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A Human Papillomavirus Whole Genome Plasmid Repository: A Resource for HPV DNA Quality Control Reagents

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Abstract

Well characterized reference reagents are useful for assay validation, proficiency/competency assessment, daily run controls, and to improve inter-laboratory comparisons. Synthetic human papillomavirus (HPV) DNA fragments and plasmid clones are available, but synthetic fragments include limited segments of the HPV genome and many HPV plasmids have interrupted coding regions or contain partial genomes. As a result, they are not compatible with all HPV DNA detection and typing assays. To address this need, we are establishing an HPV plasmid repository of HPV clones containing the whole genome of each type with no interruptions in coding regions. To date, HPV plasmid clones for 16 HPV types, (including all vaccine types and 14 types in clinical assays: HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) have been constructed using a Gibson assembly method and validated by sequencing and the Novaplex HPV typing assay. The newly constructed HPV whole genome plasmids can serve as a quality control reagent resource for HPV DNA assays and are available for public health and research laboratories.

Keywords

human papillomavirus; whole genome plasmid; quality control reagents; HPV typing

Introduction

Human papillomaviruses (HPVs) are double-stranded circular DNA viruses with a genome of approximately 8 kilobase pairs [1]. HPV DNA detection and typing using molecular assays are used in routine surveillance and vaccine impact monitoring studies. Clinical HPV DNA tests are used in cervical cancer screening [2, 3]. The growing interest in HPV DNA tests for diagnosis of HPV infection is reflected by commercial availability of more than 200 HPV DNA tests as of 2020 [2]. However, peer-reviewed publications describing

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Data and Material Availability: CDC-generated HPV plasmids are available to national and international researchers/institutions for non-commercial use under standard materials transfer agreement and can be requested by emailing to Hem Thapa (qkt1@cdc.gov) or Troy Querec (hep0@cdc.gov). Nucleotide sequences for CDC-generated HPV plasmids are provided as a supplementary excel file.

Disclaimer: The findings and conclusion in this study is of authors and do not necessarily represent the official position of the Centers for Diseases Control and Prevention. All authors declare no potential conflicts of interest.

analytical and/or clinical evaluation are not available for 82% of commercial HPV tests [2]. Since laboratories use different assays for HPV detection and typing, quality assurance is required to ensure HPV typing results are accurate, reproducible, and comparable [4]. Well characterized HPV DNA reference reagents are important to allow laboratories to evaluate and compare assays, to monitor performance, and to assess competency of laboratorians [5, 6]. However, a natural source of biological material to generate type-specific HPV reference reagents is not available [7]. Clinical samples are by nature limited in quantity and often have mixed infection with multiple HPV types, making them an unsuitable resource for reference reagents. Furthermore, HPV DNA reference reagents from culture models or human cell lines containing full-length HPV genomes are not available for all HPV types.

HPV experts recommended use of recombinant plasmids containing full-length HPV genomes formulated in a background of human genomic DNA as an alternative source of HPV DNA reference reagents [5, 7]. Plasmid-based international standards for HPV were first established in 2008 for HPV16 and HPV18 with additional standards developed in 2019 for 7 HPV types (HPV6, 11, 31, 33, 45, 52, 58) targeted by vaccines [5, 8]. These international standards are intended to be used as a secondary reference calibrator to allow in-house reagents to be quantified in international units to allow uniform reporting of the results independent of the assay used for HPV detection [8]. Although plasmids for nine HPV international standards contain full-length genome of individual HPV types, plasmids for seven HPV types (HPV6, 11, 16, 18, 31, 33 and 58) have interruptions at HPV genes, and vector backbones used for cloning vary between HPV types. For example, the HPV16 international standard plasmid contains the full-length HPV16 genome but has the L1 gene split into two regions of the vector backbone (Figure S1) [5].

Additional HPV plasmids are available but many of these plasmids including ones received in our lab (Table 1) have interruptions at coding regions or contain a partial HPV genome, and HPV inserts are cloned into different vector backbones. Synthetic HPV DNA fragments are used as an alternative source of control reagents in HPV assays [9], but they typically include only a small portion of the HPV genome. Since HPV DNA detection and typing assays target various HPV coding genes, HPV plasmids with gene interruptions may be unsuitable for some assays. This is particularly problematic for proprietary assays that do not provide details on targeted regions. Availability of uninterrupted HPV full-length genome plasmids would solve this problem. We describe our approach and progress in establishing a standardized HPV plasmid repository of clones containing the uninterrupted full-length HPV genome in a standardized vector.

Materials and Methods

HPV DNA templates for construction of CDC HPV Plasmids

HPV plasmids received in our lab from other institutions (hereafter referred to as non-CDC HPV plasmids) and synthetic DNA fragments were used as DNA templates for construction of HPV whole genome plasmids ((hereafter referred to as CDC HPV plasmids). Detail information about HPV DNA templates is described in the Supplementary Information.

Plasmid Maps

Plasmid maps were generated with SnapGene software (GSL Biotech, USA) using vector backbone sequences available from SnapGene's website and based on the information available for individual vector backbones from the literature. The maps of both non-CDC and CDC HPV plasmids contains HPV sequences confirmed by Sanger sequencing. The annotation and numbering of nucleotide position of HPV sequence is based on the HPV reference genome sequence available from the PapillomaVirus Episteme (PaVE; https://pave.niaid.nih.gov/) database [10].

Construction of pGEMT Easy-01 plasmid

The pGEMT Easy-01 plasmid used in this study as vector DNA template for construction of CDC HPV plasmids was derived from pGEMT Easy vector (Promega, USA). The pGEMT Easy vector is supplied by the manufacturer as a DNA fragment linearized at base 60 with EcoRV restriction enzyme and a T nucleotide added to both 3' ends. The vector was circularized in a 10 μL ligation reaction using reagents from the pGEMT vector system that contained 50 ng of pGEMT Easy vector, 5 μL of ligation buffer, 1 μL of T4 ligase and 2.5 μL of 2x GoTaq master mix (Promega, USA). The ligation reaction was carried out at 25°C for two hours, reaction product transformed into Mix and Go *E.coli* DH5α strain (Zymo Research) and positive clones were confirmed by sequencing. The circularized pGEMT Easy-01 vector contains additional two nucleotides at base 61 and 62 when compared to linearized pGEMT Easy vector (Figure S2).

Construction of CDC HPV whole genome plasmids

HPV whole genome plasmids were constructed by assembly of overlapping DNA fragments of HPV and vector backbone using NEBuilder HiFi DNA assembly master mix kit (New England Biolabs, USA) following the manufacturer's recommendation but with modifications. Laboratory workflow for construction of HPV whole genome plasmids is shown in Figure 2 using HPV58 as an example and the detail method is described in the Supplementary Information.

Validation of CDC HPV plasmids using PGMY primers

HPV plasmids were tested with PGMY09/11 primers followed by agarose gel electrophoresis as described before [11]. PCR experiments were done in a reaction volume of 20 μL and contained 10 ng of HPV plasmids as template, 0.25 μM of five forward (PGMY11A-E) primer mix, 0.25 μM of thirteen reverse (PGMY09-F-HMB01) primer mix, and 10 μL of 2x PrimeSTAR Max DNA polymerase master mix. The PCR conditions were as follows: initial denaturation at 98°C for 2 min, 33 cycles of 98°C for 15 sec, 55°C for 15 sec, extension at 72°C for 5 sec to amplify 450 bp amplicon or 50 sec with non-CDC HPV58 plasmid, and final extension at 72°C for 5 min.

Validation of CDC HPV plasmids using Novaplex II HPV28 Detection Assay

HPV whole genome plasmids were tested using Novaplex II HPV28 Detection Assay (Novaplex; Seegene Technologies, USA). The Novaplex is real-time multiplex fluorescent-based PCR assay that allows for identification of 28 HPV types (HPV6, 11, 16, 18, 26, 31,

33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68, 69, 70, 73, and 82) using Seegene's propriety technology. HPV plasmids were diluted in nuclease-free DNA suspension buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA) containing 20,000 copies/μL human genomic DNA (high molecular weight DNA from blood; Roche Diagnostics) to generate three different concentrations (100, 10, and 2 copies/μL) of plasmid DNA. HPV typing with Novaplex was done using 5μL of HPV plasmids at different concentrations or control samples (human genomic DNA at 20,000 copies/μL or DNA suspension buffer) using endpoint-CMTA program, and results were interpreted by following the manufacturer's protocol. The Novaplex assay was repeated at least three times to check for reproducibility.

Results

Analysis of non-CDC HPV plasmids

Non-CDC HPV plasmids for 16 HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) received in our lab were sequenced to determine the HPV inserts and generate maps for each plasmid (Figures 1, 3 and S3-S16). The inserts were cloned in 11 different plasmid backbones. As shown in Table 1, plasmids for 15 HPV types contain full-length genomes with HPV35 partial genomes cloned into two different plasmids. Several nucleotide substitutions were identified in 10 types compared to the HPV GenBank accession with the highest level of sequence identity. With the except of HPV45, HPV52 and HPV66, interruptions were identified in at least one HPV gene. For example, the full-length HPV58/pLink plasmid has an interruption in the L1 gene that is split into two regions by the vector backbone (Figure 1B). Amplification of HPV58/pLink plasmid with PGMY primers, targeting a conserved region of L1 gene, generated a 4.2 kb amplicon, approximately 10 times larger than the amplicon from a plasmid containing the contiguous HPV58 L1 gene (Figures 1C-1D).

Construction of CDC HPV whole genome plasmids

The laboratory workflow for construction of HPV whole genome plasmids, shown in Figure 2, was successful for the 16 HPV types attempted to date (Figures 3 and S3-S16). However, HPV31 was challenging as propagation of the Gibson assembly reaction products into DH5α cells resulted in bacterial clones containing truncated versions of HPV31 with HPV inserts ranging from 625 to 1568 bp (Figure 3). This problem was solved by changing the cell line for propagation of the HPV 31 Gibson assembly products to *E.coli* Stbl2 cells. To date uninterrupted whole genome plasmids were constructed for 16 HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) using same pGEMT Easy-01 vector backbone (Table 1) and sequence verified by Sanger sequencing. Large scale purification of these HPV plasmids was achieved with DNA yields ranging from 145 to 770 μg per 300 ml of *E.coli* culture (Table S2).

Validation of CDC HPV plasmids with PGMY primers and the Novaplex HPV typing assay

PCR with degenerate PGMY09/11 primers resulted in PCR amplicon of expected size (~450 bp) for all 16 HPV whole genome plasmids indicating no interruption in the L1 gene (Figure S17). HPV plasmids were further validated by Novaplex assay by testing different

concentrations of plasmids in background of 100,000 copies/reaction of human genomic DNA. The assay reproducibly detected and unambiguously typed each HPV plasmid at 500, 50 and 10 copies/reaction (Table 1).

Discussion

Our goal was to provide a set of HPV plasmids that could be used in the validation of current and new HPV DNA assays, even if regions targeted by these assays were not known. This requires full-length HPV whole genome plasmids without any interruptions in coding genes. However, most of the HPV DNA international standards including the plasmids used in the global HPV LabNet typing proficiency studies [12–17] have interruption of HPV genes [5, 8].

Gene interruptions or partial genome inserts in many of the available HPV plasmids occur as a result of traditional restriction-ligation based cloning approaches. Many studies cloned restriction enzyme digested DNA fragments from clinical samples into plasmids to isolate new HPV types [18, 19]. New cloning techniques have been developed over the past years that could be adopted depending on the nature of cloning project [20]. The Gibson Assembly technique first developed by Daniel Gibson in 2008 is now widely used for cloning genes, genetic pathways, and genomes [21–23]. Since the method allows for assembly of large DNA constructs (up to 900 kb) and cloning of DNA fragments into any plasmid backbone independent of the restriction sites, we adapted the method to develop an HPV plasmid repository with the eventual aim to construct HPV whole genome plasmids for all known HPV types.

In this study, the laboratory workflow for construction of HPV whole genome plasmids was established and plasmids for 16 HPV types (HPV6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) were constructed. CDC-generated HPV plasmids containing the full-length HPV genome for each type with no gene interruptions were made in the same vector backbone to standardize quantitation and sequencing. The size of overlapping HPV DNA fragments varied from 380 bp to 7117 bp and cloning of full-length HPV genome was achieved by assembly of 2–3 overlapping HPV DNA fragments with vector backbones. Although our assembly reaction used agarose gel purified PCR fragments and lower concentration of vector backbones to minimize non-specific assembly of DNA fragments, bacterial colonies with empty vector were still observed. The proportion of bacterial clones containing self-ligated vector varied for each HPV type and could be due to the presence of repetitive sequences in the HPV genome as was observed previously for DNA fragments with high GC content [23, 24].

Only HPV31 presented cloning challenges as propagation in the standard DH5α bacterial cell line only yielded clones with truncated HPV genomes. These could have been generated from recombination events, and propagation in *E.coli* Stbl2 [25], a strain widely used for cloning of unstable inserts such as retroviral sequence and direct repeats, solved the problem. This suggests the HPV 31 genome is unstable. As other HPV types to be cloned in the future could also be unstable, we have shifted our workflow to propagation in *E.coli* Stbl2.

Nucleotide substitution(s) in the HPV sequence of CDC-generated HPV plasmids is a carryover from templates used for construction of overlapping HPV DNA fragments and is not due to the experimental workflow used for generating these plasmids. Furthermore, large scale purification of all 16 HPV plasmids gave a good DNA yield and sequencing showed no mutation(s) during propagation indicating stability of these plasmids. CDC-generated HPV plasmids performed as expected when tested using degenerate PGMY09/11 primers and the Novaplex HPV typing assay. PGMY primers which target the conserved region of L1 gene have been widely used in HPV detection assays and are known to detect a broad range of HPV types [11]. Novaplex assay detects 28 HPV types, but HPV DNA region targeted by this assay is proprietary.

In summary, the CDC plasmid repository includes 16 types to date. These materials are available upon request. The potential users of HPV plasmids include public health, research, and clinical laboratories desiring defined samples for competency assessment, quality control and assay development. These plasmids are significant improvement on currently available HPV plasmids as they contain HPV whole genome with no gene interruption and can be used as HPV DNA reference materials. Some applications of CDC HPV plasmids include positive controls for HPV DNA assays, template for cloning HPV genes, and as reference materials for validating new assays to evaluate sensitivity and specificity, quality control of assay reagents, generating panels for proficiency/competency assessment, and comparing inter-laboratory results. Limitations to this study are that the HPV plasmids were not evaluated using a wide range of HPV DNA assays and validation was not repeated in laboratories outside of CDC. Although we plan to test these plasmids using additional HPV assays in future, we believe these materials will be universally applicable to all DNA-based HPV typing assays as sequencing results showed these plasmids to contain full-length genome of each HPV type. Further use of these materials by HPV scientific community may provide more insights into suitability of CDC HPV plasmids on different testing platforms. The methods presented here can be applied to generate and improve DNA reference reagents for all known HPV types, as well as other pathogens, and provide additional tools for quality improvement in laboratory detection of known and emerging pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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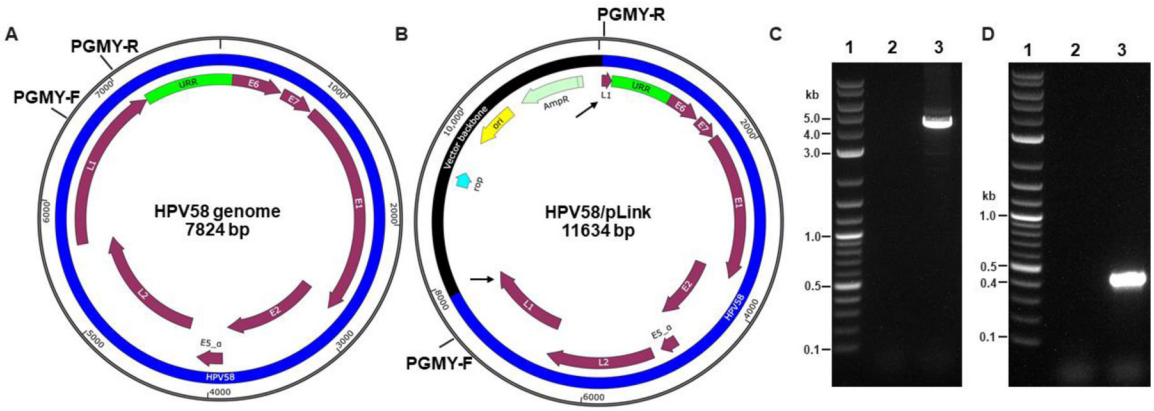

Figure 1.

HPV58 genome and HPV58 plasmids. **(A)** Map of HPV58 circular genome based on sequence from PaVE database. PGMY primers bind to contiguous HPV58 L1 sequence to give 449 bp PCR amplicon. **(B)** Map of non-CDC HPV58 (HPV58/pLink) whole genome plasmid. The L1 gene is split into two regions of the vector backbone (black arrows) and the primer binding sites for PGMY primers in L1 sequence is highlighted. **(C)** PCR with PGMY primers using non-CDC HPV58 plasmid as DNA template. **Lane1**: DNA standard, **lane 2**: control PCR reaction without DNA template and **lane 3**: PCR reaction with non-CDC HPV58 plasmid resulting in 4259 bp PCR amplicon. **(D)** PCR with PGMY primers using CDC HPV58 (HPV58/pGEMT Easy-01) plasmid as DNA template. **Lane1**: DNA standard, **lane 2**: control PCR reaction without DNA template and **lane 3**: PCR reaction with CDC HPV58 plasmid resulting in 449 bp PCR amplicon.

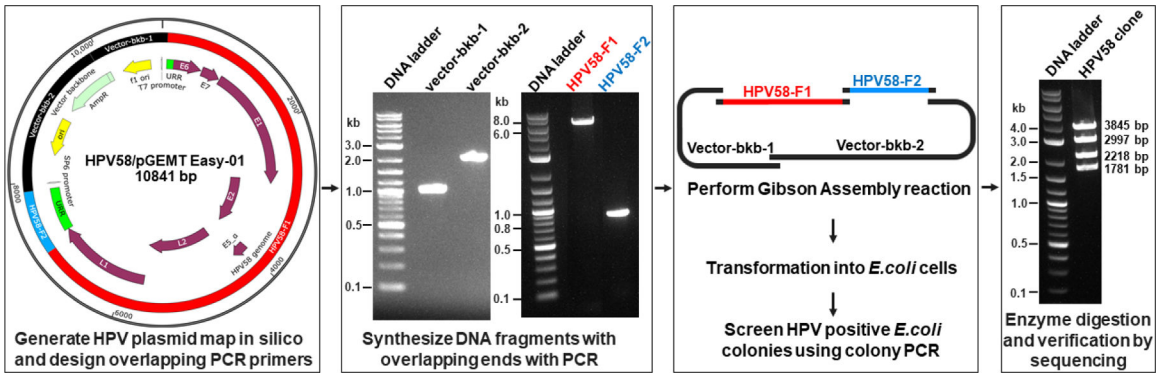

Figure 2.

Laboratory workflow to construct CDC HPV whole genome plasmids in a standard pGEMT Easy-01 vector using a Gibson assembly method. HPV58 is shown here as an example.

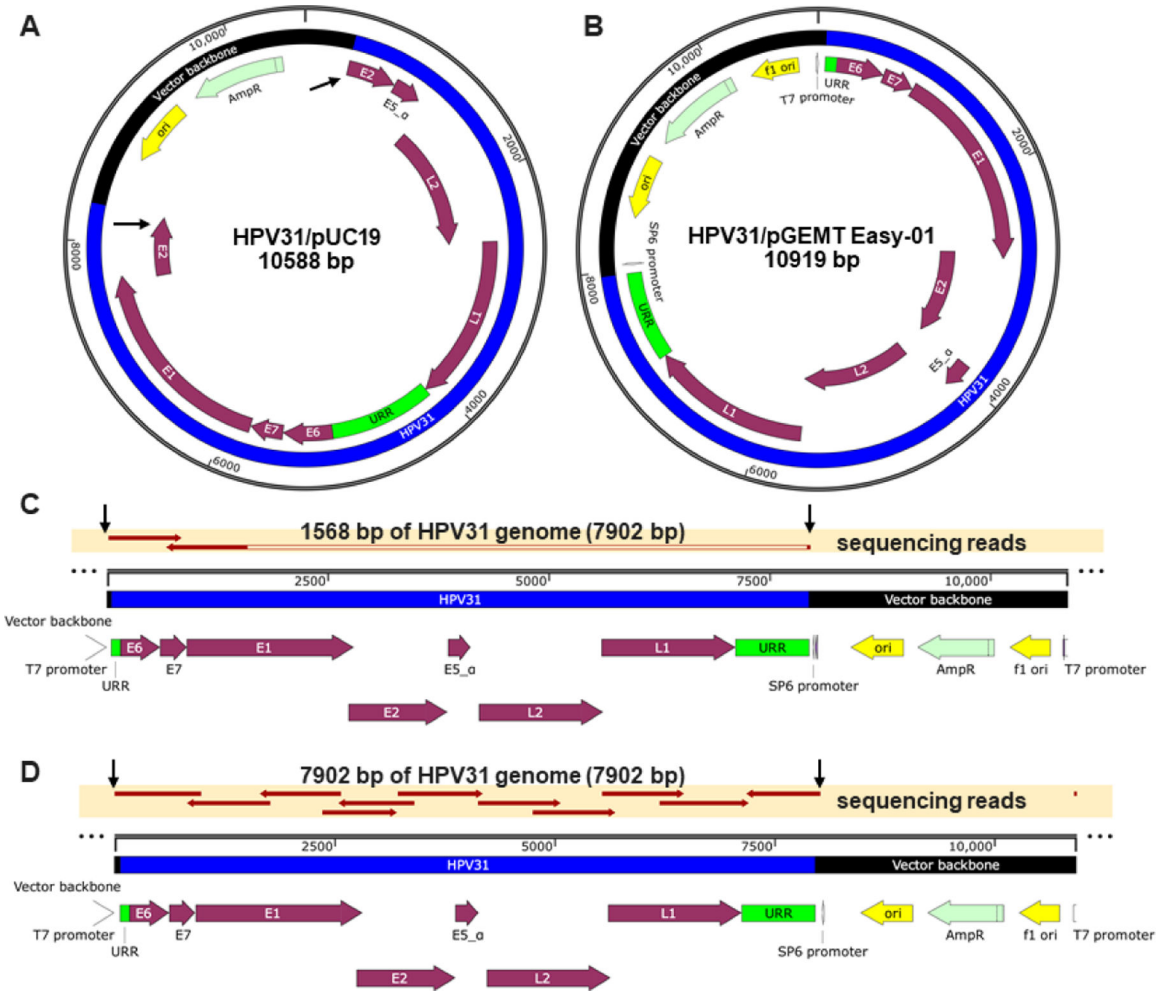

Figure 3.

Construction of CDC HPV31 whole genome plasmid. (**A**) Map of non-CDC HPV31 (HPV31/pUC19) whole genome plasmid. The E2 gene is split into two regions of the vector backbone (black arrows). (**B**) Map of CDC HPV31 (HPV31/pGEMT Easy-01) whole genome plasmid without interruption at coding genes. (**C**) Propagation of Gibson assembly reaction product to construct CDC HPV31 plasmid in *E.coli* DH5α strain resulted in clones containing partial genome sequence of HPV31 genome. Representative example of a clone containing 1568 bp of HPV31 sequence is shown. (**D**) Propagation of Gibson assembly reaction product in *E.coli* Stbl2 strain resulted in clones containing whole genome sequence of HPV31. Representative example of a clone containing full length HPV31 genome is shown.

Table 1

Summary of HPV plasmids described in this study

HPV Type	Non-CDC Plasmids[a]	Whole genome CDC plasmids with no gene interruptions[b]	GenBank Accession[c]	Substitution(s) in Plasmids[d]
HPV6	Whole genome in pUC19 Interruption at L2 gene	HPV6/pGEMT Easy-01 Validated with 500, 50 & 10 copies	JN252323.1	T221A, A2163G, G **Insertion** between A2163-C2164, A2156G, T7467G
HPV11	Whole genome in pBR322 Interruption at L1 gene	HPV11/pGEMT Easy-01 Validated with 500, 50 & 10 copies	MK313768.1	C138A, C323T, C4521A, A4887C
HPV16	Whole genome in pBluescript SK- Interruption at L1 gene	HPV16/pGEMT Easy-01 Validated with 500, 50 & 10 copies	U89348.1	T4194 **Insertion**
HPV18	Whole genome in pBR322 Interruption at E7 gene	HPV18/ pGEMT Easy-01 Validated with 500, 50 & 10 copies	AY262282.1	None
HPV31	Whole genome in pUC19 Interruption at E2 gene	HPV31/pGEMT Easy-01 Validated with 500, 50 & 10 copies	HQ537666.1	C2484T, TGGTAT **Deletions** between T4137-A4138
HPV33	Whole genome in pLink322 Interruption at E1 and E2 genes	HPV33/pGEMT Easy-01 Validated with 500, 50 & 10 copies	M12732.1	A81C, T7908 **Deletion**
HPV35	Partial genome in two pBR322 Interruption at E1 and L2 genes	HPV35/pGEMT Easy-01 Validated with 500, 50 & 10 copies	X74477.1	None
HPV39	Whole genome in pSP65 Interruption at L1 gene	HPV39/pGEMT Easy-01 Validated with 500, 50 & 10 copies	M62849.1	None
HPV45	Whole genome in pGEM4 No interruption at coding genes	HPV45/pGEMT Easy-01 Validated with 500, 50 & 10 copies	X74479.1	C4899G, G4900C
HPV51	Partial genome in pUC13 Interruption at E6 gene	HPV51/pGEMT Easy-01 Validated with 500, 50 & 10 copies	KF436866.1	A7204G
HPV52	Whole genome in pUC19 No interruption at coding genes	HPV52/pGEMT Easy-01 Validated with 500, 50 & 10 copies	MZ374367.1	T4208C
HPV56	Whole genome in pT713 No interruption at coding genes	HPV56/pGEMT Easy-01 Validated with 500, 50 & 10 copies	X74483.1	A **Deletion** between T2909-G2910, C3820 **Insertion**
HPV58	Whole genome in pLink Interruption at L1 gene	HPV58/pGEMT Easy-01 Validated with 500, 50 & 10 copies	D90400.1	None
HPV59	Whole genome in pUC9 Interruption at E6 gene	HPV59/pGEMT Easy-01 Validated with 500, 50 & 10 copies	EU918767.1	A164G, G317A, G1198A, C1471T, G3202T, G3288A, A3466C, A3835C, A3907G, T4058C, A4096C, A4130G, G4355A, A4368G, C4727T, G4746A, A4862G, A4896G, G5031A, T5484C, A6361G, A6691G, G6763A, C6958T, C7078T, C7142T, C7549A, G7590A, A7722C
HPV66	Whole genome in pBR322 No interruption at coding genes	HPV66/pGEMT Easy-01 Validated with 500, 50 & 10 copies	U31794.1	None
HPV68	Whole genome in pBluescript SK- Interruption at E7 gene	HPV68/pGEMT Easy-01 Validated with 500, 50 & 10 copies	DQ080079.1	None

[a] Non-CDC plasmids correspond to HPV plasmid templates used for construction of CDC-plasmids.

[b] CDC-plasmids correspond to new HPV whole genome plasmids constructed in this study.

[c] GenBank accession number corresponds to HPV sequence with highest percent identity when HPV insert from CDC-plasmid is used as query for Nucleotide BLAST in NCBI database.

[d] Discrepancies in HPV inserts of both non-CDC and CDC-plasmids when compared to HPV sequences from corresponding GenBank accession numbers.