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Detecting episomal or integrated human papillomavirus 16 DNA using an exonuclease V-qPCR-based assay

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ABSTRACT

Screening for human papillomavirus (HPV) integration into host cell chromosomes typically requires large amounts of time and reagents. We developed a rapid and sensitive assay based on exonuclease V (ExoV) and quantitative polymerase chain reaction (qPCR) to determine HPV genome configurations in cell lines and tissues. We established the assay using genomic DNA from cell lines known to harbor integrated or episomal HPV16. DNA was incubated with ExoV, which is specific for linear DNA, and the DNA fraction resistant to digestion was measured by qPCR. The percent of DNA resistant to ExoV digestion was calculated relative to undigested DNA for determination of episomal or integrated HPV16. The ExoV assay was accurate, capable of distinguishing episomal from integrated HPV16 in cell lines and tissues. Future applications of the ExoV assay may include screening of HPV genome configurations in the progression of HPV-associated cancers.

1. Introduction

Human papillomaviruses (HPVs) carry a circular, chromatinized double-stranded DNA genome, approximately 8 kb in length, packaged in a non-enveloped capsid. HPV is an epitheliotropic virus completing its entire productive life cycle as a circular episome in the differentiated squamous epithelium. HPV infects basal epithelial cells, gaining access through a micro-abrasion or wound. Within basal cells, HPV is maintained as a low copy, circular episome that is replicated alongside cellular DNA (Pyeon et al., 2009). As infected basal cells divide and exit the cell cycle, host epithelial cell differentiation induces the expression of HPV oncogenes E6 and E7, proteins necessary for S-phase entry (Doorbar, 2005; McCance et al., 1988). An S-phase like environment is critical for the productive phase of the viral lifecycle, defined by the replication of the HPV genome by host machinery, with thousands of circular copies of viral DNA per host cell produced (Bedell et al., 1991). Terminal differentiation stimulates the expression of HPV structural genes L1 and L2, which encapsidate the HPV genome. Progeny virions are then released at the epithelial surface for transmission.

To date, 240 HPV types have been identified (Egawa and Doorbar, 2017). HPV types that infect mucosal epithelia are categorized as either low-risk (non-oncogenic) or high-risk (oncogenic) types. Persistent infection with high-risk types of HPV, including HPV16 and HPV18, is associated with almost all cervical carcinomas and an increasing number of oropharyngeal squamous cell carcinomas (Burd, 2003; de Martel et al., 2017; Gooi et al., 2016; Walboomers et al., 1999). In its associated cancers, HPV integration is frequently observed. While the majority of HPV-positive cervical cancers harbor integrated HPV, the percent of integrated HPV-positive oropharyngeal squamous cell carcinomas is more variable, as the identification of integrated virus is influence by the methods used for detection (Jiang et al., 2015; Olthof et al., 2014; Parfenov et al., 2014; Wiest et al., 2002). *In vitro* studies have also shown that HPV integration occurs in long-term cell culture (Pett et al., 2006). Such integration events result in an abortive HPV infection, with the integrated viral genome no longer available for packaging and transmission to a new host. In addition, HPV integration is thought to contribute to oncogenesis through deregulated expression of viral oncogenes E6 and E7. HPV E6 and E7 inactivate the cell cycle checkpoint proteins p53 and pRB, thereby increasing cell proliferation and genetic instability over time (Groves and Coleman, 2015; McBride and Warburton, 2017; McCance et al., 1988; Moody and Laimins, 2010). The mechanism for how HPV genomes integrate into host chromosomes is not understood. HPV can integrate as either a single copy (type 1 integrant) or as multiple concatemeric copies carrying full-

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length genomes (type 2 integrant). Type 1 integration frequently involves the loss or truncation of the HPV E2 gene, a negative regulator of E6 and E7, resulting in the deregulation of HPV E6 and E7 expression (Groves and Coleman, 2015; McBride and Warburton, 2017).

Identifying the HPV genome status both *in vitro* and *in vivo* is critical to understanding the cellular effects that result from distinct HPV genome configurations and their implication in cancer development, progression, and treatment. Southern blots are the standard used to determine HPV genome configurations. However, Southern blot analysis is not only time and labor intensive, but also requires amounts of DNA that may not be achievable from small tissue biopsies (Hubbard, 2003). Limiting DNA quantity has prompted development of polymerase chain reaction (PCR)–based assays that can be applied to study viral integration in HPV-associated cancers (Carow et al., 2017; Scarpini et al., 2014). Amplification of papillomavirus oncogene transcripts (APOT-PCR) is a standard approach that analyzes changes in the length of HPV transcripts from integrated and episomal viral genomes using reverse-transcription PCR and gel electrophoresis (Klaes et al., 1999). While useful, the APOT assay requires isolation of high-quality RNA and several enzymatic steps for analysis in PCR. In the APOT assay, detection of fused transcripts from integrated HPV forms can be missed when excess episomal forms are present (Dona et al., 2013). Another approach directly detects integrated papillomavirus sequences (DIPS-PCR) using DNA template and ligation-mediated PCR to amplify the viral-cellular junctions of integrated HPV genomes (Luft et al., 2001). Addition of sequencing provides nucleotide-level resolution of the viral sequences lost and allows for assessment of viral clonality by determining the viral integration site on host chromosomes. However, this method is also labor-intensive and does not measure the presence of episomal forms, which are often present with HPV integrants as mixed forms. Whole genome, exome, or RNA sequencing methods overcome these limitations but are expensive and require complex bioinformatic analysis that may not be available to many investigators. A straight-forward method that is often used to determine the HPV physical state in tumor samples measures the ratio of E2/E6 DNA using quantitative PCR (qPCR). This method is based on observations that the E2 region is frequently lost following integration. A limitation of the E2/E6 ratio analysis is distinguishing type 2 HPV integrants from episomal HPV, due to preservation of E2 sequences in type 2 integrants and episomes (Yoshinouchi et al., 1999).

Here, we describe a rapid and sensitive enzymatic approach combined with qPCR to determine the HPV genome status within HPV-positive tissues and cell lines. Genomic DNAs were subjected to digestion by exonuclease V (ExoV), an enzyme that preserves nicked and supercoiled DNAs but degrades linear DNAs. Because episomal genomes are circular and integrated forms are linear, ExoV selectively digests integrated HPV DNA. With qPCR, the fraction resistant to ExoV digestion is calculated as a readout for the presence or absence of episomal HPV genomes. Using the ExoV assay, we have accurately determined the HPV genome configuration within cell lines and tissues grown in organotypic raft culture. The ExoV assay provides a rapid and sensitive method for routine monitoring of HPV16 genomic states that can be applied to both the laboratory and clinical settings.

2. Results and discussion

2.1. Establishment of an ExoV assay to detect circular and linear DNA

To establish a qPCR-based assay capable of distinguishing circular and linear DNAs, we employed exonuclease V (ExoV), which is a RecBCD complex isolated from *Escherichia coli* with an enzymatic activity that preserves nicked and supercoiled DNA but degrades linear DNAs. A mixture of circular plasmid DNA with cellular genomic DNA was digested with ExoV and quantitative PCR (qPCR) was used to measure DNA levels (Fig. 1A). The percent resistance was calculated as the amount of DNA detected after ExoV digestion relative to the amount in undigested samples. A high percent resistance would be consistent with circular DNA, while low percent resistance would indicate linear DNAs digested by ExoV. Specific primers to a 5 kb (luciferase gene) and 10 kb plasmid (Epstein-Barr virus BMRF1 gene), as well as primers specific to 16 kb human mitochondrial DNA (mtDNA) were used in qPCR (Table 1). Primers to human ribosomal 18S DNA (rDNA) were used to measure enzyme processivity and efficiency in degrading linear DNA, as the 18S rDNA locus is a tandemly repeated 45 kB cluster with approximately 400 copies dispersed on 5 chromosomes (Stults et al., 2008).

To establish the assay, we added 5 kb plasmid DNA (pGL3) either directly to purified HaCaT DNA or to HaCaT DNA lysates prior to purification using a Qiagen column. We observed that the 5 kb plasmid showed a 95% resistance to ExoV when added to purified DNA. The percent resistance dropped to 76% when the plasmid was added to the lysate prior to purification (Fig. 1B). The reduction in percent resistance to ExoV could result from breakage of DNA molecules either passing through the column or from residual nuclease or topoisomerase activity in the DNA lysate. To determine if larger DNAs would be more prone to DNA breakage, the experiment was repeated adding a 10 kb plasmid (Epstein-Barr Virus (EBV) BamM). The 10 kb plasmid was 80% resistant to ExoV digestion when added post-DNA purification but was reduced by an additional 18% when purified together with cellular DNA (Fig. 1C). The decrease in percent resistance was proportionately similar to what was observed with the 5 kb plasmid. Endogenous 16 kb circular mtDNA was approximately 52% resistant to ExoV treatment (Fig. 1D). Although some loss of DNA integrity was evident during DNA isolation and purification, the ExoV assay detected circular DNA up to 16 kb in size. In all samples, the ExoV resistant fraction of rDNA was below 4%, confirming the enzyme's efficiency in digesting linear DNA. Together, these data demonstrated the ExoV assay can discriminate circular DNA up to 16 kb in size from linear chromosomal DNA.

2.2. Use of the ExoV assay for detection of circular HPV genomes

Human papillomaviruses have 8 kb genomes that are maintained as circular, chromatinized, extrachromosomal elements (episomes) following infection. The genome remains circular throughout the HPV lifecycle; however, aberrant viral integration is frequently observed in HPV-positive cancers (Parfenov et al., 2014; Pett and Coleman, 2007). Viral integration is also observed following transfection of the viral genome in cell culture, requiring routine monitoring of HPV-immortalized cell lines (Dall et al., 2008; Pett et al., 2006). Here, we tested if the ExoV assay could be applied to determine the HPV genome configuration of HPV-positive cells. We analyzed various HPV16-positive cell lines that included immortalized human foreskin keratinocytes (HFKs) or human tonsillar epithelial cells (HTEs) established by transfection of the HPV16 genome. We also analyzed UMSCC47 cells, a cell line derived from a head and neck tumor known to carry approximately 18 copies of HPV16 integrated into the cellular genome (Akagi et al., 2014; Brenner et al., 2010; Olthof et al., 2015). We compared our ExoV results to Southern blot analysis, the standard laboratory method used to visualize HPV genome configurations (Hubbard, 2003).

For Southern blot analysis, genomic DNA was digested with HindIII, which does not digest the HPV16 genome, or BamHI, which has a single restriction site on the HPV16 genome. Episomal HPV16 is identified by comparing the uncut supercoiled band in HindIII digestion to the linearized 8 kb band following BamHI digestion. Higher molecular weight bands located above the supercoiled band are either nicked/open circular or concatemeric forms of HPV DNA. UMSCC47 DNA digested with HindIII showed an HPV DNA fragment greater than 10 kb that migrated at a higher molecular weight when DNA was digested with BamHI. This pattern was consistent with integrated HPV16, as expected for the UMSCC47 cell line (Fig. 2A). HPV-positive HFKs established following HPV16 transfection showed the expected pattern for circular DNA, with the supercoiled band in the HindIII undigested HPV DNA that migrated

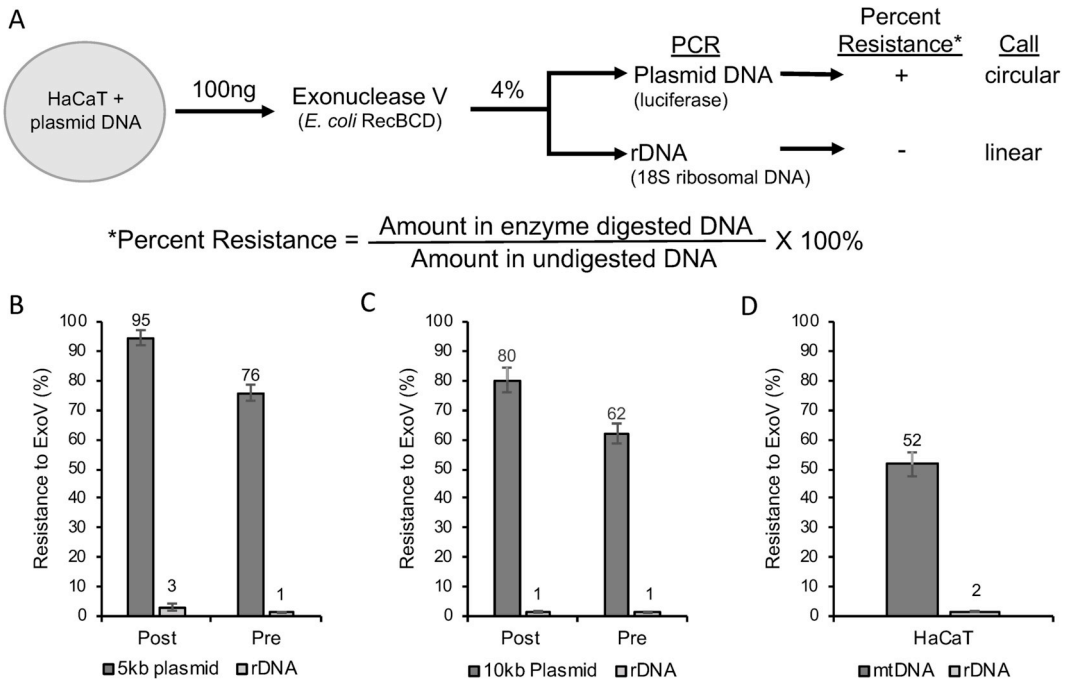

Fig. 1. Establishing size constraints of the Exonuclease V (ExoV) assay with plasmid DNAs. A) Schematic of the ExoV assay. Plasmid DNA served as a circular, episomal DNA, while 18S ribosomal DNA (rDNA) served as a multi-copy linear DNA control. The percent of DNA resistant to ExoV digestion was calculated relative to undigested DNA and used to determine the physical state of the DNA target. B) Purified HaCaT genomic DNA (post) or unpurified HaCaT lysates (pre) were supplemented with $1.4x10^7$ copies of a 5 kb plasmid (pGL3) and analyzed by ExoV digestion. The percent resistance to ExoV digestion for the 5 kb plasmid was calculated in qPCR using primers to the luciferase gene. The average percent resistance and standard error of the mean of 4 biological replicates are shown. C) HaCaT genomic DNA (post) and HaCaT DNA lysates (pre) with $1.4x10^7$ copies of a 10 kb (Epstein-Barr virus BMRF1) plasmid was analyzed in the ExoV assay. The percent resistance to ExoV digestion was calculated in qPCR using primers to the BMRF1 gene. The average percent resistance and standard error of the mean of 3 biological replicates are shown. D) The DNA percent resistance of mitochondrial DNA (mtDNA) to ExoV digestion was determined from purified HaCaT DNA using primers specific to mtDNA. The average percent resistance and standard error of 3 biological replicates are shown.

Table 1
Oligonucleotides used in qPCR analysis.

Gene Name	Sequence (5′-3′)
Luciferase	F: GAAAGGCCCGGCGCCATTCT
	R: TTCATAGCTTCTGCCAACCG
EBV BMRF1	F: CAGGCTGAGGAACGAGCAG
	R: CAACGAGGAAGCCGTCTTG
Ribosomal 18S	F: GCAATTATTCCCCATGAACG
	R: GGGACTTAATCAACGCAAGC
E2	F: CCATATAGACTATTGGAAACACATGCGCC
	R:CTGTAGTTGCAGTTCAATTGCTTGTAATGC
E5	F: TACGTCCGCTGCTTTTGTCT
	R: AACGCAGAGGCTGCTGTTAT
E6	F: GAGAACTGCAATGTTTCAGGACC
	R: TGTATAGTTGTTTGCAGCTCTGTGC
L2	F: TGCATCGGCTACCCAACTTT
	R: ACCCGACCCTGTTCCAATTC

at 8 kb following BamHI digestion (Fig. 2A).

Using the same DNA samples as used in the Southern blots, we analyzed the HPV genome configurations using the ExoV assay with E6-specific primers for amplification in qPCR. In UMSCC47 cells, HPV E6 DNA percent resistance was below 1%, consistent with linear, integrated DNA (Fig. 2B). HPV-transfected HFKs exhibited HPV E6 percent resistance of 63%, reflecting the presence of circular, episomal HPV DNA. rDNA percent resistance remained below 4% for all DNAs analyzed, indicating complete enzymatic digestion of linear DNA. Together these results demonstrate that the ExoV assay can distinguish episomal and integrated HPV-positive epithelial cell lines.

2.3. The ExoV assay confirmed the HPV16 genome status in organotypic raft tissues

As the ExoV assay requires low amount of input DNA, analysis of HPV in tissues could be used as a screening approach for HPV integration of fresh biopsy samples in clinical and laboratory settings. We examined if the ExoV assay can be applied to monitoring HPV genome configurations in tissue samples using HPV16-positive human tonsillar epithelial cells (HTE) grown in organotypic raft culture (Asselineau and Prunieras, 1984; Meyers et al., 1992). Prior to growth in organotypic raft culture, the HPV genome configuration for HPV-positive HTE cell lines grown in monolayer was determined by Southern blot. HTE cell line 16D contained episomal HPV16, while 16E contained integrated HPV16 (Fig. 2C). Using the ExoV assay examining the HPV16 E6 region, the HTE 16D rafts showed an average 71% resistance indicative of circular, episomal HPV genomes in three raft tissues tested (Fig. 2D). In contrast, HPV E6 percent resistance was below 1% for HTE 16E rafts, as expected for linear, integrated HPV (Fig. 2E). rDNA percent resistance for all rafts tested using the ExoV assay was below 9%, confirming ExoV enzymatic activity (Fig. 2D and E). We extended our analysis to include immortalized HFK cell lines that had been infected with replication-competent or defective HPV genomes grown in organotypic raft culture. Replication-defective HPV with a translation termination linker (TTL) mutation in E1 or E2 E39A mutation are unable to be maintained episomally, such that immortalized cell lines have integrated HPV. For the HPV16 E1 and E2 mutants examined, a low HPV E6 DNA copy number was observed in undigested samples, consistent with cell lines carrying an integrated HPV genome. Cells infected with wildtype virus maintain HPV as a circular episome, although integration can also occur. However, growth in raft culture would replicate/amplify the episomal DNA, which would not occur with cases with integrated DNA.

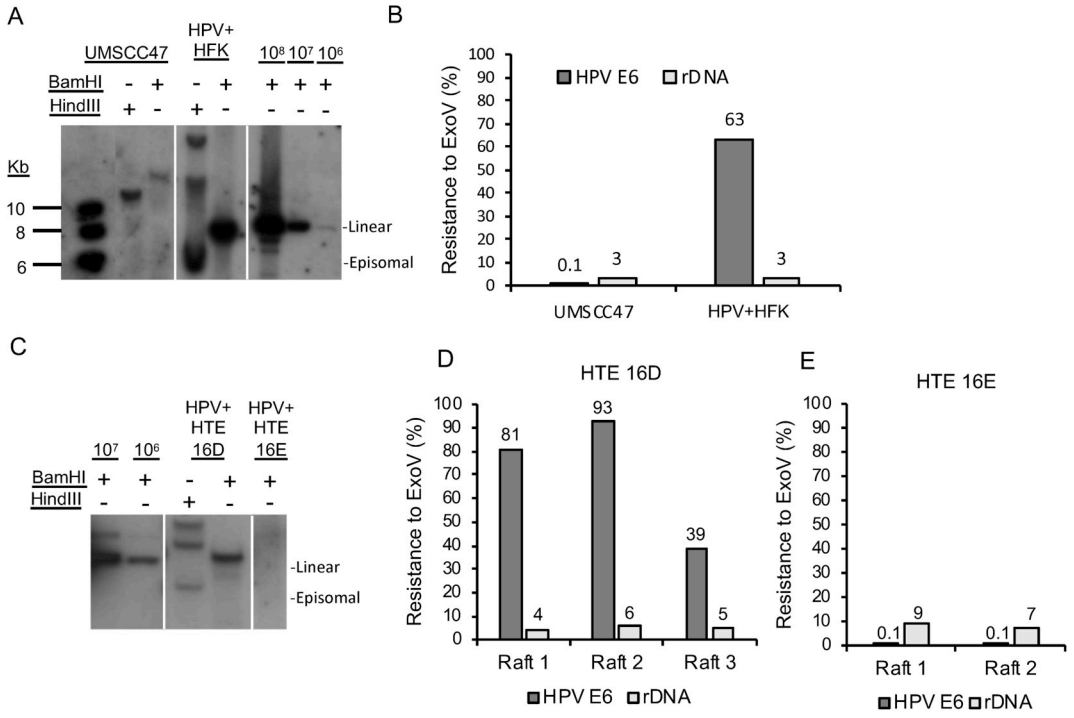

Fig. 2. Application of the ExoV assay to monitor the physical state of the HPV16 genome in HPV16-positive keratinocytes grown in monolayer and organotypic raft culture. A) Genomic DNA from UMSCC47 and HPV-transfected (HPV + HFK) grown in monolayer were digested with BamHI or HindIII and analyzed by Southern blot. White lines depict where the image was cropped. Samples were compared to $7x10^6$,$7x10^7$,$7x10^8$ copies of an HPV16-positive pUC plasmid linearized with BamHI as copy number controls (equivalent to 10, 100, 1000 HPV copies per cell). B) Percent resistance to ExoV digestion of HPV16 E6 DNA from UMSCC47 and HPV + HFK cell lines. C) Southern blot analysis of HTE 16D and 16E cells grown in monolayer. DNAs were either digested with BamHI or HindIII. An HPV16-positive pUC plasmid digested with BamHI loaded at $7x10^6$,$7x10^7$,$7x10^8$ copies served as copy number controls. White lines depict where image was cropped. HTE16E image was taken from a longer exposure. D) Percent resistance to ExoV digestion of HPV16 E6 DNA from the HTE cell line 16D carrying episomal HPV grown in three separate organotypic raft cultures. E) Percent resistance to ExoV digestion of HPV16 E6 DNA from the HTE cell line carrying integrated HPV16 grown in two separate organotypic raft cultures.

Table 2
ExoV analysis of raft DNA derived from keratinocytes infected with wildtype or replication-defective HPV16.

Samples (DNA)	% E6 DNA Resistance	% rDNA Resistance	Undigested DNA (Ratio)			HPV State
			E2/E6	E5/E6	L2/E6	
WT -1	59	2.2	1.2	1.1	1.1	Episomal
WT -2	85	3.4	1.2	1.1	1.3	Episomal
WT -3	66	4.5	1.1	1.1	1.2	Episomal
E1 TTL -1[a]	1.5	2.1	0.4	0.3	0.3	Integrated
E1 TTL -2[a]	2.4	8.5	0.6	0.3	0.3	Integrated
E1 TTL -3[a]	1.8	7.6	0.4	0.4	0.3	Integrated
E2 E39A -1[a]	3.7	2.1	0.3	0.3	0.2	Integrated
E2 E39A -2[a]	1.6	3.0	0.6	0.7	0.7	Integrated
E2 E39A -3[a]	1.3	2.0	0.3	0.3	0.2	Integrated
UMSCC47	0.9	2.5	1.5	1.1	1.4	Integrated

[a] Ct values around 30 in undigested DNA samples.

Indeed, analysis of wildtype HPV-infected HFK cell lines grown as rafts showed resistance to ExoV digestion that ranged from 59 to 85% (Table 2). In contrast, HFK cell lines infected with E1 or E2 replication-defective mutants showed percent resistance to ExoV typically below that of rDNA, indicating that HPV was integrated, as expected, in these rafted samples (Table 2). Thus, the ExoV assay can be used to determine HPV16 genome configurations in fresh tissue samples and may be applied to monitoring the HPV16 genome status in patient samples.

2.4. The ExoV assay is unaffected by multiple rounds of DNA thawing

The detection of circular DNA using the ExoV assay depends on the integrity of the sample. Introduction of double-stranded DNA breaks from tissue or DNA storage and processing would result in loss of circular templates. Repeated thawing and freezing of DNA samples can result in DNA degradation and compromise the accuracy of the ExoV assay (Shao et al., 2012). To determine how freeze/thaw cycles affect the detection of circular DNA by the ExoV assay, we subjected HPV-transfected HFK genomic DNA supplemented with the 5 kb plasmid to 10 freeze/thaw cycles. We observed little reduction in the detection of the 5 kb or the HPV episomal DNA (Fig. 3). Larger circular 16 kb mtDNA showed a slight reduction in the percent resistance to ExoV only after 6 freeze/thaw cycles. Our results indicate that small circular DNA, including the HPV16 genome, can tolerate several freeze/thaw cycles. Thus, frozen samples can be analyzed using the ExoV assay. Addition of a plasmid reporter at the time of DNA harvest can serve to monitor DNA integrity over time. Although not tested, it is unlikely that the ExoV assay will work with formalin fixed, paraffin embedded (FFPE) tissues as DNA degradation occurs during storage that would compromise detection of any circular episomal DNA (Guyard et al., 2017).

2.5. Detection of mixed HPV forms using the ExoV assay

Mixed HPV forms consisting of both episomal and integrated HPV genomes can be difficult to differentiate by a number of established assays. In addition, HPV can integrate in a concatemeric configuration, where multiple genomes are joined in a head-to-tail repeated manner (type 2 integrant) that can be difficult to distinguish from circular intact genomes in various assays. To determine if the ExoV assay can quantify mixed HPV forms, DNAs previously supplemented with the 5 kb plasmid carrying either type 2 integrated DNA from UMSCC47 cells or episomal DNA from HPV16 + HFK cells were mixed at various ratios (Fig. 4A). The 5 kb plasmid DNA was used as a standard for DNA

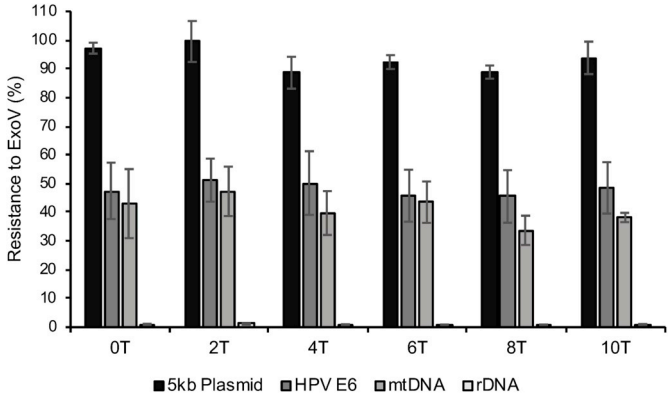

Fig. 3. Effect of frozen storage on sensitizing circular, episomal DNA to ExoV digestion. A) Genomic DNA from HPV-transfected HFKs supplemented with $1.4x10^7$ copies of the 5 kb plasmid (pGL3) was frozen at −80 °C for 30 min and thawed on ice for 15 min up to 10 rounds. The percent resistance to ExoV digestion was calculated for the 5 kb plasmid, HPV16 E6 DNA, mitochondrial DNA (mtDNA), and 18S ribosomal DNA (rDNA) at every second interval. The average percent resistance and standard error of the mean from 3 biological replicates are shown.

integrity. The percent resistance of the 5 kb plasmid ranged between 59% and 73%, with the inconsistency likely due to slight pipetting errors during mixing of the spiked DNA samples. HPV16 E6 DNA showed an increase in the ExoV percent resistance that was proportional to the ratio of circular/episomal DNA versus linear/integrated DNA, reaching a maximum percent resistance of 46% for the HPV-positive HFK DNA. (Fig. 4A). The proportional increase in HPV E6 DNA showed a linear correlation with an R^2 value of 0.97 (Fig. 4B).

The HPV-positive HFK cell lines examined showed on average a ~50% resistance to ExoV when grown as a monolayer (Figs. 2 and 3). The reason for incomplete percent resistance in these samples may reflect loss of DNA integrity of the HPV episome due to isolation of DNA from HFK cell lines or represent pooled populations containing both integrated and episomal HPV. Considering these caveats, the ExoV assay is unable to estimate the absolute amount of episomal DNA versus integrated DNA. Instead, the ExoV assay can be simply used for detecting if a sample carries episomal or integrated HPV DNA. The percent resistance for the linear rDNA would be used to set the value for discrimination of linear from circular DNA in a given sample.

2.6. Accuracy of ExoV assay in determining the physical state of the HPV genome compared to other established methods

To determine the accuracy of the ExoV assay as a screening tool for analyzing HPV genome configurations, a panel of HPV-positive cell lines was evaluated. Three HFK lines were generated by HPV infection (HFK WT infected) using the extracellular matrix to cell transfer method as previously described (Bienkowska-Haba et al., 2018). Eight HFK lines were generated by transfection of HPV16 genomes (wildtype, WT, or E5 TTL mutant). UMSCC47 was included as a control for integrated HPV DNA (Table 3). Southern blot analysis showed 7 cell lines as having integrated HPV16, and 5 cell lines showed an episomal banding pattern (Figs. 2 and 5, Table 3). For the ExoV assay, samples were called episomal if the E6 DNA percent resistance was at least 10% and 3-fold above that of rDNA. ExoV analysis confirmed all 5 episomal samples by Southern blot analysis (HFK lines 1, 2, 4, 5, 8; Table 3). ExoV analysis of samples with integrated HPV16 DNA by Southern blot identified 5/7 cell lines as having integrated HPV16 DNA (UMSCC47, HFK lines 3, 6, 7,10; Table 3). However, ExoV analysis called HFK lines 9 and 11 positive for episomal HPV16 DNA with 20% and 29% resistance to ExoV digestion, respectively. In both cases, the rDNA percent resistance was below 3. To ensure that the episomal E6 DNA signal represented intact genomes, we also measured the percent resistance of E2, E5, and L2 regions of the HPV genome. All samples, including HFK lines 9 and 11, showed a similar percent resistance across the HPV genome, further validating the episomal and integrated calls based on the ExoV assay (Table 3). Overall, the ExoV assay showed an 83% concordance in detecting episomal and integrated HPV16 DNA compared to Southern blot analysis.

The ExoV assay is highly sensitive to detection of episomal DNA, raising the possibility that the episomal calls for HFK lines 9 and 11 were false positives. However, Southern blot analysis has its own set of limitations that include an inability to detect low copy number episomes (with a limit of ~10 copies per cell, Fig. 5). To address this concern, we measured the E6 DNA copy number in undigested DNA samples. HFK lines 9 and 11 had a low copy number near or below the limit of detection on the Southern blot, indicating that the discrepancy is likely a failure of the Southern blot in detecting episomal signals for these samples (Table 3).

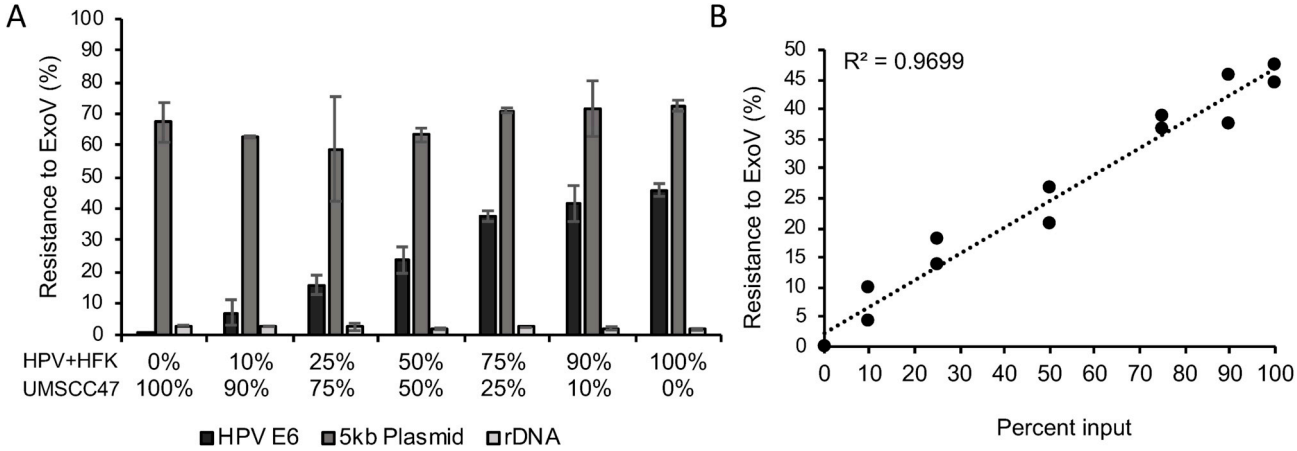

Fig. 4. Estimating the limit of detection of episomal HPV16 DNA in the ExoV assay. A) Genomic DNA from HPV-transfected HFKs containing episomal HPV were mixed with DNA from UMSCC47 cells containing integrated HPV. Before mixing, HFK and UMSCC47 genomic DNAs were supplemented with 5 kb (pGL3) plasmid. The average percent resistance to ExoV digestion is shown for the 5 kb plasmid, HPV16 E6 DNA, mitochondrial DNA (mtDNA), and 18S ribosomal DNA (rDNA). Error bars represent the standard deviation of the mean from 2 biological replicates. B) A linear correlation in the percent resistance of HPV16 E6 DNA relative to the percent input was observed. Each point represents the percent resistance to ExoV digestion of 2 technical replicates from 2 independent biological experiments.

Table 3
Accuracy of ExoV assay in determining the physical state of the HPV genome compared to other assays.

Cell Line	Southern Blot Analysis	HPV Copy Per Cell[a]	ExoV digested DNA Percent Resistance[b]					Undigested DNA (Ratio)			Combined Analysis
			E6	E2	E5	L2	rDNA	E2/E6	E5/E6	L2/E6	
UMSCC47	Integrated	18	0.1	0.1	0.2	0.1	4.2	1.0	0.9	0.8	Integrated Type 2
1 HFK WT infected	Episomal	26	36	35	42	43	1.5	1.1	1.0	1.6	Episomal
2 HFK WT	Episomal	70	15	11	14	10	1.4	0.8	0.8	0.8	Episomal
3 HFK WT	Integrated	0.1	0	6	0	0	0.8	0.03	0.08	0.01	Integrated Type 1
4 HFK WT infected	Episomal	41	22	18	20	19	5.7	0.9	0.8	0.8	Episomal
5 HFK WT infected	Episomal	58	19	13	15	13	2.7	0.9	0.9	0.9	Episomal
6 HFK WT	Integrated	0.4	1	2	1	1	2.5	0.9	0.9	0.9	Integrated Type 2
7 HFK E5 mutant	Integrated	5	4	3	5	4	1.1	0.9	0.7	0.9	Integrated Type 2
8 HFK E5 mutant	Episomal	37	44	34	43	42	1.3	1.0	1.0	1.0	Episomal
9 HFK E5 mutant	**Integrated**	**3**	**20**	**14**	**24**	**22**	**0.8**	**1.0**	**0.8**	**0.8**	**Mixed**[c]
10 HFK WT	Integrated	3	1	1	3	2	3.8	1.2	0.3	0.2	Integrated Type 1
11 HFK E5 mutant	**Integrated**	**15**	**29**	**21**	**27**	**26**	**2.3**	**1.1**	**0.8**	**1.0**	**Episomal**

[a] HPV copy number per cell was calculated from the ratio of relative HPV E6 DNA to human ribosomal 18S DNA and normalized to UMSCC47 HPV copies per cell.
[b] Episomal calls were based on percent resistance for HPV (E6, E2, E6, and L2) being greater than 10% and being at least 3-fold over rDNA.
[c] Mixed calls were determined by having less than 1 episome per cell (ExoV percent resistance times the HPV copies per cell).

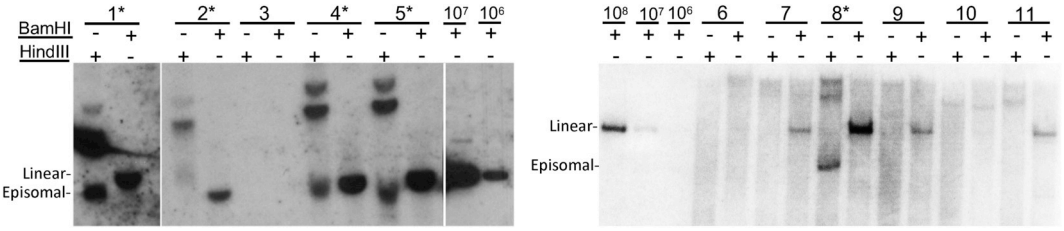

Fig. 5. Southern blot analysis to determine the HPV16 genome configuration in a panel of HPV-positive keratinocyte cell lines. Southern blot analysis of genomic DNA from HFKs either infected with wild type HPV (samples 1, 4, 5) or transfected with wild type or mutant HPV genomes (samples 2, 3, 6–11) were digested with BamHI or HindIII. An HPV16-positive pUC plasmid was digested with BamHI and loaded at $7x10^6$,$7x10^7$,$7x10^8$ copies as copy number controls (equivalent to 10, 100, and 1000 copies per cell). Sample 1 was run on a separate gel; while copy number standards and samples 2–5 were run on the same gel. *represents samples with episomal HPV on Southern blot analysis.

Loss of HPV viral sequences can occur following integration, with the ratio of E2 to E6 DNA used to determine the HPV genome state in clinical samples. Thus, samples with episomal HPV retain E2 and E6 with a ratio greater than 0.9, while integrated HPV samples with a loss of E2 DNA have a E2/E6 ratio less than 0.1 (Boulet et al., 2009; Choi et al., 2018; Lorenzi et al., 2017). Using this analysis, most HPV-positive cell lines met the criteria for having episomal DNA (ratio > 0.9 for E2/E6; Table 3). However, this method cannot distinguish a type 2 integrant from episomal HPV as observed for the UMSCC47 cell line, known to harbor type 2 integrated HPV16 DNA (Table 3). We included the copy number ratio of HPV16 E2, E5, and L2 regions to detect integration at other sites of the HPV genome. A loss of viral sequences relative to E6 was observed for HFK cell lines 3 and 10, consistent with viral integration. The HFK line 10 showed that viral integration retained E2 but lost the E5 and L2 region of the HPV genome, which was consistent with the absence of a signal for full length genomes on the Southern blot. For HFK lines 9 and 11, the ratio analysis was inconclusive and showed ratios consistent with episomal or type 2 integrated HPV. However, if we combined the ExoV percent resistance and copy number analysis, HFK line 9 had less than 1 copy of episomal HPV per cell, likely being a mixed sample with integrated and episomal DNA. HFK line 14 had at least 3 copies of episomal HPV per cell, illustrating the increased sensitivity of the ExoV assay in detecting samples with episomal DNA when compared to Southern blotting.

3. Conclusions

While it is not a prerequisite for cancer progression, HPV integration has been observed in a majority of HPV-positive cervical cancers and a portion of oropharyngeal squamous cell carcinomas (McBride and Warburton, 2017; Pett and Coleman, 2007). HPV has also been observed to frequently integrate in cell culture as cells are passaged in the laboratory over time. Southern blots are routinely used to screen for HPV genome configurations. However, Southern blotting requires amounts of DNA that may not be achievable from small tissues biopsies or tissues grown in culture, while also being labor and time intensive. To rapidly and efficiently determine HPV genome configurations within tissues and cell lines, we developed a simple ExoV-qPCR based assay that is rapid and sensitive, requiring nanogram quantities of genomic DNA. The ExoV assay, when combined with copy number analysis of the HPV genome, accurately detected episomal and integrated forms of HPV in both cell lines and tissues grown in organotypic raft culture.

A limitation of the ExoV assay is that it may be prone to calling samples as having episomal DNA (false positives) due to its high sensitivity in detecting circular DNA. As such, samples should be extracted in clean work areas and negative controls included to monitor for unintended plasmid contamination. Any plasmids carrying the target region amplified in PCR would confound the data and increase the false positive calls. As a sentinel for contamination, HaCaT DNA was purified in parallel with most of the samples analyzed and was negative for HPV in our PCR assays. In addition, we improved the accuracy of the ExoV assay in identifying episomal samples by measuring the copy number at various regions across the HPV genome, which informed on the structure of the HPV DNA, monitored potential contamination, and validated the intact state of episomes detected. Such analysis has biological implications as a recent RNA-seq study identified structural variants that were consistent with being HPV episomal hybrids carrying partially deleted viral DNA interspersed with human DNA in head and neck tumors (Nulton et al., 2017). Although we observed that small genomes like HPV were rather stable tolerating several rounds of freeze/thawing,

addition of an unrelated plasmid could be used to monitor DNA integrity of stored samples. Although the ExoV assay was only applied to screening the HPV genome, the assay can be adapted to examine other small DNA viruses adopting circular, episomal configurations in their life cycles. Future applications of the ExoV assay may include screening of HPV genome configurations in the progression of HPV-associated cancers.

4. Materials and methods

4.1. Cell and organotypic raft culture

Human tonsillar epithelial cells (HTE) and human foreskin keratinocytes (HFK) were isolated and transfected with the HPV16 genome as previously described using cre-loxP recombination to generate circular full length HPV16 genomes (Bodily et al., 2011; Guidry et al., 2019; Wilson and Laimins, 2005). HPV-infected HFKs were generated from extracellular matrix enhancement of infection as previously described (Bienkowska-Haba et al., 2018). Immortalized outgrowths represent a pooled population. HFKs were co-cultured with mitomycin C-treated NIH 3T3 J2 feeder fibroblasts and grown in E medium supplemented with 5% fetal bovine serum (FBS, GIBCO) as previously described (Meyers and Laimins, 1994). HTEs were also co-cultured with mitomycin C-treated NIH 3T3 J2 feeder fibroblasts and grown in E medium supplemented with 5% FBS and 10 μM Rho kinase inhibitor Y-27632 (Tocris). 3T3 J2 fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum (HyClone). The 3T3 J2 fibroblasts were treated with 8 μg/mL of mitomycin C (Santa Cruz) in 10% FBS-supplemented DMEM for 2–4 h, washed with 1X PBS three times, and kept in 10% FBS-supplemented DMEM. Treated J2 fibroblasts were used within 2 weeks after mitomycin C treatment. UM-SCC-47 (UMSCC47) cells were cultured in DMEM + GlutaMAX (Gibco, 1 g/L D-glucose, 110 mg/L sodium pyruvate) supplemented 10% FBS (Brenner et al., 2010). HaCaT cells were cultured in DMEM + GlutaMAX supplemented with 5% FBS. All cells were grown in a 37 °C humidified incubator supplied with 5% CO_2. Organotypic rafts were cultured as previously described (Guidry et al., 2019).

4.2. Plasmid and genomic DNA isolation and ExoV digestion

Plasmid DNA was isolated using the NucleoSpin Plasmid kit (Macherey-Nagel). All DNA isolations followed manufacturer's instructions. Total cellular DNA from rafts, UMSCC47, and HaCaT cells were isolated using the QIAamp Blood Mini Kit (Qiagen). HTEs and HPV-transfected HFKs grown in monolayer were isolated using phenol chloroform extraction and ethanol precipitation (Fehrmann et al., 2003). HPV-infected HFK DNA was isolated using the Nucleospin Blood Quick Pure kit (Macherey-Nagel). Approximately, $7x10^8$ copies of plasmid DNA were added to purified genomic DNA isolated from $5x10^5$ HaCaT cells, (equivalent to ~5 μg (μg) added post-purification) or to $5x10^5$ HaCaT cells lysed in 200 μl of Qiagen AL lysis buffer and 200 μl 1X phosphate-buffered saline (PBS, Corning) that was subsequently purified using a QiaAmp Blood mini column (pre). Genomic DNA was stored in 10 mM tris buffer at 4 °C to avoid damage by freeze-thawing DNA. For ExoV digestion, 100 ng of genomic ± plasmid DNA was treated with or without 3.3 units of ExoV (RecBCD, NEB) in a 10 μl reaction volume and incubated for 1 h at 37 °C. Digestion was followed by heat inactivation at 95 °C for 10 min. DNA was then kept on ice or stored at −20 °C until qPCR analysis. In the mixing experiment, approximately 5 μg of either UMSCC47 or HPV-positive HFK genomic DNAs were supplemented with $7x10^8$ copies of the 5 kb plasmid and then mixed at ratios starting at 10% HFK + 90% UMSCC47 up to 100% HFK or UMSCC47, with the final DNA amount totaling 100 ng.

4.3. Southern blot analysis

Southern blots were prepared as previously described (Bodily et al., 2011; Fehrmann et al., 2003; Southern, 1975). Briefly, 5 μg or 10 μg of total cellular DNA was digested with either BamHI (cuts HPV16 once) or HindIII (does not cut HPV16) restriction enzymes (NEB) and resolved on a 0.8% agarose gel with 0.5 μg/ml of ethidium bromide. $7x10^6$, $7x10^7$, and $7x10^8$ copies (equivalent to 10, 100, and 1000 copies per cell) of an HPV16-positive pUC plasmid linearized with BamHI was included on the Southern blot analysis for estimation of viral copy number. DNA was transferred onto a nylon membrane (GE Water and Process Technologies or GeneScreen Plus) and hybridized with full length HPV16 genome labeled with α-^{32}P dCTP using the Rediprime II Random Prime Labelling System (Amersham Biosciences) or the Invitrogen RadPrime DNA Labeling System according to the manufacturer's instructions. Radioactive signals were visualized by autoradiography (Amersham Hyperfilm).

4.4. qPCR analysis

DNA from a 4 ng equivalent (4 μl of 1:10 dilution from the ExoV digested/undigested reaction) was quantified by qPCR using a 7500 FAST Applied Biosystems thermocycler with SYBR Green PCR Master Mix (Applied Biosystems) and 300 nM of each primer in a 15 μl reaction (Primer sequences in Table 1). qPCR cycling conditions were as previously described: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, followed by melting curve analysis (Bienkowska-Haba et al., 2018; Guidry et al., 2019). No template controls (HaCaT alone or water as template) were included to control for PCR contamination. Relative DNA amounts were calculated based on a standard curve generated using a 10-fold dilution spanning 5 logs that started at 100 ng of either undigested UMSCC47 (Figs. 1–3) or HaCaT DNA containing 1.4 x 10^7 copies of pUC plasmid containing the full HPV16 genome (Fig. 4 and Tables 2 and 3). The efficiency of primers was accounted for in the standard curve calculations, with all qPCRs having slopes ranging between −3.1 and −3.4. The percent resistant fraction to ExoV digestion was calculated from amount of DNA relative to the standard curve after ExoV digestion divided by amount of DNA relative to the standard curved in the undigested sample times 100. The copy number was calculated based on the standard curve generated from a 10-fold dilution series of HaCaT DNA supplemented with an HPV16 positive plasmid for E6, E2, E5, and L2 primers. HPV copy number per cell was relative to the rDNA (18S) copy number, and normalized to UMSCC47 DNA, set at 18 copies per cell (Akagi et al., 2014; Olthof et al., 2015).

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