is present in a reaction, its corresponding probe is consumed, and in comparison with the NC, the respective melting peak either is reduced or disappears from the melting curve. The change in the melting peak of a probe indicates which target has been amplified and hence present in a sample. The NC melting curve is marked in green, and the target melting curve (PC) is marked in red. (**A**) HR-HPV 59, (**B**) HR-HPV 31, (**C**) HR-HPV 16, (**D**) HR-HPV 18, and (**E**) HR-HPV 52. Note the double-headed arrows which depict the distinct change in the melting profile. HR-HPV DNA plasmids were used at a concentration of 3000 copies per PCR.

(GE) of the other HPV types (HR-HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) included in the panel in both samples with single and multiple infections, with at least 97% specificity. The study design and findings of the 2014 HPV LabNet study were recently described by Eklund et al. (2018).

2.11. Analytical specificity

A panel of bacteria, viruses, and fungi was evaluated for potential cross-reactivity, including 20 LR-HPV types and other organisms that can be present at the site of clinical sample collection (Supplementary Table 3). HR-HPV DNA plasmids were used at 3000 copies per MPA reaction as PC samples.

2.12. Verification of repeatability

To verify that the Papilloplex® HR-HPV assay conforms to established acceptance criteria for repeatability (Bustin et al., 2009), the assay was assessed on 3 consecutive runs, performed by the same

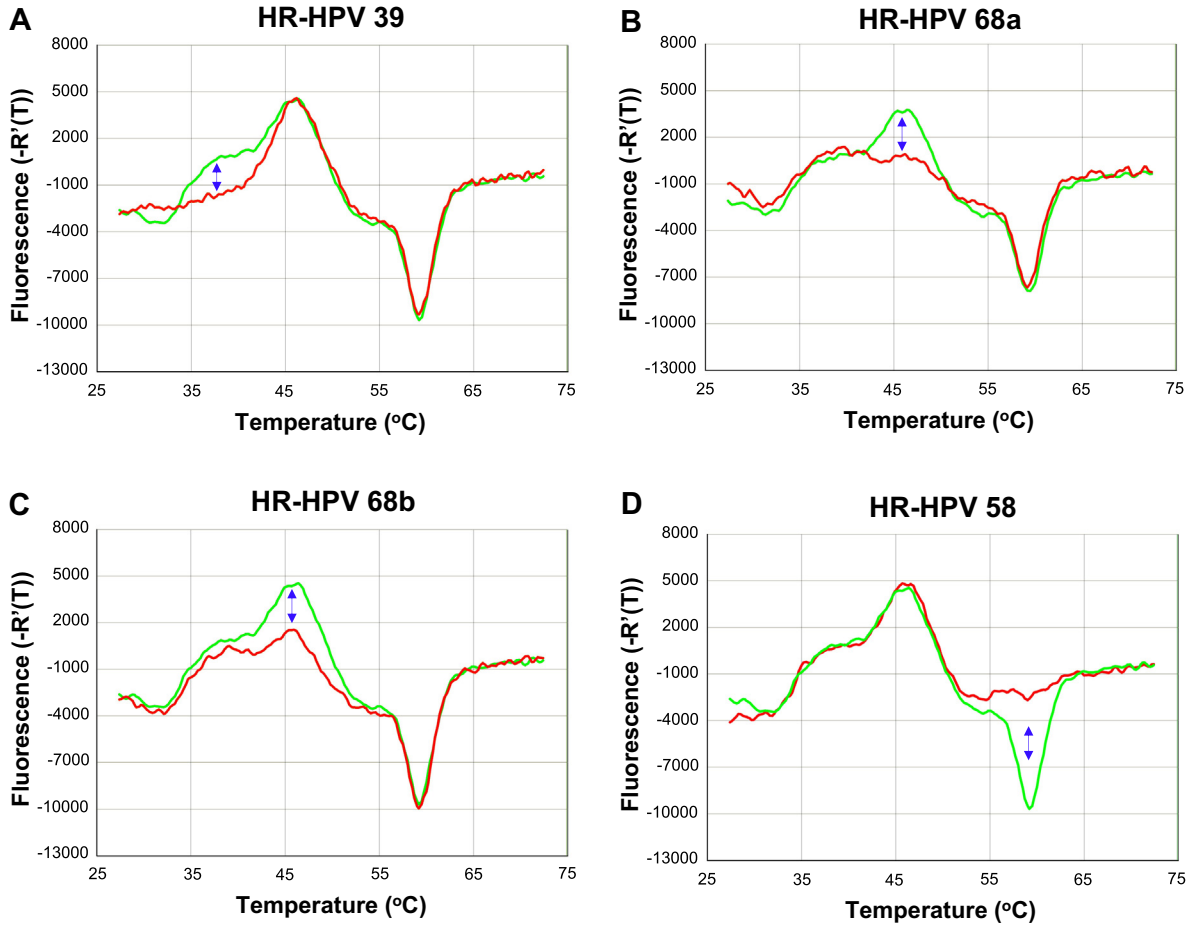

Fig. 4. Melting curve analysis (HEX detection channel) of the amplification reactions of HR-HPV targets. When an HR-HPV target is present in a reaction, its corresponding probe is consumed, and in comparison with the NC, the respective melting peak either is reduced or disappears from the melting curve. The change in the melting peak of a probe indicates which target has been amplified and hence present in a sample. The NC melting curve is marked in green, and the target melting curve (PC) is marked in red. (**A**) HR-HPV 39, (**B**) HR-HPV 68a, (**C**) HR-HPV 68b, and (**D**) HR-HPV 58. Note the double-headed arrows which depict the distinct change in the melting profile. HR-HPV DNA plasmids were used at a concentration of 3000 copies per PCR.

operator on the same ABI 7500 Fast Real-Time PCR System, using the same lot of reagents. All control plasmids were tested at 3 different concentrations: 10^3 (low), 10^4 (medium), and 10^5 (high) copies per reaction, with 5 replicates of each concentration. Pooled genotyping data were analyzed for % of correct HR-HPV types in total and within each run. Variation in Ct values of each sample and dilution was calculated [standard deviation and % coefficient of variation (CV)] and compared against acceptance criteria.

2.13. Verification of reproducibility

To confirm that the Papilloplex® HR-HPV assay conforms to established acceptance criteria for reproducibility (Bustin et al., 2009), the assay was performed for 12 consecutive days, 3 runs per day (36 runs in total), executed by 3 operators on 2 ABI 7500 Fast Real-Time PCR Systems, using 3 production lots of the Papilloplex® HR-HPV assay. Each operator used a combination of reagent lot and instrument and undertook 12 runs in total. The control plasmids were tested at 3 different concentrations: low [1–10 times the limit of detection (LoD), 10^3 copies/reaction], medium (10–100 times the LoD, 10^4 copies/reaction), and high (>100 times the LoD, 10^5 copies/reaction). Pooled genotyping data were analyzed for % of correct HR-HPV types in total and within each variability group. The % agreement between variability groups was also calculated. Variation in Ct values of each sample within and between each variability group was assessed (standard deviation and % CV) and compared against the acceptance criteria.

3. Results

3.1. Differential detection of 14 HR-HPV types within a single-tube real-time PCR

Each HR-HPV type was successfully detected and differentiated by the Papilloplex® HR-HPV assay (Figs. 2–4). Each probe of the Papilloplex® HR-HPV assay detected only the specific HR-HPV genotype, with no cross-reactivity (Supplementary Table 1). HR-HPV type identification and differentiation were also successful in test samples containing multiple HR-HPV types detected within the same channel even when the characteristic peaks of these types were adjacent (Fig. 5).

3.2. Analytical sensitivity (LoD and assay range)

The claimed LoD per MPA reaction at which ≥95% of replicates provided a positive result (≥95% accuracy) was 100 copies for HR-HPV 16 and 18 and 1000 copies per MPA reaction for HR-HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68a/b. The analytical sensitivity and differential HR-HPV specificity of the Papilloplex® HR-HPV genotyping kit were further validated through the participation in the WHO HPV LabNet Proficiency Study (2014) which covered all 14 HR-HPV types addressed by the assay (Supplementary Table 2). The data generated from the WHO HPV LabNet study (2014) showed that the Papilloplex® HR-HPV assay is proficient for the detection of all 14 HR-HPV genotypes included in the test and offers robust simultaneous HPV genotyping. No

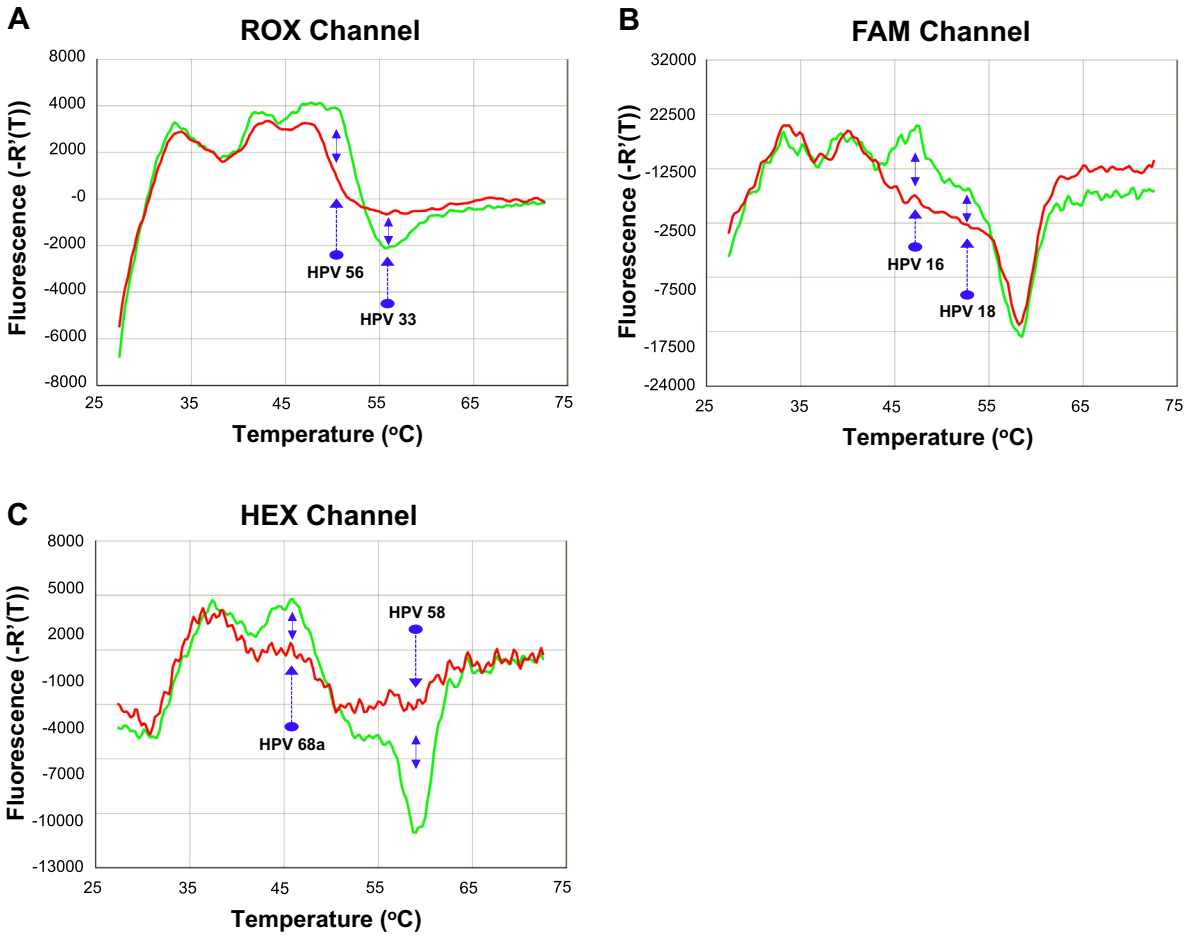

Fig. 5. Melting curve analysis of the amplification reactions of 2 positive HR-HPV targets adjacent in melting curves. Melting curves showing a sample positive for (**A**) HR-HPV 56 and 33 (ROX fluorescent channel), (**B**) HR-HPV 16 and 18 (FAM fluorescent channel), and (**C**) HR-HPV 68a and 58 (HEX fluorescent channel). The NC melting curve is marked in green, and the target melting curve is marked in red. Note the double-headed arrows which depict the distinct changes in the melting peak of the corresponding probes. HR-HPV DNA plasmids were used at a concentration of 3000 copies per PCR.

false positive types were detected by the assay (Supplementary Table 2).

The assay range was verified by testing 20 replicates at the high end of the range (10^6 DNA copies of HR-HPV per reaction, one 10-fold dilution higher than the claim of 10^5 DNA copies), 20 replicates in the middle of the range (10^4 DNA copies of HR-HPV per reaction), and 20 replicates at the low end of the range (3 × LoD) which gave 300 DNA copies per reaction for HR-HPV types 16 and 18 and 3000 DNA copies per reaction for all other HR-HPV types detected by the assay. The verified assay range (the % accuracy was ≥95%) is 100 to 10^5 DNA copies per MPA reaction for HR-HPV types 16 and 18, and 1000 DNA copies to 10^5 per MPA reaction for all the other HR-HPV types.

3.3. Analytical specificity (cross-reactivity)

The Papilloplex® HR HPV assay was specific to the targeted HR-HPVs only and showed no cross-reactivity with any of the organisms tested (Supplementary Table 3). All tested samples showed no amplification, and the melting curve profiles were comparable to the NC melting curves.

3.4. Verification of repeatability

The Papilloplex® HR-HPV genotyping assay conforms to established acceptance criteria for repeatability (Bustin et al., 2009). All HR-HPV types, at all 3 concentrations, were correctly identified in each replicate of each run, resulting in 100% correctly identified genotypes in total and 100% agreement between the runs. Total % CV of Ct values of the low, medium, and high copy number samples was smaller than 2.5%.

3.5. Verification of reproducibility

The Papilloplex® HR-HPV assay conforms to established acceptance criteria for reproducibility (Bustin et al., 2009). For all targeted HR-HPV types a ≥98% positive detection rate was observed at low, medium, and high concentrations (Supplementary Table 4). No significant variability between operators, runs, and batches was recorded at any concentration tested. The % CV of Ct values across operators, instruments, and batches of the low, medium, and high copy number samples were smaller than 10%.

4. Discussion

One of the most important scientific discoveries over the past 30 years is the causal link between HPV infection of the cervix and cervical cancer (Crosbie et al., 2013). It is widely accepted that the most common HPV types in cervical cancer are HR-HPV types 16 (57%) and 18 (16%) (Li et al., 2011) with small regional variations (Guan et al., 2012). However, recent meta-analysis studies have provided evidence that additional HR-HPV genotypes including HR-HPV 58 (5%), 33 (5%), 45 (5%), 31 (4%), 52 (3%), and 35 (2%) are also linked to the development of cervical cancer (Guan et al., 2012; Li et al., 2011) and are the cause of 20% of current colposcopy referrals. The implication of HR-HPV infections in human carcinogenesis underlies the clinical

importance of sensitive diagnostic tests that offer differential detection of HR-HPV genotypes.

This study evaluated the principles and analytical performance of the commercially available GeneFirst Papilloplex® HR-HPV assay, a new CE-IVD–marked real-time PCR test, based on patented MPA detection technology. Papilloplex® HR-HPV provides the simultaneous detection and differentiation of 14 HR-HPV genotypes including 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 types in a single closed-tube reaction. The results from this study showed that the Papilloplex® HR-HPV molecular diagnostic assay provides robust analytical performance and generates reproducible and reliable results. The assay showed an analytical sensitivity of 100 copies for HR-HPV 16 and 18 and 1000 copies per MPA reaction for all other HR-HPV types. The test was shown to be highly specific to the 14 HR-HPV types included in the test; each probe detected/amplified only the corresponding HR-HPV type. The analytical sensitivity and differential HR-HPV specificity of the Papilloplex® HR-HPV genotyping kit to concurrently detect and discriminate all 14 HR-HPV DNA types were further validated through the participation in the WHO HPV LabNet Proficiency Study (2014) (Eklund et al., 2018). No cross-reactivity with any of the 20 LR-HPV DNA types or microorganisms tested was observed. Furthermore, highly reproducible results were demonstrated. The assay conformed to established acceptance criteria for repeatability and reproducibility analytical procedures. Several aspects of the assay design ensure necessary quality assurance of the test results. The test features an internal human DNA control for specimen adequacy and quality of DNA extraction. In addition, external positive and negative controls are included in each run to assess run validity.

Application of highly sensitive and accurate HPV diagnostic tests suitable to discriminate all 14 HR-HPV DNA types may have major clinical significance and help to identify the associated risk of developing high-grade CIN. Moreover, type-specific HPV methods are valuable for the effectiveness of HPV vaccinations, improve risk stratification of patients with HPV in cervical screening programs, but also enable screening intervals to be extended. Recent clinical evaluation studies, using cervical liquid-based cytology samples with known clinical outcomes, provided compelling evidence that the Papilloplex® HR-HPV assay detects reliably CIN2+ in a disease-enriched retrospective cohort (Bhatia et al., 2018). The study showed comparable results to well-established clinically validated HPV genotyping assays including the Hybrid Capture 2 HPV DNA Test™ (Qiagen), the RealTime High Risk HPV test (Abbott), the Linear Array® HPV test (Roche), and the Optiplex HPV genotyping assay (formerly the Multiplex HPV genotyping kit; DiaMex) (Bhatia et al., 2018).

The competitor IVD assays vary in their ability to offer simultaneous HPV genotypic assessment. They do not i) distinguish between HPV types (e.g., Hybrid Capture 2 HPV DNA Test, Qiagen; Aptima® HPV assay, Hologic), ii) offer partial genotyping (e.g., RealTime High Risk HPV test, Abbott; Cobas® HPV Test, Roche; HPV Harmonia, Liferiver), iii) distinguish all 14 HR-HPV types (e.g., PapilloCheck®, Greiner; Anyplex™ II HPV HR Detection, Seegene), or iv) determine both HR-HPV and LR-HPV genotypes (e.g. Linear Array® HPV test, Roche; Optiplex HPV genotyping assay, DiaMex). In addition, the competitor assays use a variety of detection technologies (e.g., Luminex® suspension array technology followed by hybridization, real-time PCR, methylation PCR, target amplification followed by sandwich capture assay, microarray, NASBA amplification, TMA amplification). Moreover, some assays require dedicated/specific equipment. The Papilloplex® HR-HPV test is compatible with real-time PCR equipment commonly used in routine clinical and research laboratories and so does not require a specific locked-down platform. The ability of the assay to perform simultaneous multiplex detection and differentiation of 14 HR-HPV types, together with an endogenous human control target, within a single closed-tube format reduces time and the risk of contamination associated with more “open” genotyping systems. The assay is also amenable to several DNA extraction chemistries and requires a small amount of input DNA.

Overall, the development and application of MPA proprietary technology (Fu et al., 2012) that underpins the CE-IVD marked Papilloplex® HR-HPV assay represent a technical advancement in the field of molecular diagnostic medicine. Use of MPA is applicable in any thermocycler platform and allows the concurrent detection and differentiation of up to 36 targets (6 targets/fluorescent channel) in a single-closed tube reaction, which overcomes the current 1-channel-1-target limitation of multiplex hydrolysis probe-based PCR. The technical advances of MPA permit the assessment of multiple targets, simplify the test procedure, minimize hands-on time, and reduce the risk of sample contamination (unlike methods which require additional steps such as nucleic acid hybridisation). The results from the present study highlight the successful application of MPA technology as a valid diagnostic tool that can be applied to the diagnosis and/or monitoring of disease predisposition, including also conditions associated with single nucleotide polymorphisms, as recently described (Fu et al., 2012). Overall, GeneFirst Papilloplex® HR-HPV assay based on MPA technology demonstrated a robust analytical performance with reproducible and reliable results in the concurrent detection and genotyping of HR-HPV.

Contributions

M.M., E.B., and G.F. developed the Papilloplex® HR-HPV Genotyping assay. G.K.S., M.B., M.M., M.S., and D.K. carried out the analytical performance experiments. M.B. prepared the linearized plasmid DNA concentrations. Y.L. developed the probes. G.K.S., M.B., M.M., M.S., and D.K. were involved in data analysis. M.H. and M.S. defined regulatory strategy and compiled CE Technical file. G.F. directed the product development. G.K.S. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Acknowledgments

This work was supported by Innovate, UK (grant numbers 131003 and 101759). The authors wish to thank Dr. Hall and Dr. Wu who assisted in the proof-reading of the manuscript.

Conflict of interest

The authors are employees of GeneFirst Ltd. MPA is a patented technology (EP2307574, EP2438189, CN200980138727.3, HK1161746, AU2010255523, US2013267434).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2019.04.005.

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