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REVIEW

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Advanced technologies towards improved HPV diagnostics

Martin Bartosik[1] | Ludmila Moranova[1] | Nasim Izadi[1] | Johana Strmiskova[1,2] | Ravery Sebuyoya[1,2] | Jitka Holcakova[1] | Roman Hrstka[1]

[1]Research Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

[2]National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Brno, Czech Republic

Correspondence
Martin Bartosik, Research Centre for Applied Molecular Oncology, Masaryk Memorial Cancer Institute, Zluty kopec 7, 656 53 Brno, Czech Republic.
Email: martin.bartosik@mou.cz

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Abstract

Persistent infection with high-risk types of human papillomaviruses (HPV) is a major cause of cervical cancer, and an important factor in other malignancies, for example, head and neck cancer. Despite recent progress in screening and vaccination, the incidence and mortality are still relatively high, especially in low-income countries. The mortality and financial burden associated with the treatment could be decreased if a simple, rapid, and inexpensive technology for HPV testing becomes available, targeting individuals for further monitoring with increased risk of developing cancer. Commercial HPV tests available in the market are often relatively expensive, time-consuming, and require sophisticated instrumentation, which limits their more widespread utilization. To address these challenges, novel technologies are being implemented also for HPV diagnostics that include for example, isothermal amplification techniques, lateral flow assays, CRISPR-Cas-based systems, as well as microfluidics, paperfluidics and lab-on-a-chip devices, ideal for point-of-care testing in decentralized settings. In this review, we first evaluate current commercial HPV tests, followed by a description of advanced technologies, explanation of their principles, critical evaluation of their strengths and weaknesses, and suggestions for their possible implementation into medical diagnostics.

KEYWORDS

CRISPR-Cas, human papillomavirus, isothermal amplification techniques, lab-on-a-chip, lateral flow assay, nanomaterials

1 | INTRODUCTION

Infection with human papillomavirus (HPV) is believed to be the most common sexually transmitted infection in the world. It is estimated that over 80% of individuals will experience HPV infection at some point in their lives.[1] Most of >200 known types of HPV do not pose significant health risk, and if so, they are mostly manifested by skin or genital warts.[2] A distinct subset of HPVs termed high-risk (hrHPVs, e.g., HPV16, 18, 31, 33, or 45), however, may induce malignant transformation of epithelial tissue, leading to the formation of precancerous lesions and ultimately cancer—especially cervical cancer (CC),[3] but to a lesser degree also anogenital tumors[4] or head and neck cancer (HNC).[5] Infection with hrHPV does not necessarily result in cancer; the virus is often cleared by the immune system (within 2–3 years) without the formation of lesions.[6] It is the persistent infection associated with the integration of viral DNA into

the host genome that is responsible for a slow transformation of the epithelium.[7]

Historically, there have been several milestones in the screening and prevention of CC. For instance, introduction of the Pap (Papanicolaou) test as a routinely used cytological test saved thousands of lives and caused CC, the number one cancer killer of women in the US in early 1900s, to drop out from the top 10.[8] However, in a well-known ATHENA trial, HPV testing was shown to be a more sensitive strategy for CC screening than the Pap test alone, that is, HPV testing had a lower false-negative rate for predicting cervical lesions.[9] This implies that by using HPV molecular diagnostics, more women can be identified and directed for further surveillance or treatment, which is a reason why HPV testing is now being considered as an additional screening method for CC in many developed countries.[10]

Another milestone is the HPV vaccination, which has helped bring down the prevalence of CC in the US alone by 64% in females aged 14–19 years.[11] There are currently three approved prophylactic vaccines, that is, Gardasil® (quadrivalent vaccine introduced in 2006 targeting HPV6, HPV11, HPV16 and HPV18), Cervarix® (bivalent vaccine since 2007 targeting HPV16 and HPV18) and Gardasil 9® (nonavalent vaccine since 2014 targeting HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV45, HPV52 and HPV58).[12] Clinical trials targeting young women have shown similar and very high efficacies for all three vaccine types, such as FUTURE trials for Gardasil (98% efficacy),[13] PATRICIA trial (short for PApilloma TRIal against Cancer In young Adults) for Cervarix (92.9% efficacy)[14] and NCT00543543 study for Gardasil 9 with 97% efficacy.[15] Moreover, randomized, double-blind clinical trial demonstrated that the quadrivalent HPV vaccine efficiently prevents infection with HPV6, 11, 16, and 18 and the development of related external genital lesions also in young men,[16] and a similar trial is underway to show efficacy for the nonavalent vaccine in men.[17] HPV vaccines are considered very safe, with only mild side effects, such as pain and swelling at the injection site, fever or headache. On the other hand, the implementation of the vaccines is far from universal or equitable, mainly due to high vaccine costs, recent supply shortage, or inadequate delivery and storage infrastructure, but also due to the COVID-19 pandemic that has affected existing HPV vaccination programs and halted the introduction of new programs,[18] or because of vaccine hesitancy and associated lack of community engagement.[19]

Less frequent, but certainly not less interesting, is determining HPV status in HNC. The virus is implicated as the causative agent of certain HNC subtypes, especially oropharyngeal carcinomas (OPC)[20] where we witnessed in past years a steep increase in incidence in non-smokers under the age of 50.[21] A meta-analysis on >12 000 HNC cases published in The Lancet Oncology revealed that ~32% of all cases were HPV-positive, with HPV16 being the most frequent subtype (>80%).[22] It was shown that HPV positivity in HNC strongly correlates with a better prognosis[20] suggesting a different biological basis than in HPV-negative tumors, and thus different approach to therapy management.[23] Indeed, information about HPV status in these cases could be helpful in selecting suitable treatment.

HPV diagnostics is performed with commercially available HPV tests, described in greater detail in Section 2. These tests, however, are relatively expensive, time-consuming, and require advanced instrumentation, limiting their more widespread application especially outside the laboratories at the point-of-care, or in low-resource settings.[24] Not surprisingly, a plethora of novel state-of-the-art technologies have emerged with the aim of reducing overall cost, time, or material consumption in HPV detection. These include for example, various isothermal amplification techniques (IATs) as rapid alternatives to classical PCR (polymerase chain reaction, described in Section 3), dot blots and lateral flow assays with colorimetric readout as simple tools ideal for low-resource settings (Section 4), CRISPR-Cas technology (acronym for clustered regularly interspaced short palindromic repeats—CRISPR-associated proteins) adapted for biosensing research that greatly improves specificity of detection (Section 5), microfluidic, paperfluidic and lab-on-a-chip (LOC) technologies that integrate all reaction steps into a single device (Section 6), or nanomaterials that increase sensitivity of the measurement (Section 7). In this review, we show that these technologies are promising candidates in current HPV diagnostics, but we also report the obstacles that these technologies must overcome to compete with standard methods of detection to be applied in clinical routine.

2 | OVERVIEW OF COMMERCIAL HPV TESTS

In clinical practice, cervical cytology (commonly known as Pap smear or Pap test) is a routine CC screening for the detection of abnormal cervical epithelial cells that may indicate precancerous lesions or cervical carcinoma. Due to the low sensitivity of Pap tests, molecular HPV diagnostics which identifies presence of an infection with high-risk types of HPV has been approved as an additional technique for CC screening. In fact, there is a gradual transition from cytological testing alone to a combination of cytology and molecular testing or to primary HPV screening.[25–27]

Currently, the most common way of detecting HPV is via nucleic acid amplification tests (NAATs), especially PCR. NAATs are highly sensitive and specific and can detect the presence of HPV DNA or messenger RNA (mRNA, product of a gene transcription) in a variety of samples, including cervical smears, anal and buccal swabs, or in saliva. PCR-based tests are widely used due to their high sensitivity, specificity, and ability to detect multiple HPV types simultaneously, and are often used for CC screening, the diagnosis of genital warts, and for other HPV-related diseases. Most commercial tests used in clinical practice are based on PCR to identify HPV DNA, particularly viral late gene *L1* (responsible for the production of the major viral capsid protein), and two early genes *E6* and *E7* (acting as oncogenes that promote tumor growth and malignant transformation). Some of these DNA tests simultaneously identify several hrHPV types with oncogenic potential, but do not distinguish between them individually; other tests detect hrHPV but differentiate only some, and most

advanced tests allow full genotyping by discriminating each HPV type present in the sample.[28,29] HPV DNA testing is used to confirm HPV infection but is not indicative of progression of the infection. Hence other tests, especially those targeting either E6/E7 mRNA or viral oncoproteins, were developed to determine active transcription of virus in infected cells and to provide more accurate information on disease prognosis.[30] Indeed, some studies confirmed that HPV E6/E7 mRNA testing was more specific and had a higher positive predictive value than HPV DNA testing, making them suitable biomarkers for the detection of high-risk HPV-associated cervical precancerous lesions.[31,32]

Basic information about the most frequently used tests in clinical practice is summarized in Table 1. More detailed and comprehensive information about commercially available HPV tests can be found either in references in Table 1[40-57] or in other review papers.[33–37] Despite a large number of commercially available HPV tests, new technologies and methods are being developed that would enable faster, simpler, and cheaper identification of high-risk active HPV viruses while maintaining the necessary specificity and sensitivity. It should be noted, however, that before entering clinical practice, any new technology would have to follow international consensus guidelines for primary screening,[38] as well as to comply with VALGENT studies (VALidation of HPV GENotyping Tests[39]) or would need an approval from the US Food and Drug Administration (FDA).[35] These advanced technologies are introduced and described in following chapters, including their strengths and weaknesses.

3 | ISOTHERMAL AMPLIFICATION TECHNIQUES AS RAPID ALTERNATIVES TO PCR

PCR is an extremely versatile nucleic acid amplification technique with many diverse applications in biomedical research and far beyond. It offers numerous benefits, such as high sensitivity and specificity, relative simplicity, or option of multiplexing, and is still a major tool used in HPV diagnostics and genotyping.[58] However, PCR requires a dedicated instrument—a thermal cycler—for cycling between temperatures, is relatively time-consuming, and highly sensitive to PCR inhibitors, such as various salts or detergents. To circumvent these drawbacks, new techniques for nucleic acid amplification started to emerge at the turn of the millennium, collectively called isothermal amplification techniques (IATs). As the name implies, IATs operate at constant temperature without a need for cycling, a feature provided by special polymerases with strand displacement abilities. They achieve comparable sensitivities as PCR-based techniques but often at shorter times (in 20–30 min), and are usually resistant to PCR inhibitors. The fact that IATs operate at constant temperature without a need for thermal cycling is a major factor why they are now being increasingly coupled with various advanced technologies, such as lateral flow assays, LOC devices, or microfluidic platforms (described in next sections).

Perhaps the most well-known IAT is a loop-mediated isothermal amplification (LAMP), especially due to its association with recent

TABLE 1 Overview of the most frequently used commercial HPV tests.

Biomolecule	Test name	Company	Target	Number of genotypes
Tests without specifying HPV type				
DNA	Hybrid Capture® 2 (hc2)[31]	Qiagen (Digene)	Full HPV genome	13
DNA	Cervista HPV HR[32,33]	Hologic	*E6/E7*	14
Partial genotyping				
DNA	Cobas HPV Test[34]	Roche	*L1*	14
DNA	RealTime High Risk HPV assay[35]	Abott Molecular	*L1*	14
DNA	BD Onclarity HPV[36,37]	Becton, Dickinson and Company	*E6/E7*	14
DNA	Xpert® HPV assay[38]	Cepheid	*E6/E7*	14
DNA	Cervista HPV 16/18[39,40]	Hologic	*E6/E7*	2
Full genotyping				
DNA	Anyplex II HPV28[41,42]	Seegene	*L1*	28
DNA	INNO-LiPA® HPV Genotyping Extra II[43]	Fujirebio	*L1*	32
DNA	PapilloCheck[44,45]	Greiner Bio-One	*E1*	24
Non-DNA based tests				
RNA	Aptima HPV Assay[46]	Hologic	E6/E7 mRNA	14
protein	OncoE6 HPV test[47,48]	Arbor Vita Corporation	HPV16/18 E6 oncoprotein	2

COVID-19 pandemic when numerous LAMP tests in a reverse transcription mode (RT-LAMP) were introduced for SARS-CoV-2 RNA detection.[59] LAMP technique uses Bst polymerase and four to six primers to rapidly amplify either DNA or RNA (under 1 h) at elevated temperatures between 55°C and 70°C. It is commonly used for amplification of bacterial and viral nucleic acids, and thus HPV has been targeted by LAMP in various studies.[60–73] Despite its advantages, LAMP is highly sensitive to contamination (similarly to PCR), requiring ideally sterile DNA-free and RNA-free workspace. Moreover, design of LAMP primers is not trivial, and needs to be performed in a special software (such as PrimerExplorerV5, https://primerexplorer.jp/e/), but even this does not guarantee that the primers will be functional. Usually, more set of primers need to be tested to find the most suitable one. Often, LAMP is coupled with colorimetric detection which utilizes pH indicators that change color when the pH of the solution turns more acidic.[70–73] This exactly happens in enzymatic amplification reactions (i.e., PCR and IATs) that generate hydrogen ions during incorporation of dNTPs into the growing DNA or RNA strand, decreasing the pH of the solution. Color changes are easily visible by naked eye and thus do not require any equipment, but the sensitivity of such detection is rather limited. For instance, in a study by Zhong et al., authors differentiated among 6 HPV subtypes in samples from patients with condyloma acuminatum, where positive samples were visible with the naked eye by changing the color of a hydroxynaphthol dye from violet to blue.[63] HPV LAMP products can be determined also with turbidimetry measurements, where magnesium pyrophosphate is generated during the LAMP reaction and makes the reaction mixture cloudy. Increased turbidity caused by magnesium pyrophosphate particles is measured optically at certain wavelength by calculating the amount of light that did not pass through the sample. Turbidimetry is a very quick and simple method without a need for any expensive lasers and fluorophores, but its accuracy can be negatively affected by bubbles in the sample, size of the particles, or particle sedimentation. Sensitivity of turbidimetry itself depends on selected light source and nature of the examined substances, but when connected to LAMP, it was demonstrated to detect positive amplification with as little as 10 copies of the target sequence per sample.[65,66]

Although colorimetric readout is quick and straightforward, it suffers from low sensitivity and ambiguous results. More sensitive approach is to use for example, Real-Time LAMP or digital LAMP, which are LAMP variants that resemble Real-Time PCR and digital PCR, respectively. In former case, the Real-Time LAMP was recently coupled with endovaginal MRI to improve accuracy for early-stage CC detection in the group of 27 CC patients and 14 negative controls.[74] Authors have targeted not only HPV16 and HPV18 DNA but also hTERT, TERC/GAPDH, and MYC/GAPDH mRNA tumor biomarkers. Overall, it was the use of a spatially multiplexed LAMP assay in combination with high-resolution imaging that resulted in improved specificity for cancer detection. The latter case involving digital LAMP was combined with microfluidic slip chips, where a total number of 2240 droplets, each of 4.5 nl allowed to quantify the viral load of HPV16 and HPV18 by comparing numbers of positive wells with standard curve obtained with plasmid control.[75] The assay was tested on 15 clinical samples, showing full agreement with commercial Cobas 4800 test (Roche), but revealing also hidden coinfections.

Electrochemical (EC) end-point detection is another suitable technique used in combination with LAMP. EC methods provide an option for inexpensive, simple, and miniaturized instrumentation with the possibility of parallel measurements at electrode chips and arrays while allowing rapid and highly sensitive determinations.[76–78] For instance, a diagnostic Clinichip HPV test that employed LAMP and electrochemical DNA chip could recognize 13 clinically relevant HPV types in less than 3 h. In that study, 247 Japanese women, including 109 with normal cytology, 43 with cervical intraepithelial neoplasia of grade 1 (CIN1), 60 with CIN2/3, and 35 with invasive cervical cancer were tested for carcinogenic HPV genotypes, reaching good agreement with direct sequencing.[68] Our team has published several research articles reporting fast and simple LAMP-based assays combined with electrochemical detection (EC-LAMP), targeting HPV16/18 genotypes in both cervical cell lines and cervical smears from women with precancerous lesions (Figure 1).[79–82] After development and optimization of the assay,[80] the EC-LAMP was applied into a cohort of 61 clinical samples to evaluate its performance and compared it with PCR-based methods and with INNO-LiPA genotyping assay.[81] Good specificity and negative and positive predictive values were reached, all over 90%. Later, a DNA extraction step was eliminated by applying the EC-LAMP directly into crude lysates, whereby the cervical samples were scraped from sampling brush with sterile tweezers, then simply boiled for 5 min and introduced into the LAMP mixture for amplification.[79] This elimination not only simplified overall assay, but also decreased a risk of contamination by shortening overall sample exposition to the environment. The last generation of the assay involved a transfer of the protocol from magnetic beads directly to the gold electrode chips for easier washing and shorter hands-on time.[82] LAMP as a rapid and undemanding technique could be thus a good choice for fast determination of HPV infection.

Another IAT, the recombinase polymerase amplification (RPA), often functions with forward and reverse PCR primers, but instead of a Taq polymerase, it uses a cocktail of three enzymes—recombinase (enabling primers to pair with homologous sequences in target DNA strand), single-stranded DNA-binding protein (stabilizing single-stranded DNA structures and preventing displacement of primers), and strand-displacing polymerase (performing actual synthesis of complementary DNA). RPA is carried out at mild temperatures between 37°C and 42°C, but even a room temperature may be used at somewhat lower efficiency, making this technique especially useful in very simple devices without access to thermoblocks, such as in lateral flow assays (see Section 4). Proper mixing before and during reaction, however, must be ensured due to relatively high density of the final RPA mixture. If well-performed, RPA can be highly useful alternative to PCR due to its speed and extremely simple instrumentation involved. The major downside is the availability of RPA reagents, which are sold by only a single company (TwistDx™, https://www.twistdx.co.uk), which not only increases the overall

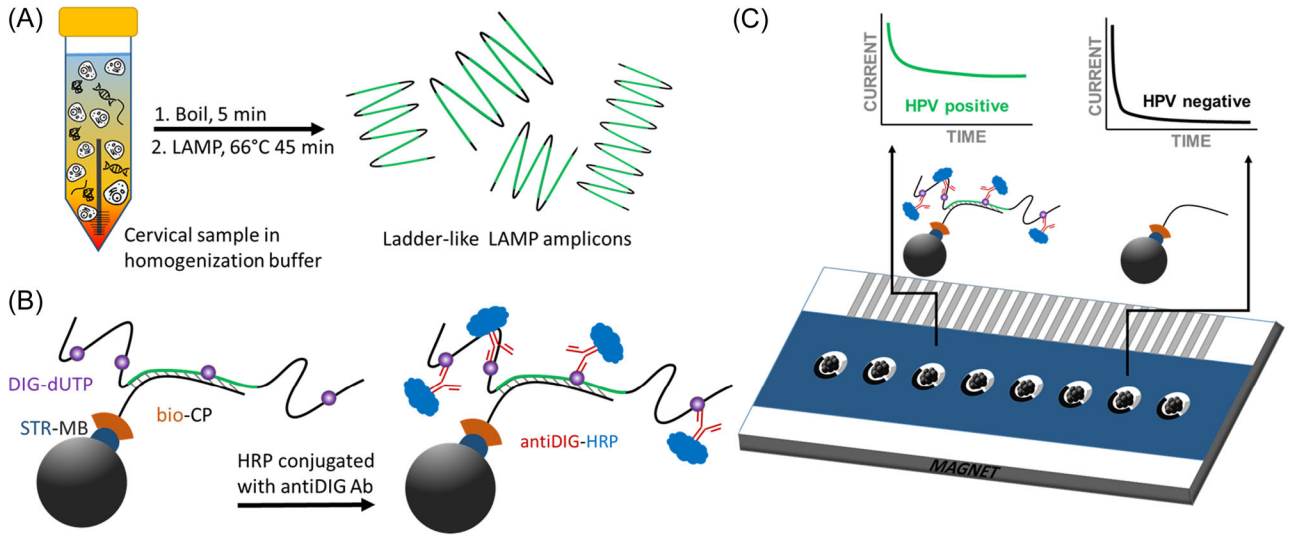

FIGURE 1 Electrochemical assay with LAMP amplification (EC-LAMP) workflow. (A) To avoid DNA extraction, clinical samples were simply boiled for 5 min, followed by the LAMP reaction. (B) Digoxigenin-tagged (DIG-dUTP) LAMP products were hybridized to biotinylated DNA capture probe (bio-CP) attached to streptavidin magnetic beads (STR-MB), which then interacted with antidigoxigenin antibody-horseradish peroxidase conjugate (antiDIG-HRP) for enzymatic reaction. (C) Electrochemical measurement of the enzymatic reaction on carbon electrode chip in 8-electrode format. Reprinted with permission from Ref.[79] Copyright 2021 Elsevier.

cost, but poses a risk in the event of prolonged disruptions in the distribution of these products.

In summary, IATs allow fast and inexpensive nucleic acid amplification with similar efficiency as PCR, requiring milder and constant temperatures without a need for PCR cycler. Since they are more tolerant to common PCR inhibitors, isothermal reaction may be often performed in crude cell or tissue lysates without preceding DNA extraction step. On the other hand, due to an ultrasensitive nature of IATs, caution must be taken to prevent contamination of the workplace and if possible, reactions should be performed in sealed environment. Furthermore, most studies so far demonstrated only a proof-of-concept, while validation within clinical studies on a larger number of samples is missing. Taken together, the advantages make IATs perfect candidates for implementation in developing countries, or in low-resource settings in general.

4 | REVERSE DOT BLOTS AND LATERAL FLOW ASSAYS—IDEAL FOR LOW RESOURCE SETTINGS

Among the numerous diagnostic techniques available, reverse dot/line blot and lateral flow assays (LFA) have gained significant attention for their simplicity, cost-effectiveness, and rapidity. The first one has proven valuable in identifying mutations related to hereditary diseases and cancer-associated genes,[83] while LFAs was mostly used for protein detection, especially antigens.[84] Below, we describe both techniques by providing their principles, examples, advantages as well as drawbacks.

Briefly, the reverse dot blot/line blot is a simple diagnostic method where the gene-specific oligonucleotide probes are bound (coated) onto the predefined dots or lines at the nylon membrane, respectively, followed by a hybridization of these probes with complementary target DNA, usually labeled PCR products.[85] The main benefits of the reverse blots are their simplicity, rapidity, accuracy, cost-effectiveness, and option of screening for multiple mutations/polymorphisms in a single hybridization reaction where results from a single sample can be located on a single strip to minimize user errors. Moreover, since PCR products (but not probes) are labeled, potential false positives occurring from binding of the probes to nonspecific sequences is less probable.[86] Despite these advantages, errors may occur during the conjugation step that may result in weak signals or false positives. Also, a visual detection with naked eye, where signal intensity varies from strong through medium, weak, very weak, and negative, may lead to false interpretation of results. Reverse blots were used to screen for HPV infection already in 1998 when Gravitt and colleagues developed a reverse line blot strip assay for screening 27 different genotypes of HPV.[87] The developed method used a sensitive and broad-spectrum PCR amplification system, followed by a single hybridization with a reverse line blot detection for analysis of 15 high-risk and eight low-risk HPV types. It was applied to a total of 359 cervical specimens, although 30 of them were eliminated due to false signals, which can be considered a major drawback of this otherwise interesting and simple assay. For each clinical sample, two multiplex PCRs were performed, which included a mix of biotinylated primers targeting *L1* region of different HPV genotypes. Although authors compared their results with their previous work utilizing dot-blot assay,[88] reaching

high concordance from 97% to 100%, validation with the gold standard was missing.

LFA, also known as lateral flow test or lateral flow device, is a rapid, simple, and low-cost paper-based analytical platform for the qualitative determination of the presence or absence of target analytes in various complex samples without the need for specialized equipment. It is mostly used for detection of protein antigens or antibodies since it relies on principles of the enzyme-linked immunosorbent assays (ELISA); two well-known examples are home pregnancy tests and rapid antigen tests for SARS-CoV-2 detection. However, LFAs have been widely used also for nucleic acid detection, employing two different formats - nucleic acid lateral flow (NALF) and nucleic acid lateral flow immunoassay (NALFIA).[89,90] NALF directly detects PCR-amplified DNA product using capture and labeled reporter oligonucleotide probes complementary to the product. Usually, capture probes are immobilized on a nitrocellulose membrane using a small molecule in conjunction with its high-affinity counterpart, like biotin-streptavidin, while reporter probes are tagged with gold nanoparticles for signal generation. Since hybridization takes place on a paper, any secondary structure formation within the three DNA sequences severely disrupts interactions, necessitating extensive optimization for each new target.[91] On the other hand, NALFIA detects hapten-labeled DNA (mostly exploiting primers tagged with digoxigenin or biotin) using capture and labeled reporter antibodies or streptavidin.[92] This architecture eliminates the need for specific optimization of the lateral flow strip, allowing the strip's reagents to be nontarget-specific.[91] A typical LFA strip consists of three pads and a detection zone (Figure 2). First, liquid sample is loaded into a sample pad and the analyte moves via capillary flow (without any external forces applied) to the conjugate release pad, where it interacts with specific antibodies conjugated to colored or fluorescent particles, mostly colloidal gold and latex microspheres.[93] The conjugate-analyte complex then moves along the strip to the detection zone to bind with immobilized antibodies or antigens at predefined lines called test line and control line. The target recognition occurs at the test line, where, as the name implies, appears a line visible by a naked eye. No line appears without a target. Control line purely indicates that the liquid migrated properly through the strip. The last pad, called absorbent pad (or absorption pad) at the end of the strip maintains the proper flow rate and stops backflow of the sample. Major challenges of LFAs include low multiplexing abilities (only few biomarkers can be detected simultaneously), lower sensitivity resulting in false negatives (especially in low viral load samples resulting in faint test lines that may be difficult to read with naked eye) and need for high-quality antibodies (to ensure accurate and reliable results due to exclusive binding of the antibodies to the target analyte and not nonspecifically to other biomolecules). LFA tests are often coupled with PCR amplification. For instance, Xu et al. developed a dual-color fluorescence-based LFA to address low multiplexing issues by detecting four common HPV types (6, 11, 16, and 18).[94] Since fluorophore-labeled detection probes for each genotype were included in the PCR reaction and capture probes were immobilized at different locations of the strip, multiple sequences could be hybridized in a single lateral flow assay. In fact, due to the presence of five test lines, the suggested assay could detect up to 13 HPV types in less than 30 min after PCR amplification. The developed assay was applied to 157 cervical samples and compared with results from GenoArray kit, achieving 98.1% (154/157) concordance between these two methods.

Besides PCR, lateral flow assays for HPV detection are increasingly combined also with IATs, mostly RPA, which was described in chapter 3.[95–98] For instance, a study by Ma et al. utilized RPA to detect 25 HPV types from as low as 100 fg of genomic DNA per reaction, using a panel of 450 cervical clinical samples. After RPA amplification, the presence of the target sequences in samples was detected by lateral flow dipstick and reversed dot blot techniques. Good concordance between the detection methods and routine cervical screening was achieved, reaching 94.7% agreement for lateral flow dipstick and 97.8% agreement for the reversed dot blot.[97] A very recent study by Kundrod et al. utilized an inventive low-cost NATflow platform (commercially available from Axxin Pty Ltd.) for RPA-based detection of HPV16 and HPV18.[98] The platform did not require DNA extraction step, but instead involved a streamlined sample preparation technique that could be directly added to the amplification reaction by using achromopeptidase enzyme (ACP) for enzymatic

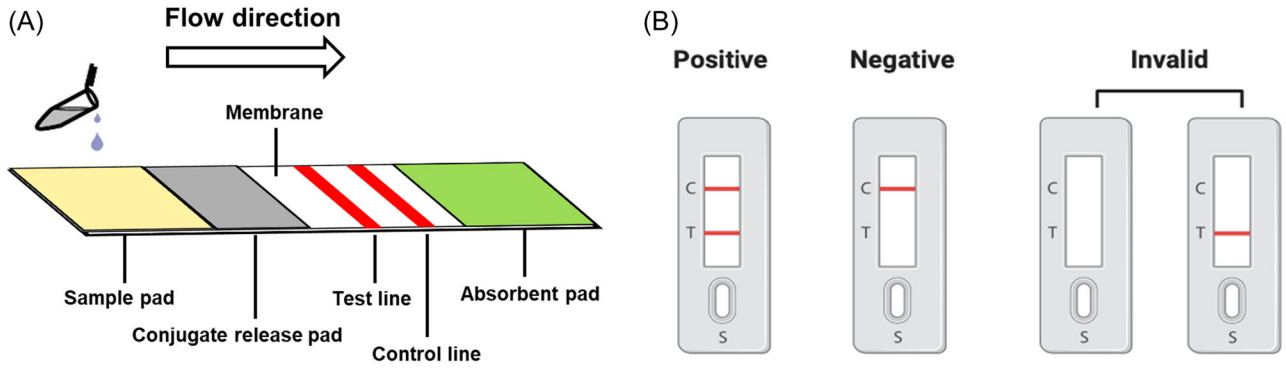

FIGURE 2 Fundamentals of lateral flow assay, showing (A) overall layout of the test strip and (B) visual detection of results based on a presence of test line (T) and control line (C). Created with BioRender.com.

lysis of the cells, reducing overall assay time to only 45 min. The performance of the test was evaluated with both provider-collected samples (30 samples collected in the U.S.) and self-collected samples in low-resource settings (55 samples self-collected in Mozambique). When compared with qPCR, the sensitivity of detecting HPV16 was 100% for samples containing a minimum of 1000 copies per reaction and 93% for those with at least 500 copies per reaction. The specificity of the test for HPV16 was 100%, the positive predictive value was 86% and the negative predictive value was 56%. When evaluating combined HPV16 and HPV18 in preserved samples, NATflow and cobas showed an overall agreement of 85%. All discordant samples had fewer than 500 copies per reaction, indicating that low viral load samples could be more difficult to determine with this platform. The projected cost of a single test was less than $5, showing a great potential for point-of-care HPV diagnostics. In another study, Rungkamoltip et al. developed an RPA-based LF strip for combined HPV16/18 DNA detection by monitoring circulating cell-free DNA (cfDNA) from serum of 39 cervical cancer samples and 29 control samples with the purpose of avoiding invasive sampling.[96] The results obtained within 30 min were compared with digital droplet PCR, showing 100% sensitivity and 88.24% specificity. However, it should be noted that circulating HPV DNA has a very low abundance in serum of women with precancerous cervical lesions and thus its use as a diagnostic biomarker is questionable. Instead, several studies demonstrated that it may act as a prognostic biomarker in blood of patients with primary tumors of cervix to monitor advanced stages or possible metastases.[99–103]

5 | CRISPR-CAS SYSTEM FOR IMPROVED SELECTIVITY

CRISPR-Cas technology (short for Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein) was introduced in 2012 by the research group of Jennifer Doudna and Emmanuelle Charpentier (awarded joint Nobel Prize in chemistry in 2020), which revolutionized not only genome editing but also molecular biology in general.[104] Soon after this discovery, CRISPR-Cas system was introduced into molecular diagnostics, including the field of biosensing. Its principle lies in a cleavage of specific nucleic acid sequences recognized by a guide RNA that is made up of two parts: CRISPR RNA (crRNA), a 17–20 nucleotide sequence complementary to the target nucleic acid, and a trans-activating RNA (tracrRNA) scaffold recognized by a catalytically active Cas protein. The final CRISPR-Cas complex then binds to the target sequence and cleaves it in a highly specific manner. This technology had a huge impact on molecular diagnostics by greatly increasing specificity of bioassays.[105] Currently, there is a plethora of different caspases, for example, Cas9, Cas12, Cas13, Cas14, and their subtypes,[106,107] that specifically cleave various target molecules such as single-stranded (ss) DNA, double-stranded (ds) DNA, ss RNA with cis- and/or trans-cleavage activity,[108] or even unwind dsDNA.[109] Accordingly, CRISPR-Cas system has been employed in detection of wide range of biomarkers, including microRNAs,[110,111] DNA methylation,[112] single point mutations,[113,114] various bacteria such as *Salmonella*,[115,116] *Yersinia pestis*,[117] or viruses such as SARS-CoV-2,[118] EBV,[119] as well as HPV.[120–142] Interestingly, CRISPR-Cas technology was recently used also in a treatment of HPV infection-associated cervical cancer. By using liposome delivery of CRISPR-Cas9, authors effectively knocked out HPV, which, in turn, induced autophagy and triggered cell death-related immune activation by releasing damage-related molecular patterns.[143]

The CRISPR-Cas system facilitates identification of individual HPV genotypes at very low copy numbers. Most of these HPV assays are frequently coupled with RPA and use fluorescence end-point detection. An interesting example was reported by Xu et al.,[133] combining a microfluidic device with RPA reaction and CRISPR-Cas12a that ensured multiplexed detection of nine hrHPV genotypes within 40 min. The so-called MiCaR platform was initiated by thermolysis of the sample (without DNA extraction), followed by a multiplexed RPA. The RPA products were then loaded into the microfluidic device and specifically cleaved with CRISPR-based system containing Cas12a, crRNA, and a fluorescence reporter. The assay targeted *L1* gene and was applied for screening of 100 cervical smear samples with 97.8% sensitivity and 98.1% specificity.[133] A similar approach was introduced by Zhao et al.[141] by combining RPA and fluorescence readout into a microfluidic dual-droplet device. This system was developed for dual detection of HPV16 and HPV18, reaching an ultralow limit of detection (1 aM, ~1 copy/reaction) in only 30 min. Moreover, when compared to standard PCR, the sensitivity of 92.3% and the specificity of 100% on a panel of 20 clinical samples was achieved. However, assay targeted *L1* gene that can be lost during HPV integration into the host genome,[144,145] leading to possible false negative results.

Although majority of CRISPR-based studies use some DNA amplification technique before detection,[122–127,129–131,133,137–142] several works reported also amplification-free strategies.[121,128,132,134,146] An interesting technology called polydisperse droplet digital CRISPR-Cas-based assay has been introduced by Xue et al.[134] whereby the reaction mixture containing CRISPR-Cas components and a fluorescent dye-labeled reporter probe was vortexed with oil to generate multiple droplets. The final protocol included both Cas12a (cleaving dsDNA) and Cas13a (cleaving ssRNA), coupled to fluorescent labels generating green (FAM) and red (HEX) positive droplets, respectively, for a dual detection assay in one mixture. Hence, the assay could independently detect HPV18 DNA from 23 cervical smears (Cas12a, HEX) and also SARS-CoV-2 RNA from 32 nasopharyngeal swabs (Cas13a, FAM). Limit of detection for HPV was 162 pg/μl of genomic DNA within 20 min of reaction time. Assay sensitivity and specificity reached 100% for both assay targets, but it should be noted that number of tested samples was too low for relevant statistical analysis.

Overall, CRISPR-Cas technology has rapidly entered biosensing research and has already made a huge impact by increasing specificity of individual assays in a relatively inexpensive manner. However,

several technical challenges persist, including off-target activity (i.e., cleavage at other than recognition site) or sometimes lower efficiency of cleavage by caspases.[147] Moreover, when applied to the field of biosensing, most works relied on pre-amplification step for increased sensitivity, prolonging overall protocol time. Nevertheless, combination with quick IATs, as shown for example, in a work by Ganbaatar who coupled 20 min of RPA reaction with 10 min of CRISPR-Cas12a cleavage,[122] could be a promising pathway for its possible use in low-resource settings.

Results from above studies using CRISPR-Cas system implies that it offers good sequence resolution at a single nucleotide level, but discrimination of individual genotypes would require very precise design of gRNA. Although HPV is a small virus, there is quite large intra-host sequence variability within highly conserved sequences, and up to a 1000 unique variants were identified within individual samples.[148] Our experience thus shows that it is better to use techniques which recognize longer sequences by using primers or probes, or to use CRISPR-Cas system at those instances where single mismatches play a crucial role.

6 | MICROFLUIDICS AND LAB-ON-A-CHIP TECHNOLOGY—INTEGRATING INDIVIDUAL STEPS INTO A SINGLE DEVICE

Microfluidic technologies, sometimes referred to as "lab-on-a-chip" provide an opportunity to create devices or chips that could compete with classical techniques in biomedical and chemical research.[149–152] These low-cost devices can process microliters of solution via capillary channels in a high-throughput manner, thus reducing the requirement for samples and reagents. Importantly, all reactions and washing steps are performed in sealed reaction systems, ideal for preventing cross-contamination. LOC technology represents rapidly growing field in DNA diagnostics, and numerous devices were introduced also for HPV analysis.[153–167]

For example, an interesting innovation was an automated diagnostics of high-risk HPVs by using machine learning coupled with single-cell droplet PCR and multiplexed microfluidics chip.[158] Images of droplets to confirm presence of amplified HPV products were captured by inverted fluorescent microscope with CCD camera, followed by an analysis using Circle Hough Transform technique for circle detection and transfer learning of LeNet neural network (termed droplet-net). Although the assay was performed only on cervical cancer cell lines and still required relatively expensive instrumentation, it could pave the way for future automation of point-of-care diagnostics with the potential to improve accuracy and decrease overall time and cost. As we mentioned above, sealed reaction systems may prevent aerosol contamination which is common in nucleic acid amplification techniques. To further decrease the risk of contamination, Mou et al. reported a femtoliter-sized microfluidic hybridization assay without DNA amplification, yet reaching ultralow attomolar detection limits.[153] The principle was similar to the hc2 commercial kit, relying on an antibody that captures DNA/RNA hybrids, but it used femtoliter-sized droplets for concentrating enzyme-catalyzed fluorescent products into a small volume to increase detectable signal, and magnetic beads for accelerating reaction time. Interestingly it reached better sensitivity than hc2 by detecting samples with low viral load. On the other hand, the whole study was conducted with only 20 HPV-positive clinical samples without using negative controls.

The physics of microfluidics relies on the pumps or pressure to generate fluid flow, requiring either special instrumentation or electricity. This, consequently, limits microfluidics-based diagnostics in low-resource settings. To resolve this issue, paper-based microfluidics (paperfluidics) introduced by Whitesides group in 2007[168] has recently garnered much attention due to its ability to passively transport fluids through capillary action, which circumvents the problem of pumps or other fluid handling equipment. Such paperfluidic platform for HPV analysis was constructed by using solely paper and adhesive sheets.[163] It integrated all the steps, including DNA extraction, LAMP amplification, and lateral flow detection of HPV16 DNA via immunochromatographic strips at the panel of ten cervical samples. Although two out of five negative samples exhibited faint positive test lines, most probably due to self-priming of LAMP primers, this low-cost disposable paperfludic chip could represent a viable option for quick diagnostics of HPV infection once the issue of false positives is resolved. A combination of above-mentioned principles, that is, hybrid capture strategy to avoid DNA amplification and paper-based assay without a need for pumps, has been very recently reported by Richards-Kortum group.[161] The assay comprised two-dimensional paper network (2DPN) and a point-of-care sample preparation protocol to detect HPV16 DNA from exfoliated cervical cells within an hour. It was conducted not only in controlled laboratory settings using cervical samples from Salvador but also in field settings in Mozambique on self-collected samples. As expected, the accuracy of the field testing on self-collected samples was lower compared to the laboratory testing, which was attributed to a higher sample turbidity in the case of self-collection. As authors noted, additional pretreatment of self-collected samples will be needed to eliminate false positive results, but otherwise, this study holds great promise for remote low-resource HPV diagnostics.

Despite the advantages that microfluidic platforms offer, including options of miniaturization, portability and sealed environment to prevent contamination, there are still challenges that limit them to reach their full potential. For instance, manufacturing process is quite complex, not fully standardized and as such is not ready for mass production.[149] Moreover, microfluidic devices face challenges when coupled with other platforms, especially those for imaging recordings, and require trained personnel and stringent, frequent quality checks that limit their applications especially for low resources environment.[169] In this sense, paper-based microfluidic devices are considered the most promising candidates for low resource settings as they are low-cost, easy-to-use, disposable, and virtually equipment-free. However, the areas of improvement also exist, such as high sample retention within paperfluidic channels and higher limits of detection associated with the traditional visual detection, both of them leading to relatively low sensitivity of measurement.[170]

7 | NANOMATERIALS ENHANCING ASSAY SENSITIVITY

Great progress in nanobiotechnology and in nanomaterial science have enabled massive development of bioassays and biosensors at the nanoscale. Nanomaterials show unique mechanical, optical, electrical, and even magnetic features due to their small dimensions, and enable ultralow detection limits as a result of their large surface-to-volume ratios.[171–174] Distinct nanostructures were introduced, such as gold or silver nanoparticles, carbon nanotubes, graphene oxide, quantum dots, polymeric nanocomposites, or even various nanorods or nanowires, which serve either as carriers for bioreceptor immobilization or as signal-generating reporters. Frequently, multiple nanomaterials are combined together to create complex architectures in a single assay to obtain as low detection limits as possible, but often without any application in real clinical settings. As such, this pursue for ultrahigh sensitivity is questionable if not demonstrated on clinical material. Moreover, nanomaterials are still relatively costly and their possible negative health and environmental impact has been a matter of numerous discussions.[175] Various studies have reported penetration and accumulation of nanomaterials into cytoplasm or even cell nucleus.[175–179]

Application of nanomaterials for HPV analysis is relatively common, but as we discussed above, plenty of reports do not show feasibility on clinical material.[180–184] Instead, many of them use "spiking" whereby synthetic sequences are inserted into the serum to show the percentage of recovered sequences and thus to evaluate possible matrix effects. Many nanomaterial-based papers that actually used cervical samples from women tested their assays on a relatively low number of patients.[185–190] For instance, Hong et al. employed carboxylic group-functionalized magnetic nanoparticles for split electrochemiluminesce assay for detection of HPV16 based on gold nanocluster probes. The introduction of various nanomaterials led to an ultralow detection limit of 6.8 aM, and the assay was applied to 27 cervical smears with substantial agreement when compared to hc2 assay. In another small-cohort study, Sun et al. developed a photoelectrochemical biosensor array (PEBA) for multiplexed detection of nine HPV genotypes (Figure 3).[185] Authors reported the detection limit of 0.1 copies/μl and analyzed a total of 40 clinical samples, including 20 HPV-positive and 20 HPV-negative samples. Relatively large cohort of 209 cervical samples was used for validation of a novel nanoparticle-assisted PCR assay (nanoPCR) for detection of E6 genes of HPV16 and HPV18.[191] Although authors did not calculate sensitivity and specificity of their nanoPCR assay, it displayed 10-fold more sensitive detection than that of a conventional PCR, used only few microliters of the sample and allowed the reaction to reach more quickly the target temperature, hence shortening overall assay time.

Despite huge advances in nanotechnology, clear benefits of its application for HPV diagnostics have yet to be demonstrated especially for low resource settings in terms of simplicity or cost-effectiveness, and potential health and environmental risks need to be addressed. In addition, nanomaterials often show lower recognition efficiencies for target analytes in complex biological environments, slow kinetics of binding processes due to heterogeneous interfaces, and questionable stability of nanomaterial-based surfaces, which are all issues that need to be taken into an account when designing biosensing technologies.[192]

8 | CONCLUSION AND RECOMMENDATIONS

The recent COVID-19 pandemic has undoubtedly accelerated development of novel technologies for detection of viral infections, including HPV. Commonly, HPV is detected at the nucleic acid level via PCR-based assays, which is considered gold standard. However, new analytical tools have emerged in last years that could outcompete them in terms of simplicity, cost, or time efficiency. Various isothermal amplification techniques were introduced as interesting alternatives to PCR that obviate a need for thermal cycling, hence providing an opportunity to construct simple and rapid diagnostic tools and devices for point-of-care testing. This is nicely evidenced by for example, numerous lateral flow assays coupled to RPA deployable in field settings, CRISPR-based systems that improve specificity of detection, microfluidic and LOC devices integrating all reaction steps and final readout, as well as diverse nanomaterials which greatly enhance sensitivity of the measurement. In some cases, LFA devices or CRISPR-based systems may still benefit from PCR amplification, but even here the IATs are gaining ground. Major drawback of these technologies, however, is their insufficient application into clinical material to prove their usefulness. Until recently, many studies used only spiking of synthetic HPV sequences into serum to mimic real settings or utilized cancer cell lines with well-defined HPV status. A reason for spiking or for using only cell lines is perhaps a lack of access to high-quality biomaterial from patients that successfully passed pre-analytical procedures used in sample collection, processing, storage, and shipping, significantly influencing nucleic acid integrity. It is worth noting that a number of academic and/or commercial biobanks exist, including European research infrastructure for biobanking (BBMRI-ERIC), International Society for Biological and Environmental Repositories (ISBER), or European, Middle Eastern, or African Society for Biopreservation and Biobanking (ESBB), which offer human samples and associated data of standardized quality for research purposes. Another significant drawback is that most of these technologies were not yet fully tested in low-resource settings outside well-equipped laboratories or hospitals. Only few relevant studies, mostly utilizing LFA combined with RPA[98] or paperfludic device with hybrid capture technology,[161] showed performance in low-resource settings in developing countries. Authors of the former study even estimated a cost of a single test to be around $5, a very affordable price due to their extraction-free and PCR-free approach.[98] Other authors did not estimate projected costs of their assays, but it can be expected that by implementing CRISPR-Cas system, microfluidic devices or nanomaterials, overall price per test would increase. Furthermore, noncolorimetric-based (other than naked-eye) end-point detection systems (e.g.,

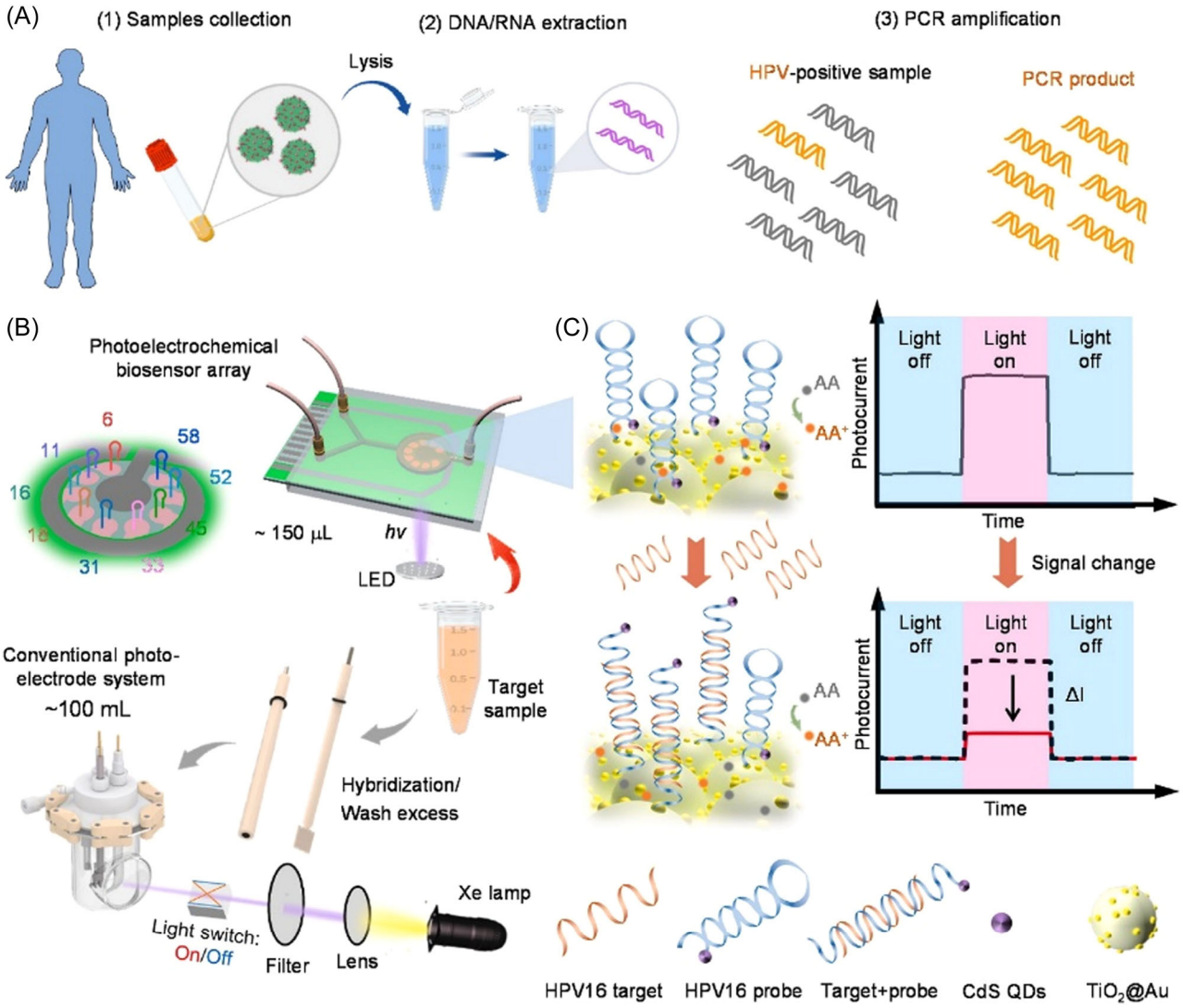

FIGURE 3 Schematic illustration of the photoelectrochemical biosensor array (PEBA) platform. (A) The clinical samples pretreatment and amplification. (B) Schematic illustration of the PEBA setup for HPV genotyping. (C) Schematic illustration of the fabricated PEBA for detecting HPV-related genes. QD, quantum dot. Reprinted with permission from Ref.[185] Copyright 2023 Elsevier.

fluorescence, luminescence or electrochemical) require initial investments in terms of new instrumentation.

In this review, we tried to highlight those studies where authors included clinical samples obtained from patients, mostly cervical smears. However, HPV can be detected also non-invasively, either in self-collected vaginal swabs, or in a liquid biopsy format, that is, in urine, blood, or saliva. While cervical smears require a pelvic examination by a gynecologist, collection of a vaginal specimen for HPV testing can be performed by the patients themselves, a feature that is especially attractive in resource-limited areas, making the HPV testing with self-collected samples as a possible primary screening alternative.[193] A large meta-analysis of 12 studies showed good accuracy between patient-collected vaginal samples and those obtained by a clinician, but due to highly heterogeneous data and a variety of specimen collection devices that have been used in individual studies, no recommendation was provided.[194]

Urine is especially attractive since it permits frequent self-collection and the sampling of large populations to measure for example, the impact of HPV vaccination programs.[195] Moreover, urine sampling, unlike cervical sampling, is a more preferred choice and better accepted by women,[196] which may lead to increased population coverage in screening programs. On the other hand, urine testing faces challenges such as lower HPV load, presence of PCR inhibitors and contaminating pathogens, and possible higher rate of false positives due to HPV infection of the urinary tract or the lower genital tract. Although many studies have shown correlation between HPV detection in cervix and urine,[195,197–203] they were often very discrepant due to diverse methodologies used during sampling, storage, sample preparation, and DNA extraction, and further optimization and standardization is required.

Regarding the blood analysis, most authors focus on the role of HPV circulating DNA as a prognostic biomarker in blood of patients

with primary tumors to monitor advanced stages or possible metastases.[99–103] Circulating HPV thus does not seem as a suitable biomarker for eventual screening programs due to low abundance of HPV in precancerous lesions.[204,205] Salivary testing is another noninvasive option allowing for early HPV diagnostics of HNC, especially for risk stratification of patients with head and neck squamous cell carcinoma (HNSCC), or for possible monitoring of recurrence after treatment.[206] However, contradictory conclusions were drawn when evaluating sensitivity of salivary testing and its usefulness as a predictive indicator.[203,207] Apparently, more studies are needed to prove salivary HPV as potentially valuable biomarker for detection of HNSCC. Unfortunately, the potential of advanced technologies described in this work has not yet been fully utilized in noninvasive diagnostics of HPV in urine, blood or saliva, or in self-collected samples in general, such as in vaginal swabs. This combination, however, is highly attractive and we envision that in near future, new analytical tools will become available enabling simple and rapid HPV diagnostics with good precision at the point-of-care or in low-resource settings.

AUTHOR CONTRIBUTIONS

All authors reviewed the available literature and wrote individual parts of the manuscript. Martin Bartosik and Roman Hrstka edited the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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ORCID

Martin Bartosik http://orcid.org/0000-0002-4728-1626
Ludmila Moranova http://orcid.org/0000-0003-3117-5535
Nasim Izadi http://orcid.org/0000-0003-2401-9630
Johana Strmiskova http://orcid.org/0009-0001-1541-0198
Ravery Sebuyoya http://orcid.org/0000-0002-3769-6676
Jitka Holcakova http://orcid.org/0000-0002-7323-8920
Roman Hrstka http://orcid.org/0000-0002-6139-2664

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