Countable Labs.

Quantitative detection of high-risk HPV subtypes in oropharyngeal cancer using single-molecule PCR.

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Abstract

HPV-positive oropharyngeal squamous cell carcinomas are rising, yet detection often occurs in later stages of disease due to lack of early-stage symptoms. Currently, P16 immunohistochemistry is the standard for detection, but it is not a direct measurement of HPV presence; further, assessment of P16 levels is subjective, lacks strain specificity, and yields equivocal results in approximately 10% of cases. These often require slow follow-up testing such as *in situ* hybridization. A faster, subtype-resolved method with better objectivity would be an improvement over current technologies.

We utilized Countable PCR, a PCR technique that enables direct counting of single molecules across a dynamic range up to 10^6, to develop a 17-plex panel that differentiates and quantifies: the 2 highest-risk HPV strains for oropharyngeal cancer (HPV-16, HPV-18), 1 common region for 14 high-risk subtypes in parallel (HPV- 16, 18, 31, 33, 35, 39, 45, 51, 51, 56, 58, 59, 66, and 68), and 1 internal control (*RPP30*) with high precision and sensitivity. Samples categorized as either positive, negative, or equivocal based on p16 immunostaining at USC were re-tested using Countable PCR.

The broad dynamic range of Countable PCR enabled minimal optimization for the panel, even in samples containing high HPV load. Further, Countable PCR was able to resolve equivocal samples in just a few hours, rather than the days required for *in situ* hybridization. Use of this Countable PCR 17-plex panel also allowed identification of all HPV subtypes while existing protocols based on real-time PCR can only identify HPV-16 and HPV-18 while lumping all other high-risk subtypes as a single entity.

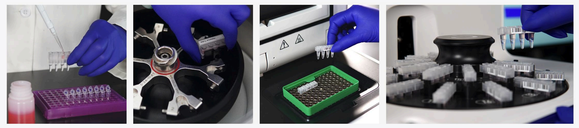
This proof-of-concept validation experiment demonstrates the value of a quantitative detection panel that is able to delineate HPV infection across multiple subtypes within a few hours.

Method

Countable PCR as an alternative approach for early HPV detection.

Countable PCR partitions DNA molecules across approximately 30 million uniform compartments within a transparent gel-like matrix, enabling **direct single-molecule counting** without dependence on PCR efficiency or standard curves. This approach may provide an **alternative to p16 immunohistochemistry and conventional qPCR** for early, subtype-resolved HPV detection.

- Directly quantifies HPV DNA, offering earlier detection of high-risk HPV compared to marker-based methods such as p16 immunohistochemistry.
- Differentiates HPV-16 and HPV-18 from the remaining 12 high-risk subtypes within a single assay, allowing subtype-level interpretation.
- Maintains equivalent analytical performance in multiplex format, supporting accurate detection of all 14 high-risk HPV subtypes in one reaction.
- Enables simultaneous quantification of viral and human targets, supporting normalization and future development of minimally invasive monitoring assays.



01. Setup
Prepare PCR amplification reaction.

02. Generate compartments
Load PCR reaction into spin column and centrifuge to generate compartments.

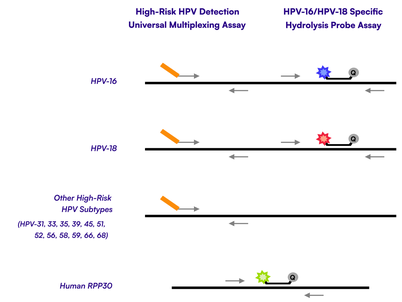
03. Amplify
Use a standard thermocycler to amplify single molecules within compartments.

04. Count
Count single molecules using the Countable system.

Figure 1. Countable PCR workflow. In Countable PCR, target molecules are distributed across 30 million compartments in an optically clear matrix. Every DNA molecule is physically separated into its own compartment and amplified without interference. This enables true single molecule counting, clean multiplexing, and data that remains precise across every run.

Countable Labs 17-plex High-Risk HPV panel utilizes a dual-chemistry design for broad-range detection and specific quantification.

This 17-plex panel utilizes 4-color detection system, with each color corresponding to a separate detection channel (Ch). A Universal Multiplexing assay detects the presence of all 14 high-risk HPV subtypes, including HPV-16 and HPV-18, on Ch03. Concurrently, specific hydrolysis probe assays are employed for the individual quantification of the highest-risk subtypes, HPV-16 (Ch01) and HPV-18 (Ch04). The panel also includes the quantification of the human *RPP30* gene (Ch02) with a hydrolysis probe to serve as an internal control.



Method, cont.

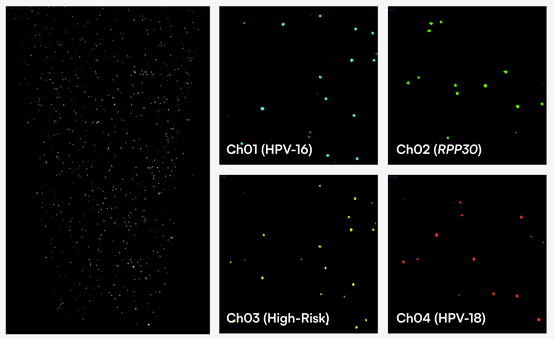


Figure 2. Light sheet images of a Countable PCR reaction containing synthetic HPV-16 and HPV-18 DNA, along with human genomic DNA. A composite image (left) shows the positive compartments from a mid-plane slice of the tube. Zoomed-in images (right) display pseudo-colored positive compartments from each channel.

Results

Analytical performance of a 17-plex single-molecule High-Risk HPV panel.

Analytical validation was performed using synthetic DNA constructs (HPV-16, HPV-18, and HPV-31 (as a proxy for the other 12 high-risk subtypes)) from ATCC and ~80 ng of NA12878 human genomic DNA (Coriell). Target counts in single-plex reactions were equivalent to those in the 17-plex panel. 5-log serial dilutions of HPV-16 and HPV-18 synthetic DNA were measured in the background of human genomic DNA and demonstrated high linearity. Finally, the Limit of Blank (LoB) for HPV-16 and HPV-18 assays were determined by averaging counts from 16 reactions containing 150 ng of human genomic DNA without HPV targets. All counts were determined with the Countable Control Software on the Countable System.

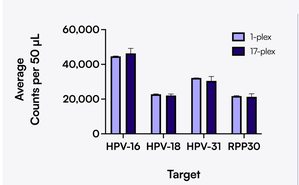


Figure 3. Multiplex assay performance. Synthetic HPV-16, HPV-18, and HPV-31 targets at various concentrations were tested. ~80 ng of human genomic DNA was tested for the *RPP30* target. Bars represent average counts per 50 µL and error bars represent standard deviations across N=3 replicates.

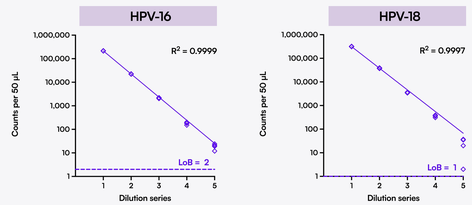


Figure 4. Linearity and LoB of HPV-16 and HPV-18 hydrolysis probe assays. Linearity was evaluated using a 5-log serial dilution series of synthetic HPV DNA spiked in the background of 80 ng of human genomic DNA (N=4). Across the dynamic range, both assays showed high linearity. The Limit of Blank (N=16) was determined to be 2 and 1 counts per 50 µL for HPV-16 and HPV-18 assays, respectively, highlighting the panel's ability to detect low viral loads.

Subtype-resolved, quantitative HPV detection may enhance molecular screening of oropharyngeal squamous cell carcinomas.

The 17-plex high-risk HPV panel was evaluated for detection of viral DNA in 46 de-identified, formalin-fixed oropharyngeal squamous cell carcinoma samples. Samples were collected and DNA was extracted at the University of Southern California, and Countable PCR reactions were performed at Countable Labs. Approximately 15 ng of DNA per sample, quantified by fluorescence spectrometry using Qubit (Thermo Fisher Scientific), was added to each reaction and tested in duplicate (N = 2). Countable PCR results were compared to p16 immunohistochemistry findings provided by USC.

Results, cont.

Table 1. High-risk HPV subtype-specific quantification and detection for 46 oropharyngeal squamous cell carcinoma samples. Heat map showing the average Counts per 50 µL (N=2) across 4 targets: HPV-16, human reference (*RPP30*), a pooled high-risk set, and HPV-18. The reported p16 status from immunohistochemistry are on the left column along with Countable PCR results on the right column. Approximately 15 ng of DNA from each sample was used per replicate.

p16 status	Countable PCR	Sample ID	HPV-16	*RPP30*	High Risk	HPV-18
p16 Positive	HPV-16 Positive	D1368	1,563,953	10,835	1,541,759	2
		D1627	1,362,525	12,287	1,368,798	2
		D1622	595,807	12,988	618,005	7
		D1600	475,438	12,062	481,382	1
		D1611	387,288	13,014	403,050	0
		D1361	381,174	11,936	394,447	0
		D1617	312,378	9,056	332,917	0
		D1636	296,094	11,680	314,067	0
		D1597	268,472	8,541	280,748	1
		D1621	163,232	11,949	167,909	1
		D1366	140,072	10,132	142,583	0
		D1634	115,797	6,624	120,987	0
		D1362	100,989	7,296	103,485	0
		D1603	97,129	11,961	104,755	1
		D1626	28,138	12,028	30,272	1
		D1605	20,635	12,875	23,271	1
		D1601	12,941	11,462	15,221	0
		D1610	4,609	13,970	6,296	1
	HPV-16 and Other High-Risk Positive	D1360	45,128	12,209	223,088	0
		D1607	42,931	13,878	109,687	0
	Other High-Risk Positive	D1619	0	11,072	815,898	0
		D1613	2	7,716	488,309	1
		D1623	4	16,465	3,141	1
p16 Negative	High-Risk Negative	D1598	13	10,284	172	10
		D1612	7	11,292	230	1
		D1365	4	7,039	45	0
		D1632	2	9,038	127	1
		D1592	3	8,694	154	0
		D1593	1	11,165	248	3
		D1620	2	13,978	52	2
		D1630	1	16,025	33	0
		D1614	1	12,214	70	1
		D1625	1	11,830	35	1
		D1363	1	11,616	59	0
		D1364	5	11,855	95	0
		D1584	6	12,841	180	0
		D1586	0	6,928	217	0
		D1588	7	13,556	183	2
		D1594	2	13,986	211	0
		D1629	0	9,506	143	0
		D1631	2	10,025	58	1
		D1633	1	13,098	134	1
		D1628	3	8,658	172	1
		D1369	0	4,578	25	0
Equivocal	HPV-16 Positive	D1618	18,309	10,105	20,430	0
Not tested	High-Risk Negative	D1608	4	13,083	124	13

1,500,000
1,000,000
500,000
0

Countable PCR provides absolute molecule counts for each target across a six-log dynamic range, enabling quantification of both viral and reference targets.

While p16 immunohistochemistry classifies samples only as positive, negative, or equivocal, the 17-plex Countable PCR panel provides **subtype-specific resolution**, clearly distinguishing HPV-16 and HPV-18 from the other 12 high-risk subtypes. This allows differentiation of samples containing HPV-16 alone, HPV-16 with an additional high-risk subtype (non-16/18), or other high-risk subtypes (non-16/18).

Two samples (D1628 and D1369) labeled as *equivocal* by p16 immunohistochemistry showed quantitative profiles consistent with high-risk negative samples in Countable PCR, illustrating the panel's potential to **resolve ambiguous cases without reflex testing**.

Conclusion

These findings support the use of Countable PCR as a subtype-resolved approach for detecting high-risk HPV DNA. The 17-plex panel addresses key limitations of p16 immunohistochemistry and qPCR, offering analytical sensitivity and multiplex precision suitable for both research and clinical investigation. Key observations include:

- **Direct viral DNA detection**, allowing classification of equivocal p16 immunohistochemistry cases without the need for reflex or *in situ* hybridization testing.
- **Subtype-specific resolution**, distinguishing HPV-16 and HPV-18 from other high-risk subtypes and identifying mixed or non-16/18 infections that may inform future risk stratification.
- **Rapid, streamlined workflow**, producing results within hours and reducing overall turnaround time.
- **Potential future applications**, including saliva-based longitudinal monitoring and precancerous lesion surveillance, where quantitative tracking of viral load may support early detection and patient follow-up.

Learn more about how Countable PCR transforms rare target detection.