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Droplet digital PCR for the detection and quantification of HPV 16, 18, and 68

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ABSTRACT

Introduction: Human papillomavirus (HPV) is a frequently occurring viral infection that is typically sexually transmitted, resulting in more than fourteen million cases per year in the United States. Among different genotypes, HPV 16 and 18 are the two most common high-risk HPV types which lead to cancer. HPV 68 could be predominant due to specific characteristics of the population such as geographic location or conservative sexual habits. Molecular diagnostic methods allow for the identification of the many unique types of high-risk HPV, which can lead to better diagnoses and treatment plans for patients. The AcroMetrix™ HPV Genotype Controls are intended for research use only, not for clinical use in assessing the analytical performance of molecular test procedures designed to identify HPV 16, 18, and 68. We developed and evaluated a droplet digital PCR assay as a tool for HPV 16, 18, and 68 viral load quantification and, in addition, to calibrate AcroMetrix™ HPV genotype control HPV 16 and HPV 18 toward available WHO international standards.

Methods: HPV DNA was extracted from AcroMetrix™ HPV Genotype Controls using QIAcube automation system. Primers and FAM-labeled fluorescence probes were designed against E6/E7 region for each HPV genotype as HPV E6/E7 is the biomarker for progression of invasive carcinoma of the uterine cervix. After PCR amplification, viral loads of HPV genotypes 16, 18, and 68 were quantified using QX200 Droplet Reader ™ (BIO-RAD). Positive droplets for FAM fluorescence were read after which the viral load was calculated using Quantasoft software. Assay Specificity was assessed by side-by-side comparison of positive and negative samples.

Results: In this feasibility study, HPV DNA quantities extracted from AcroMetrix™ HPV Genotype Controls (quantitative value for positive sample) were quantified and distinguishable from AcroMetrix HPV Negative Control product. The specificity of the assays for genotyping was evaluated by the cross-reactions between the primers/probes and target fragments of known genotypes and confirmed to be only specific to its own genotype. The viral concentration for AcroMetrix™ HPV Genotype Controls were preliminarily determined to be 1.80E+5 copies/mL of HPV 16, 1.70E+5 copies/mL of HPV 18, and 8.62E+4 copies/mL of HPV 68, respectively. The AcroMetrix HPV Negative Control shows no detectable virus from HPV 16, HPV 18 and HPV 68 assays.

Conclusions: The genotype specific droplet digital PCR assays were designed to assess the feasibility of determine viral loads in AcroMetrix™ HPV Genotype Controls. Additionally, HPV 16 and HPV 18 Controls were tested in this feasibility study toward the available WHO standards, respectively. The product performance was evaluated using droplet digital PCR assays for monitoring and managing HPV infection.

INTRODUCTION

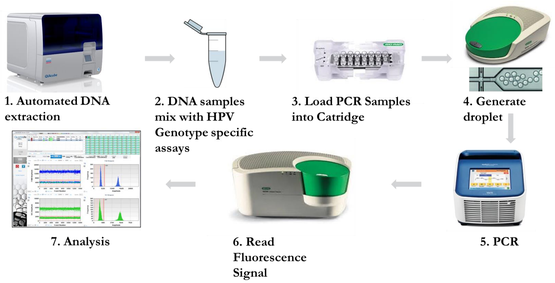


Figure 1. Workflow of HPV DNA Extraction and Genotype Specific droplet digital PCR Analysis: Cellular Extraction using Qiacube is performed at room termperatuer with approximately 30 minutes hands on preparation and 1 hour automated extraction. DNA was quantified for extraction efficiency using Qubit dsDNA HS Assay kit. Droplet digital PCR then was performed with primers and probes targeting E6-E7 region of HPV DNA to determine genotypes of HPV 16, HPV 18 or HPV 68.

RESULTS

Catalog Number	Product Name	Quantity
950075	AcroMetrix HPV-16 Genotype Control	5 X 4.0 mL
950076	AcroMetrix HPV-18 Genotype Control	5 X 4.0 mL
950077	AcroMetrix HPV-68 Genotype Control	5 X 4.0 mL
950078	AcroMetrix HPV Negative Control	5 X 4.0 mL

Table 1. AcroMetrix™ HPV Genotype Controls: Controls are available in four different variations: Genotype 16, Genotype 18, Genotype 68, and Negative. The The AcroMetrix™ HPV Genotype Controls are intended for research use only, not for clinical use in assessing the analytical performance of molecular test procedures designed to identify different genetic variants (genotypes) of high risk human Papilloma Virus (HPV).

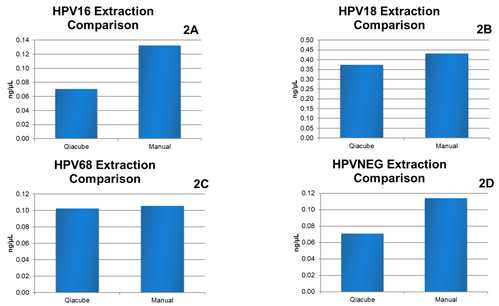


Figure 2. Extraction Method Efficiency Comparison: Manual extraction vs. Automated extraction. This study was consisting of a side by side comparison with sample extracted from Qiacube and Manual methods with yield checked by Qubit analysis. Figure 2 shows the whole cell extraction yields comparison using extraction materials (A) HPV 16 Control. (B) HPV 18 Control. (C) HPV 68 Control. (D) HPV Negative Control.

Extraction Sample	HPV 16 Assay	HPV 18 Assay	HPV 68 Assay
HPV16 Extract	N/A	No Call	No Call
HPV18 Extract	No Call	N/A	No Call
HPV68 Extract	No Call	No Call	N/A
HPVNEG Extract	No Call	No Call	No Call

Table 2. Qiacube Extraction Cross Contamination Study Set up. HPV DNA from cell extract was tested by non-specific assays, respectively, to confirm no contamination (no call) based on droplet digital PCR results. N/A: for contamination study, DNA tested with its own genotype assay was not performed.

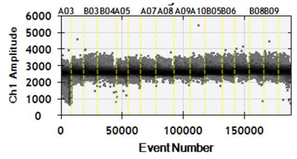


Figure 3. Qiacube Extraction Cross Contamination Study Results: No call for all non-specific assays, no contamination identified. The experiments were conducted using 4 replicates of each DNA extract from 4 different QIAcube instruments. NTC was tested for each assay as negative control.

Extracted DNA From Product	HPV16 Genotype Assay copies/mL	HPV18 Genotype Assay copies/mL	HPV68 Genotype Assay copies/mL
HPV 16 Control	1.80^5	No Call	No Call
HPV 18 Control	No Call	1.70^5	No Call
HPV 68 Control	No Call	No Call	8.62^4
HPV NEG Control	No Call	No Call	No Call
NTC	No Call	No Call	No Call

Table 3. HPV Genotype Specific Assay Specificity Assessment.

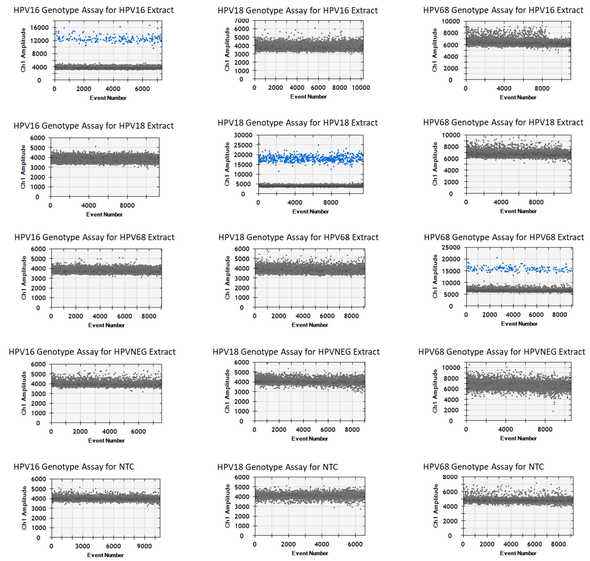


Figure 4 HPV Genotype Assay Specificity Assessment. The feasibility study confirms that the three genotype specific assays: HPV16 Assay, HPV18 Assay and HPV68 Assay are all specific to their target, and no cross-reactivity to other genotypes, respectively. The detection result is “No Call” for all other genotypes/Negative/NTC except the targeting genotype.

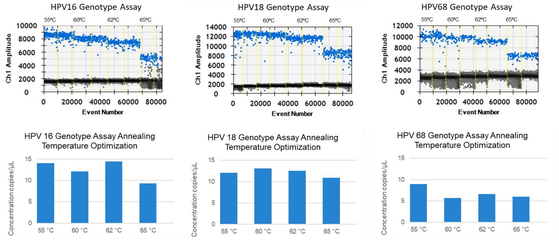
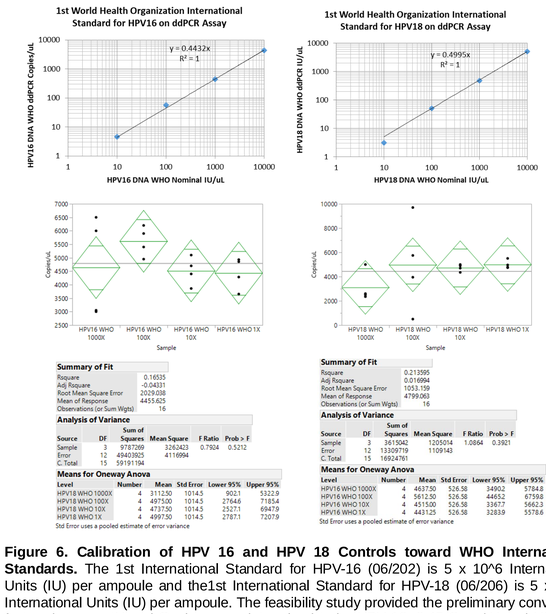


Figure 5. Optimization of Assay Annealing Temperature. Gradient PCR was used in this experiment in order to determine the optimal annealing temperature. Using the gradient function of the ABI Veriti universal block, a gradient of 55 to 65˚ C was set. This was achieved by setting the annealing temperature at 55, 60, 62 and 65 ºC, respectively for 60 s annealing. 60 ºC has been determined to be the optimized annealing temperature.



Summary of Fit

Rsquare	0.16535
Adj Rsquare	-0.04331
Root Mean Square Error	2029.038
Mean of Response	4455.625
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Sample	3	9787289	3262423	0.7924	0.5212
Error	12	49403925	4116994		
C. Total	15	59191194			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
HPV16 WHO 1000X	4	3112.50	1014.5	902.1	5322.9
HPV16 WHO 100X	4	4975.00	1014.5	2764.6	7185.4
HPV16 WHO 10X	4	4737.50	1014.5	2527.1	6947.9
HPV16 WHO 1X	4	4997.50	1014.5	2787.1	7207.9

Std Error uses a pooled estimate of error variance

Summary of Fit

Rsquare	0.213595
Adj Rsquare	0.016994
Root Mean Square Error	1053.159
Mean of Response	4799.063
Observations (or Sum Wgts)	16

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Sample	3	3815042	1205014	1.0864	0.3921
Error	12	13309719	1109143		
C. Total	15	16824761			

Means for Oneway Anova

Level	Number	Mean	Std Error	Lower 95%	Upper 95%
HPV18 WHO 1000X	4	4637.50	526.58	3490.2	5784.8
HPV18 WHO 100X	4	5612.50	526.58	4465.2	6759.8
HPV18 WHO 10X	4	4515.00	526.58	3367.7	5662.3
HPV18 WHO 1X	4	4431.25	526.58	3283.9	5578.6

Std Error uses a pooled estimate of error variance

Figure 6. Calibration of HPV 16 and HPV 18 Controls toward WHO International Standards. The 1st International Standard for HPV-16 (06/202) is 5 x 10^6 International Units (IU) per ampoule and the1st International Standard for HPV-18 (06/206) is 5 x 10^6 International Units (IU) per ampoule. The feasibility study provided the preliminary conversion factors between IU and Copies were determined to be HPV 16: 0.45 Copy/IU and HPV 18: 0.48 Copy/IU in this method for The AcroMetrix™ HPV Genotype Controls.

CONCLUSIONS

- Full-process HPV 16, HPV 18, HPV 68 and HPV Negative QC Controls allow for efficient evaluation of different commercially available HPV Assay platforms, assessing the analytical performance of molecular test procedures designed to identify different genetic variants (genotypes) of human Papilloma Virus (HPV).
- In the feasibility study, the digital droplet PCR assays were designed and optimized on assay concentration, primer/probe pairs, annealing temperature, template input concentration, etc. The optimization improved the assay performance and provided accurate analysis of HPV genotypes, facilitating HPV molecular assay development and applications .
- The feasibility study demonstrated that the droplet digital PCR genotype specific assays for HPV were sufficient to evaluate different HPV genotypes. The WHO International Standards were also tested in the feasibility study to obtain the preliminary conversion factors between IU ad Copy values.

ACKNOWLEDGEMENTS

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TRADEMARKS

The products discussed in this presentation are for research use only, not for clinical use. The droplet digital PCR method is in feasibility study phase and data is preliminary.

http://ThermoFisher.com/AcroMetrix/

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