A Highly Multiplexed Droplet Digital™ PCR (ddPCR™) Assay for Human Papillomavirus (HPV) Detection in Research Settings

Note

This study was originally carried out on the Nio™ Digital PCR System, which supports seven optical detection channels with the following fluorophores: FAM, Yakima Yellow, ATTO 550, ROX, Cy5, ATTO 700/Cy5.5, and DY-521-XL. The QX700™ HT Droplet Digital PCR System (Bio-Rad Laboratories, Inc., catalog #17010628) also supports seven fluorophores, but with a different fluorophore set: FAM, HEX, ROX, ATTO 590, Cy5, Cy5.5, and ATTO 550. Despite the optical difference in two of the fluorophores, the core digital PCR (dPCR) principles of droplet partitioning, endpoint fluorescence detection, and absolute quantification remain equivalent between platforms. Representative data were generated using the QX700 HT ddPCR System to demonstrate implementation of the workflow.

The assay used in this study is identical to the ddPLEX CrystalTag HPV 11 types ddPCR Assay available from Bio-Rad Laboratories (#12026945, #12026946). Note that this study used Nio Crystal Universal Reporters, formulated to match the fluorescent channel specifications of the Stilla™ Nio Digital PCR System. For use when running ddPLEX CrystalTag ddPCR Assays across all seven channels of the QX700 Droplet Digital PCR System, QX700 CrystalTag Reporters 7 (Bio-Rad, #12027244, #12027265) should be used instead.

This application note describes research-use-only workflows and is not intended to establish clinical performance, diagnostic utility, or applications for patient management.

Introduction

HPV, a DNA virus that infects human epithelial cells, causes a common sexually transmitted infection associated with various benign and malignant lesions, mainly of the genital and upper aerodigestive tracts (Table 1). In cervical cancer, one of the major causes of death from cancer in women,[1] about 99.7% of cases are caused by persistent high-risk human papillomavirus.[2]

For HPV-related research applications, ddPCR technology can be used for sensitive and precise nucleic acid quantification and biomarker investigation. To monitor HPV in research settings, we developed a highly multiplexed ddPCR assay compatible with our seven-color QX700 HT ddPCR System, enabling users to detect and quantify human DNA and 11 HPV types in a single reaction. This assay was developed using a color-combination multiplexing approach and a modular detection chemistry.

Table 1. Human papillomavirus (HPV) types and associated cancer risks.[3–5]

Papillomavirus	Fully Demonstrated	Most Likely Involved
Cervical cancer	HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59	HPV26, 53, 66, 67, 68, 70, 73, 82
Cancer of the cervix, vulva, vagina, penis, anus, oral cavity, oropharynx, tonsil	HPV16	—

ddPLEX CrystalTag HPV 11 types ddPCR Assay in Research Settings

A color-combination approach has been previously implemented to expand multiplexing capacity.[6] By using two probes per target, each population can be revealed in two colors, expanding the detectable number of targets per assay. The maximum multiplexing level is 21 targets when using a seven-color detection system, such as the QX700 HT ddPCR System. This approach was subsequently applied to develop a 12-plex assay targeting the major HPV types associated with cancer in research samples.

The QX700 HT ddPCR System, ddPLEX CrystalTag HPV 11 types ddPCR Assay, CrystalTag Probes, and QX700 CrystalTag Reporters 7 mentioned in this app note are for Research Use Only. Not for use in diagnostic procedures.

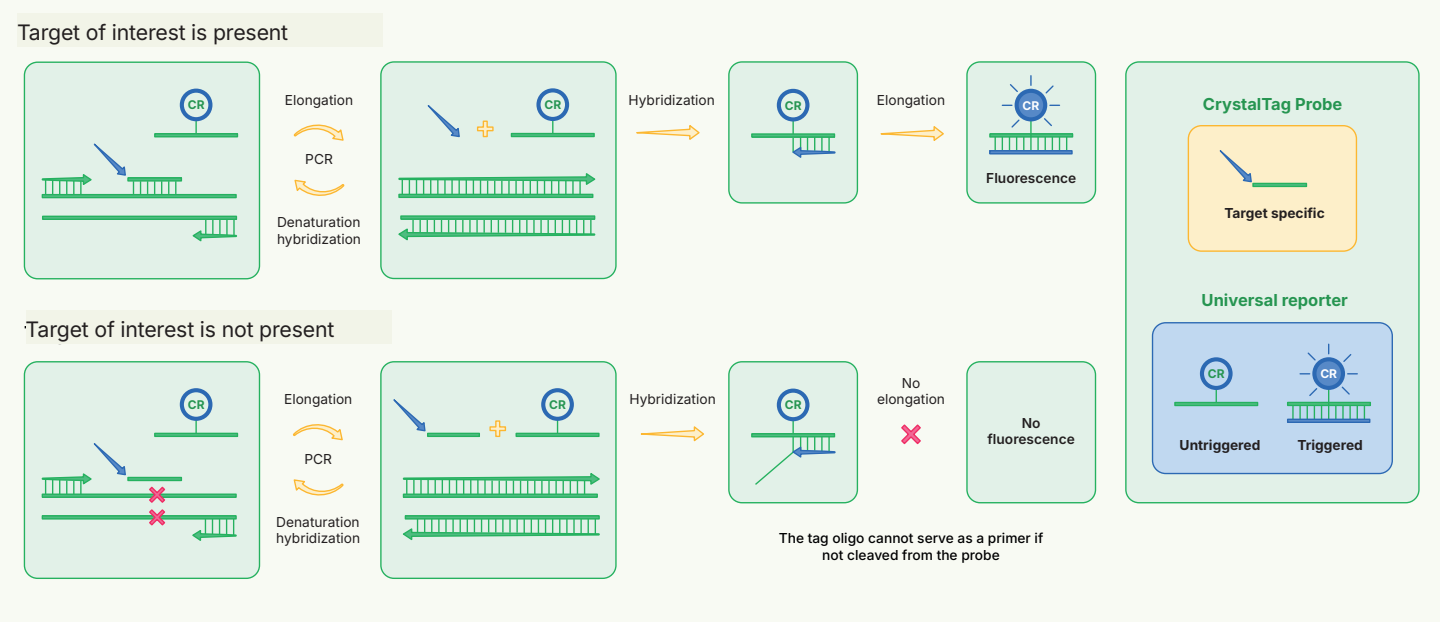


Fig. 1. CrystalTag technology research workflow. When the target sequence is present, hybridization and cleavage of the target-specific probe trigger fluorescence from the universal reporter. In the absence of the target sequence, the reporter remains untriggered, and no fluorescence signal is generated.

Table 2. Configuration of human papillomavirus (HPV) ddPCR assay in research settings.

#	Target	Color 1	Color 2
1	HPV16	HEX	Cy5
2	HPV18	ROX	ATTO 590
3	HPV31	HEX	ROX
4	HPV33	FAM	Cy5
5	HPV35	HEX	Cy5.5
6	HPV39	HEX	ATTO 590
7	HPV45	ROX	Cy5
8	HPV51	ROX	Cy5.5
9	HPV52	ATTO 590	Cy5.5
10	HPV56	Cy5	Cy5.5
11	HPV58	FAM	ATTO 590
12	ALB (human reference gene)	FAM	—

CrystalTag Technology

The ddPLEX CrystalTag HPV 11 types ddPCR Assay design is based on modular detection chemistry, which consists of non-fluorescent target-specific probes, the CrystalTag Probes, that can trigger fluorescent molecules for PCR readout. The same set of fluorescent molecules, the CrystalTag Reporters, is used for all the targets, and only the non-fluorescent target-specific molecules are changed from target to target (Figure 1).

Methods and Results

ddPLEX CrystalTag HPV 11 types ddPCR Assay Detects and Quantifies 11 HPV Types and Human DNA in a Single Reaction in Research Settings

The assay's ability to detect and quantify human DNA and 11 HPV types in a single reaction in research settings was first tested using synthetic DNA. Testing was performed to evaluate target-specific amplification behavior using a positive control containing the 11 synthetic target sequences and human genomic DNA (hgDNA) to assess amplification specificity. Individual tests using a single synthetic target sequence were also performed to confirm that no cross-over amplification occurred among the 12 targets. Representative data obtained with the QX700 HT ddPCR System are shown in Figures 2 and 3.

ddPLEX CrystalTag HPV 11 types ddPCR Assay Demonstrates Strong Quantitative Performance in Research Samples

To analyze a color-combination assay, the 1D dot plot is used to set a unique threshold for each channel to separate positive and negative droplets. QX700 ddPCR System Analysis Software colocalizes fluorescence within the same droplet and assigns and quantifies populations previously defined using the corresponding color code in the population editing tool.

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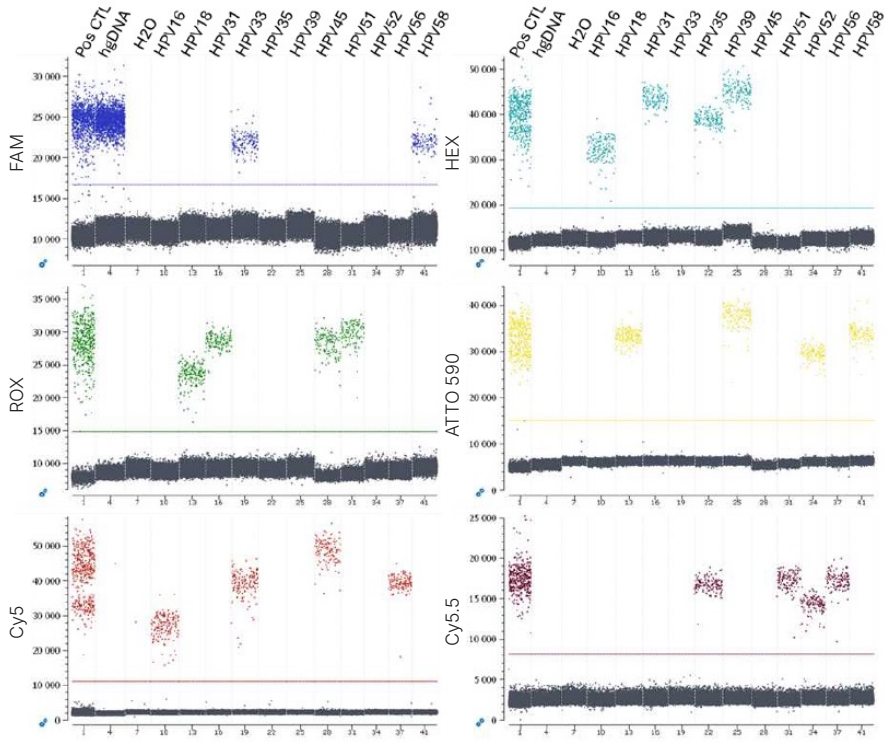


Fig. 2. 1D plots obtained during wet-lab testing on the QX700 HT ddPCR System. From left to right, the chambers contain the following samples: positive control containing the 11 synthetic target sequences and hgDNA, hgDNA only, NTC (H2O), and individual synthetic target sequences. hgDNA, human genomic DNA; NTC, no template control.

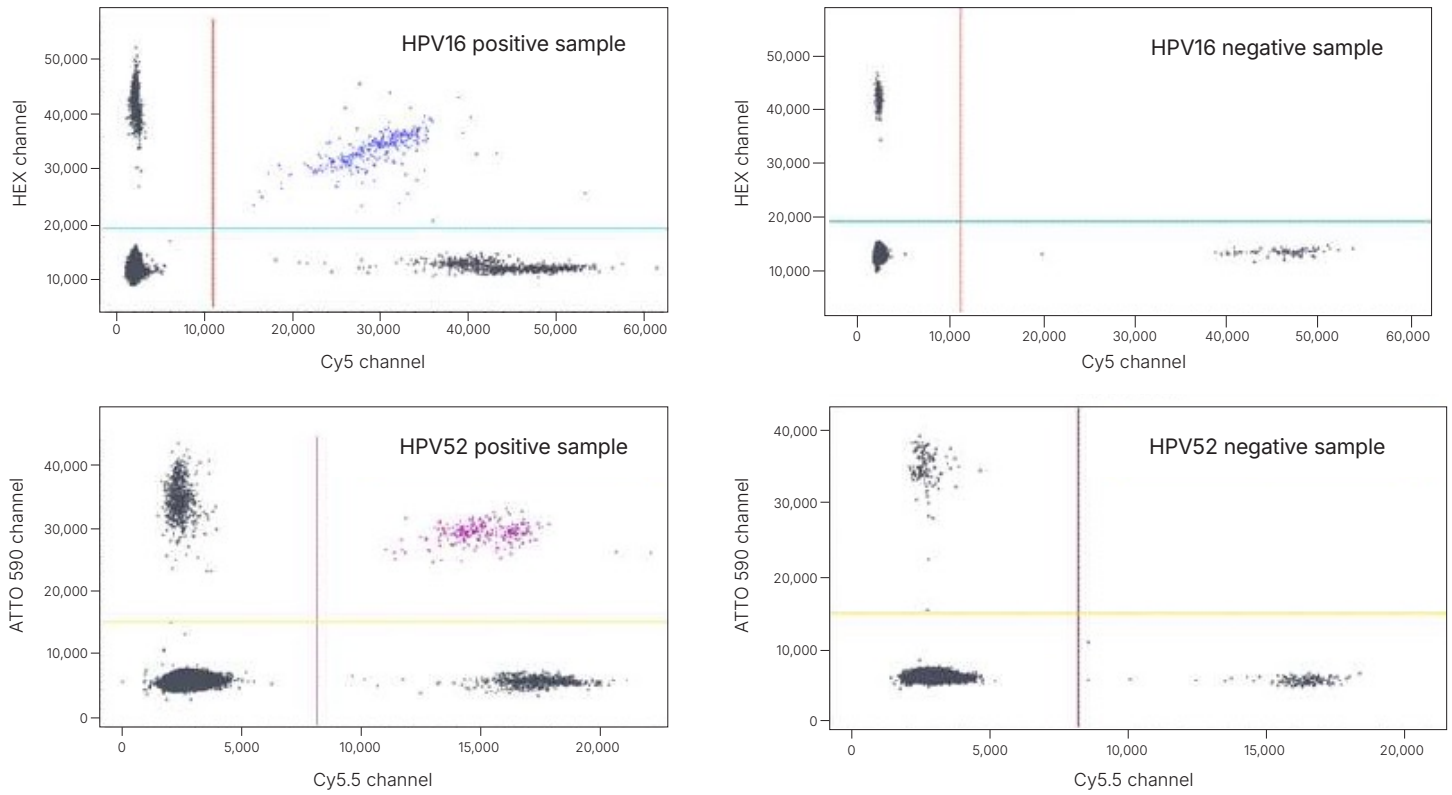


Fig. 3. 2D plots of HPV16 and HPV52 positive and negative samples analyzed with the ddPLEX CrystalTag HPV 11 types ddPCR Assay in research settings. Each target can be visualized as a double-positive population corresponding to its assigned color combination (Table 2).

To evaluate assay analytical performance, serial dilutions of HPV16 and HPV18 were prepared in a background of human DNA. The dilution series ranged from 50 to 3 copies (cp)/µL of HPV16 or HPV18 DNA in a background of 625 copies of hgDNA per reaction.

n = 4 replicates were performed for each dilution point. Linear regression analysis of measured versus expected concentrations yielded coefficients of determination (R^2) of 0.984 and 0.986 for HPV16 and HPV18, respectively (Figure 4).

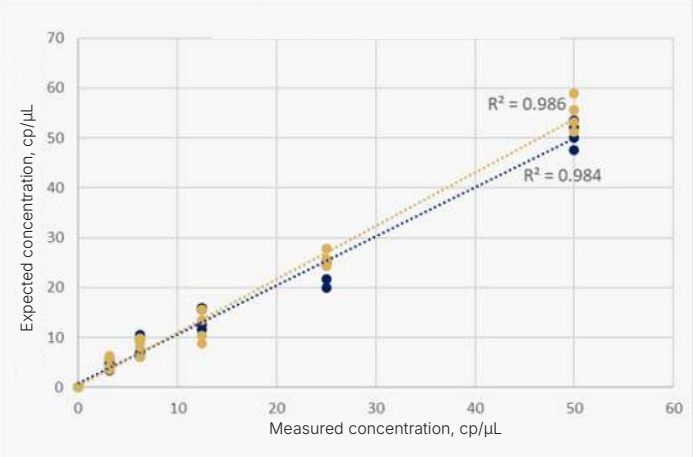


Fig. 4. Serial dilution of HPV16 and HPV18 DNA. Measured HPV DNA concentrations obtained with the QX700 HT ddPCR System are plotted against expected concentrations. HPV16 (●), HPV18 (●).

Conclusion

Using color-combination multiplexing and modular probe chemistry, a highly multiplexed ddPCR assay was developed for the detection and quantification of human DNA and 11 HPV types in a single reaction in research samples on the QX700 HT Droplet Digital PCR System. The assay demonstrated target detection across the evaluated experimental conditions and strong linearity for HPV16 and HPV18 across the tested concentration range. These results illustrate the capability of highly multiplexed ddPCR assays to detect and quantify HPV targets in research workflows.

Visit **bio-rad.com/QX700Family** for more information.

References

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