World Cancer Research Journal WCRJ 2020; 7: e1542

RECENT IMPROVEMENTS IN *IN SITU* HYBRIDIZATION FOR THE DETECTION OF HPV INFECTIONS IN CLINICAL SAMPLES

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Abstract – *Objective:* *Human Papilloma Virus (HPV) infection is well-established as a cause of cervical cancer. Importantly, early HPV detection can decrease both the frequency and mortality of HPV-related cancers.* In situ *hybridization (ISH) is a widely used method for the early detection of HPV. Yet, ISH can be expensive, time-consuming and, in some cases, insufficiently sensitive to detect nucleic acid target at low copy number, which may lead to false positive or false negative results. To address these limitations, we recently developed a novel in situ hybridization technology based on proprietary Loop RNA probes (LRPs), which provides enhanced sensitivity, high-specificity and improved cost-effectiveness.*

Patients and Methods: *Manual and automated ISH was performed on paraffin-embedded cervical cancer cell lines and cervical biopsy tissues obtained from HPV-positive and -negative patients. ISH was also performed on liquid-based cytology samples, spread in monolayer, for the detection of HPV in cervicovaginal samples.*

Results: *We compared our Loop RNA probes and reagents with commercially available kits for detecting HPV. LRPs were able to detect a single copy genome- integrated HPV, as well as HPV RNA in cell lines, patient biopsies and in liquid-based cytology samples.*

Conclusions: *Our results show that LRP technology is a powerful system for the in-situ detection of HPV DNA and RNA at low copy number, even down to a single copy of genome-integrated HPV.*

KEYWORDS: *HPV, ISH, Cervical cancer.*

INTRODUCTION

Cervical cancer is one of the most common cancers in women worldwide, with an estimated 550,000 cases and 320,000 deaths in 2018[1]. Epidemiologic and clinical data demonstrate that a subgroup of human papillomaviruses (HPV) strains play a major role in the etiology of cervical cancer[2]. When an infection with high-risk HPV strain persists, it may lead to precancerous lesions which, eventually, within 10 to 20 years, may progress to a cervical cancer. To date, a total of 13 HPV genotypes have been classified as cervical carcinogens by the International Agency for Research on Cancer (IARC)[3]. HPV genotypes 16 and 18 are responsible for about 50% of cervical intraepithelial neoplasia (i.e. CIN 2 and 3) and about 70% of cervical cancers[4,5].

Recent advances in nucleic acids sequencing and multiplex RT-PCR have enabled the research community to investigate in great detail the mechanisms that regulate HPV gene expression and replication during the early and late phases of viral infection. Importantly, these studies have characterized biomarkers of viral infection. For example, it has been learned that the E2 gene is highly expressed during the early phase of HPV infection, and its expression can be used as an immediate early marker of viral

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infection. It has also been learned that the oncogenic potential of HPV can be inferred by the levels of expressions of the E6 and E7 genes, as well as the number of copies of integrated viral genomes [6,7].

Sequencing and RT-PCR are very sensitive tools and can be used to detect very small amounts of HPV RNA and DNA molecules. However, they do not provide information regarding cell morphology and localization of targets, which are important parameters in assessing disease state when evaluating cervical specimens for HPV-induced cancers. Therefore, there is an urgent need for *in situ* hybridization technologies that are able to more efficiently detect integrated HPV DNA as well as HPV RNA, while maintaining cell morphology.

In situ hybridization (ISH) is a widely used technique for the detection of nucleic acids species within cells and tissues. In contrast to sequencing and RT-PCR, it does not require the homogenization of the sample, which makes it a suitable tool for analyses in which information regarding morphology of cell and tissue is intended to be preserved. Moreover, while providing detailed quantitative information on target levels, ISH also allows the detection of a target's subcellular localization.

However, sensitivity has remained a challenge for certain ISH applications, especially those involving the detection of single-copy targets. In a clinical setting, insufficient sensitivity may increase the chances of misdiagnosis. For example, by using current ISH technologies, the detection of HPV in cervicovaginal smears can be easily missed, as infections are often caused by a single integration of the viral genome in a small number of cells within a specimen.

In an attempt to overcome this limitation, current ISH protocols employ long probes that extend the region of complementarity with the target to improve sensitivity. However, with increased probe length comes higher chances of generating off-target hybridization and a resulting increase of false positive signals and background. The strategy of increasing probe length is also inherently limited by the relatively small size of the virus. As the rate of HPV transmission is still high within the sexually active population [3], infection remains a major public health concern. As a consequence, health systems worldwide have been developing massive screening programs aimed at detecting early infections of HPV in men and women and preventing transmission within the population.

To date, the RNAscope® Technology (Advanced Cell Diagnostics-ACD) has been considered the most sensitive, commercially available *in situ* hybridization technology for detecting HPV RNA in cell lines and tissue sections[8]. However, although powerful, RNAscope® has considerable costs and requires considerable time. In addition, as the ACD RNAscope® probes for HPV are designed to detect E6/7, early stage infection of HPV may be missed. In this background, we recently developed a novel *in situ* hybridization technology based on proprietary Loop RNA probes (LRPs), which provides improved sensitivity, high-specificity and quicker turnaround times at reduce costs.

MATERIALS AND METHODS

Formalin-Fixed Paraffin-Embedded (FFPE) Slides

For C33A and SiHA cells and cancer specimens, FFPE slides were purchased from AMSBIO (Cambridge, MA, USA).

Liquid-based cytology samples analysis

Samples were collected using a brush-like device and resuspended in about 30 ml ThinPrep® collection media (Hologic, San Diego, CA, USA). 1 ml of suspension was processed for HPV detection by RT-PCR using Roche Cobas® (Roche Molecular Systems; Branchburg, NJ, USA) while the rest was spotted onto slides using a ThinPrep 5000 processor (Hologic, San Diego, CA, USA).

HPV RNA ISH probes

HPV plasmids were digested with *Hind*III, *Eco*RI, or *Bam*HI (New England Biolabs, Ipswich, MA, USA) for 2 hours and purified using Qiaquick® PCR Purification Columns (Qiagen; Hilden, Germany). To transcribe and label probes, BIOARRAY HIGH-YIELD® RNA transcript labeling kit (T7) (Enzo Life Sciences, Inc.; Farmingdale, NY, USA) was used to produce biotin labeled RNA probes. The biotin labeled RNA probes were then purified using the QIAGEN RNeasy kit (Qiagen, Hilden, Germany). Finally, RNA probes were fragmented in fragmentation buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3, 20 mM ß-mercaptoethanol), ethanol precipitated, air dried and resuspended in Tris-EDTA buffer solution (pH 7) with a volume in µl equal to the amount of probe RNA in µg, assuming a 90-100% yield.

In situ *hybridization*

HPV was detected by *in situ* hybridization using the RNAscope® kit (ACD; Newark, CA, USA) as well as the Enzo HPV RNA kit (Enzo Life Sciences Inc.; Farmingdale, NY, USA) per the manufacturer's

protocol. Automated staining was performed on the automated Leica Bond® platform. For liquid-based cytology samples, slides were post-fixed with 4% Paraformaldehyde (Sigma-Aldrich; St. Louis, MO, USA) for 20 minutes on ice. Detection of HPV using the Enzo HPV RNA kit was performed manually.

RESULTS

Loop RNA probes features

To produce antisense and sense Loop RNA probes targeting HPV DNA and RNA, regions corresponding to E2, E6, E7, and part of the E1 of the HPV genome were synthesized by *in vitro* transcription. A feature of the LRP probe design is the inclusion of multiple RNA spacer segments, which are not complementary to the target nucleic acid sequence or to the human genome and which are designed so that most of their bases can be labeled with haptens, such as biotin or digoxigenin, throughout the transcribed HPV gene regions. When such a probe is hybridized to its target sequence, the labeled spacer segments loop out, thereby facilitating access to the labels by horseradish peroxidase (HRP) or alkaline phosphatase-conjugated antibodies.

Detection of HPV RNA and integrated DNA in cell lines

In order to evaluate specificity, we performed the initial optimization of LRP in SiHa cells, which carry a defined number of integrated HPV16 copies (1 to 3) per cell. C33A cells, which are not infected with HPV, were used as a negative control. We first used an antisense probe, which is complementary to a region of the sense strand of the viral genomic DNA and, thus, also complementary to the respective RNA transcript. As shown in Figure 1A, both a punctate signal and a diffuse signal are clearly observed in SiHa cells. The punctuate signal likely originates from integrated copies of the virus DNA genome in the host genome, while the diffuse signal represents HPV RNA located within the cytoplasm[9]. As expected, no signal is detected in C33A cells (Figure 1B).

Accordingly, when a sense HPV 16 LRP was used, we were able to detect only the punctate signal

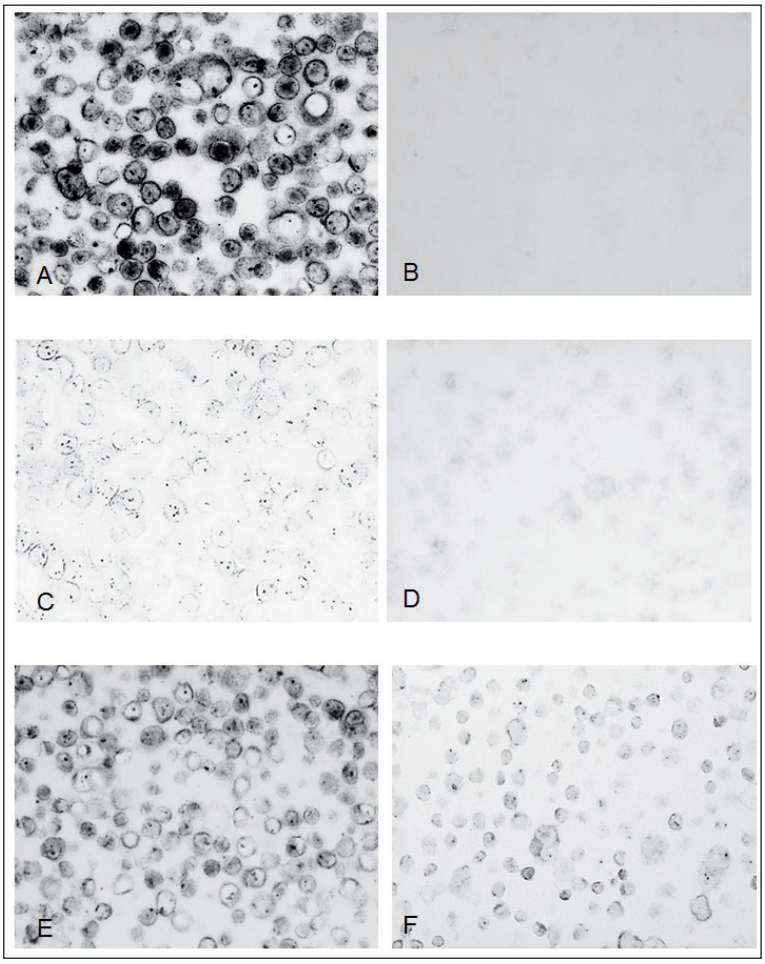

Fig. 1. Hybridization of cells using Loop RNA HPV type 16 antisense probes were tested on (***A*** and ***E***) SiHa cells and (***B*** and ***F***) HPV negative cervical cancer C33A cells resulting in detection of HPV RNA and integrated DNA (***A***). Hybridization using Loop RNA HPV type 16 sense probes was tested on (***C***) SiHa cells and (***D***) HPV negative cervical cancer C33A cells resulting in detection of integrated HPV DNA. SiHa cells without RNase treatment display both a diffuse signal and a punctate signal (***E***). ***A***, Pretreatment of SiHa cells with RNase A for 30 minutes abolished the diffuse signal. Detection was performed using SAVIEW® PLUS AP Reagent (Enzo Life Sciences, Inc, Farmingdale, NY, USA) followed by exposure to NBT/BCIP chromogen (10X magnification).

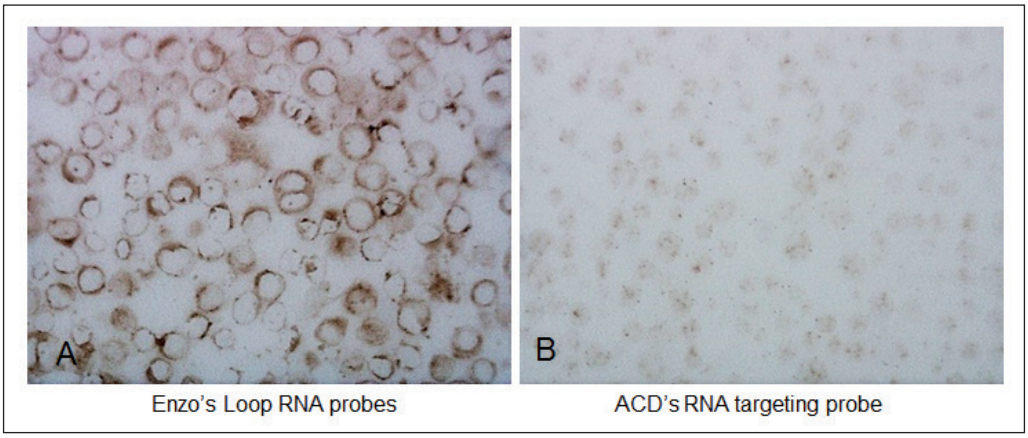

Fig. 2. The signal from the antisense Loop RNA HPV type 16/18 probes was stronger than signal from RNAscope® HPV type 16/18 probe. SiHa cells hybridized with antisense Loop RNA HPV Type 16/18 probes (***A***), and (***B***) HPV type 16/18 RNA probes and RNAscope® reagents (ACD). Detection for Enzo RNA probe was performed using SAVIEW® PLUS HRP reagent followed by exposure to DAB chromogen, while RNAscope® HPV type 16/18 detection system was used in B (10X magnification).

in SiHa cells (Figure 1C and D), demonstrating the ability of the technique to discriminate viral DNA integrated in the host genome from viral RNA. Moreover, the absence of diffuse signal when the sense probe was used confirms the fact that SiHa cells do not carry episomal viral DNA[10].

Furthermore, treating the cell preparation with RNase A prior to hybridization induces a complete disappearance of the diffuse signal when antisense HPV 16 LRP was used (Figures 1E and 1F), thereby confirming that LRPs can effectively detect HPV RNAs. Detection of integrated HPV DNA and RNA using LRPs from Enzo is comparable to that achieved by the RNAscope® protocol (Figure 2A *vs.* 2B).

Manual detection of HPV RNA and DNA in clinical samples

We next addressed the question of whether results similar to those obtained with cell lines can be obtained with tissue sections, and whether LRPs are suited for use with automated processing systems. As shown in Figures 3A and 3B, similar results were observed when a CIN1 tissue section was manually stained using an antisense LRPs and double-stranded DNA (dsDNA) probes (Enzo Life Science, Inc.) targeting HPV 16/18. LRP-generated staining is more intense than cDNA probe-generated staining (Figure 3A *vs.* 3B) suggesting that LRPs are more sensitive than dsDNA probes.

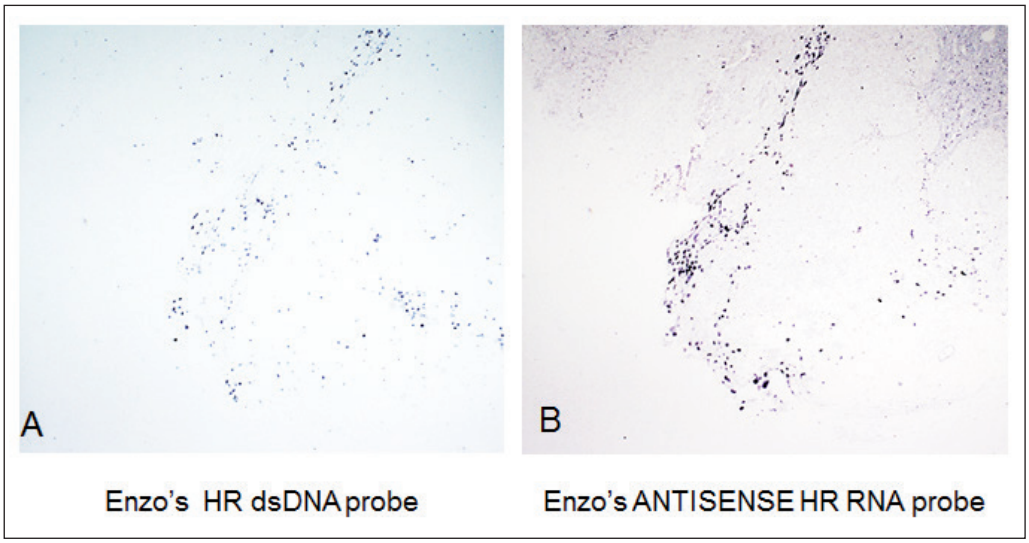

Fig. 3. Hybridization of human cervical FFPE biopsies with commercially available PATHO-GENE® HPV type 16/18 DNA probes (***A***) and antisense Loop RNA HPV type 16/18 probes (***B***). Detection was performed using SAVIEW® PLUS AP Reagent followed by exposure to NBT/BCIP chromogen (10X magnification).

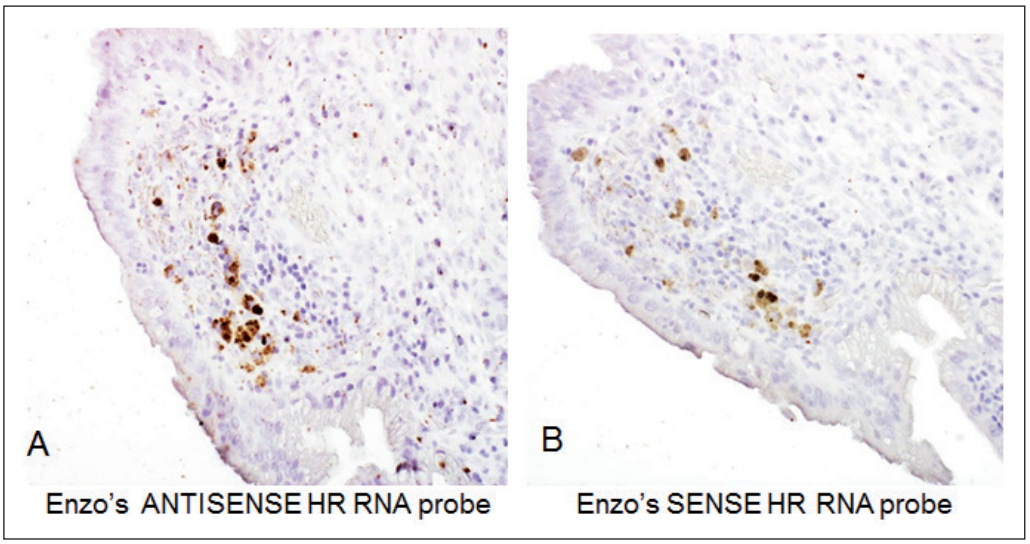

Fig. 4. Hybridization of human cervical carcinoma biopsy on Leica Bond with antisense Loop RNA HPV type 16/18 probes (***A***) results in more intense signal compared to sense Loop RNA HPV type 16/18 probes (***B***) (20X magnification). Detection was performed using the DAB chromogen.

Automated detection of HPV RNA and DNA in clinical samples

Next, antisense and sense LRPs targeting high risk HPV (16, 18, 31, 33, and 51) were used to detect HPV in cervical carcinoma biopsy specimens on the Leica BOND® platform (Figure 4A and 4B). As expected, signal obtained using antisense LRPs was more intense than that obtained using sense LRPs, probably because transcription of viral mRNAs serves as a natural amplification step that enhances viral detection by ISH, especially in those tissues where virus is present at very low copy number. Finally, weakly HPV-infected cervical carcinoma biopsies were stained using LRPs (16/18) and RNAscope® probes (16/18) on the Leica BOND® platform. We found that tissues stained with Enzo LRPs displayed equal or better signal than RNAscope® probes (Figure 5 A *vs.* 5B). All together, these results show that Enzo's LRP technology is a powerful system for detecting HPV DNA, even down to a single copy of host genome-integrated HPV, as well as HPV RNA.

ISH in liquid-based cytology samples

We also developed a LRP-based ISH method in liquid-based cytology samples, spread in monolayer. As a proof of concept, we first performed ISH using antisense LRPs targeting high risk HPV (16, 18, 31,

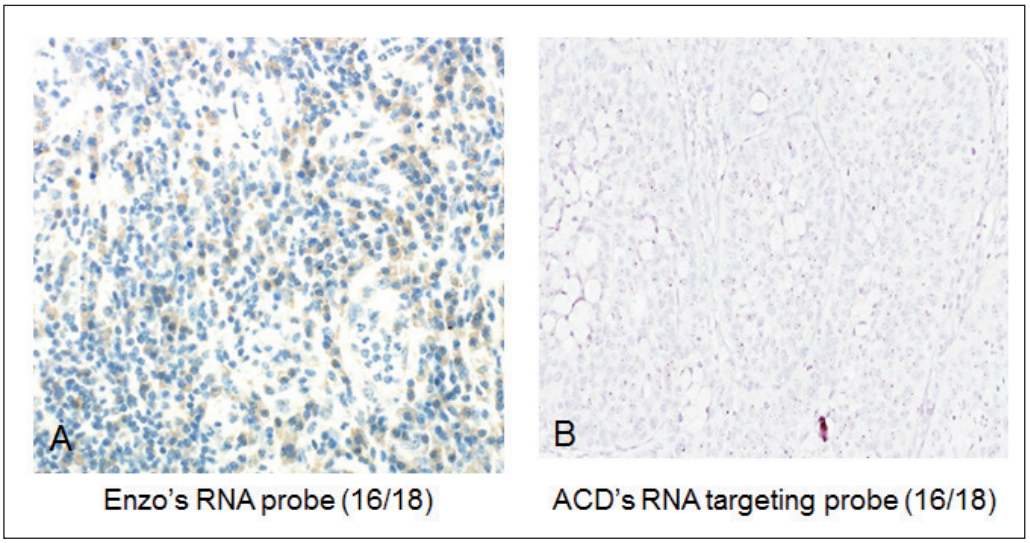

Fig. 5. Hybridization of weakly HPV infected human cervical carcinoma biopsy on Leica Bond with antisense Loop RNA HPV type 16/18 probes (***A***) results in more intense signal compared to RNAscope® HPV type 16/18 probes. ***B***, Detection was performed using the DAB chromogen (20X magnification).

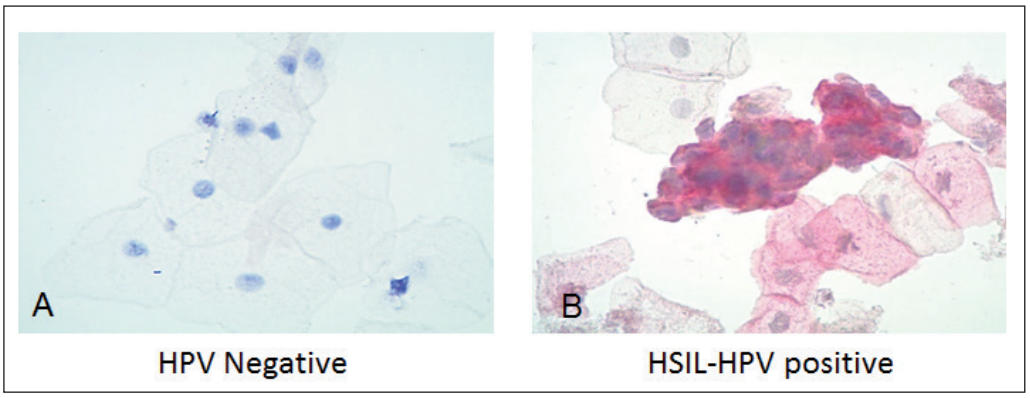

Fig. 6. Representative pictures of ISH performed in HPV negative (***A***) and HPV positive HSIL (***B***) specimens collected in ThinPrep® and spread in monolayer onto positively charged glass slides. Samples were incubated with antisense Loop RNA HPV (types 16, 18, 31, 33, and 51) probes. Detection was performed using the Enzo AP Red chromogen and hematoxylin was used to stain nuclei (20X magnification).

33, and 51) and detection with Enzo Red chromogen in HPV positive clinical samples that were previously analyzed by PCR and categorized as high-grade squamous intraepithelial lesions (HSILs). Negative samples were, of course, included in the tests either to detect possible background signal or to check whether or not cell morphology was preserved. As shown in Figures 6A and 6B, cell morphology was well-maintained. Furthermore, HSIL cells stained positive for HPV (Figure 6B) while red signal was not detected in negative clinical samples (Figure 6A). We then performed ISH on several clinical samples with increasing Ct value by PCR and compared the ISH results with those obtained by PCR. Interestingly, we were able to detect HPV in very low copy number infected cells (Ct value: 38.6, Figure 7).

DISCUSSION

Here, we demonstrate that our LRP technology is a powerful system for the *in situ* detection of HPV DNA at low copy number, even down to a single copy of genome-integrated HPV, as well as HPV RNA.

It has been previously generalized that at least 10–20 copies per cell of the target should be present in order, for the *in situ* hybridization, to detect DNA or RNA target[11]. However, we were able to clearly detect a single host genome integrated copy of HPV DNA in SiHa cells, which appeared as a nuclear punctate signal. In this regard, it is worth mentioning that SiHa cells do not carry episomal HPV[9,12]. For the same reason, we assume that the diffuse cytoplasmic signal observed can be exclusively attributed to the presence of viral RNA. We demonstrated in both cell lines as and clinical samples that detection of integrated HPV DNA and RNA using LRPs is equal to, if not better than, detection using the RNAscope® protocol. Notably, while the length of the Enzo LRP protocol is about five hours, the RNAscope® protocol requires more than eight hours for completion. Therefore, we believe that the LRP technology may be efficiently applied to routine clinical samples.

We proved that our LRP-based ISH is more sensitive than ISH using dsDNA probes. This result is not unexpected as RNA probes remain conformationally available for hybridization throughout the hybridization process with no potential binding partner but for the target, while dsDNA probes tend to renature during hybridization. RNA probes also have greater thermodynamic stability when paired with both DNA and RNA targets compared to DNA probes[13]. With respect to RNA detection, one can consider transcription of viral mRNA as a natural amplification step that enhances viral detection by ISH, especially in those tissues where virus is present at very low copy number.

Finally, we developed an ISH method using LRPs for liquid-based cytology samples, spread in monolayer, as an alternative approach for detecting HPV in cervicovaginal samples while preserving cell morphological features. In addition, we were able to detect HPV in cells with very low viral copy number, suggesting that LRP ISH may be competitive with PCR, which is considered the gold standard in HPV detection.

One potential drawback in the use of any RNA probe is sensitivity to degradation by ribonucleases (RNase). However, to avoid this problem, we developed a proprietary ready-to-use hybridization buffer for LRPs that is formulated to minimize or eradicate any possible RNase activity.

CONCLUSIONS

In summary, we developed an easy-to-use and cost-effective method for the *in situ* detection of low-copy HPV DNA and RNA in cell lines and

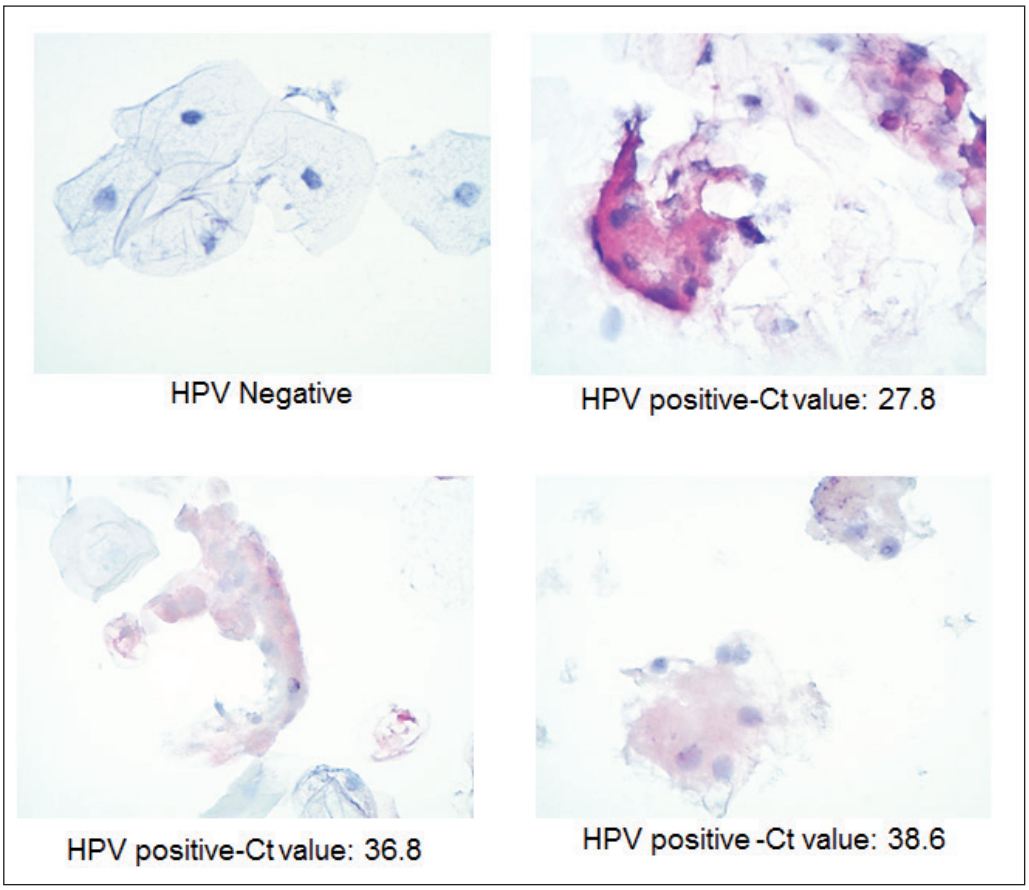

Fig. 7. Representative pictures of ISH performed in several clinical samples, prepared as in Figure 6, with increasing Ct value by Real-time-PCR. PCR was performed using Cobas® HPV test. Samples were incubated with antisense Loop RNA HPV (types 16, 18, 31, 33, and 51) probes. Detection was performed using the Enzo AP Red chromogen and hematoxylin was used to stain nuclei (20X magnification).

clinical specimens. The Loop RNA probe strategy described herein can be used to efficiently design probes with virtually unlimited potential to detect any gene and transcript of interest. Because only a single probe hybridization step is involved, the LRP ISH protocol is more straightforward and rapid than competing high-performance ISH systems. Our LRP ISH technology is suitable for the detection of HPV infection in cell lines and clinical specimens, by both manual and automated protocols.

Conflict of Interest:

The Authors declare that they have no conflict of interests.

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