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QUANTITATIVE SCREENING OF SINGLE COPIES OF HUMAN PAPILLOMA VIRAL DNA WITHOUT AMPLIFICATION

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Abstract

We describe a novel quantitative viral screening method based on single-molecule detection that does not require amplification. DNA of human papilloma virus (HPV), the major etiological agent of cervical cancer, served as the screening target in this study. Eight 100-nucleotide (nt) single-stranded (ss)-DNA probes were designed complementary to the E6-E7 gene of HPV-16 DNA. The probes were covalently stained with Alexa Fluor 532 and hybridized to the target in solution. The individual hybridized molecules were imaged with an intensified charge-coupled device (ICCD) in two ways. In the single-color mode, target molecules were detected via fluorescence from hybridized probes only. This system could detect HPV-16 DNA in the presence of human genomic DNA down to 0.7 copy/cell, and had a linear dynamic range of over six orders of magnitude. In the dual-color mode, we employed fluorescence resonance energy transfer (FRET) and added YOYO-3 dye as the acceptor. The two colors from Alexa Fluor 532 and YOYO-3 were dispersed by a transmission grating located in front of the ICCD. With this reinforced criteria for identifying the hybridized molecules, zero false-positive count was achieved. We also showed that DNA extracts from Pap test specimens did not interfere with the measurements.

Keywords

single molecule; fluorescence; quantification; human papilloma virus (HPV); hybridization

Single-molecule techniques opened up new research frontiers for the study of biological macromolecules. They provided insights into biochemistry, biophysics and biology by revealing unique behaviors of individual molecules. In the early 1990s, a rapid DNA sequencing scheme based on the detection of single, fluorescently tagged nucleotides cleaved from a single DNA fragment was proposed.^{1, 2} Fluorescence resonance energy transfer (FRET) between different chromophores enabled the investigation of protein dynamic interactions in living cells.^{3, 4} Gene expression has been analyzed at the single-molecule scale by fluorescence correlation spectroscopy (FCS).^{5, 6} Characteristics of individual biomolecules, such as activity of enzyme molecules,^{7–9} diffusion variations of single DNA molecules^{10, 11} and electrostatic behavior of protein molecules¹¹ have been studied as well. Here, we try to exploit the enormous potential of single-molecule detection in clinical diagnostic applications.

Cervical cancer is the second most common cancer among women worldwide with over 50% mortality rate,¹² resulting in 300,000 deaths annually according to the World Health Organization (WHO). In the late 1990s, high-risk subtypes of human papilloma virus (HPV) such as HPV-16 and HPV-18 proved to be the major etiological agents of cervical cancer. An overall HPV prevalence of 99.7% among patients was estimated.¹³ Although the threshold of the viral load to the cancer stage is not exactly known, it has been established that there is a

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positive relationship between viral load and cervical cancer risk.^{14–16} The major reason for cervical cancer's high mortality rate is that it does not produce any early-warning symptoms or physical changes until it is so advanced that is usually unresponsive to any treatments.

Early detection of HPV could decrease the incidence of cervical cancer and increase the curability. High sensitivity is the key. Currently, the simple, inexpensive Papanicolaou smear (Pap smear) screening is the most widely used method. It clearly helps in reducing the incidence of cervical cancer, but it suffers from a significant false-negative rate of 20–40%¹⁷ as a result of inherent screening errors.¹⁸ HPV testing based on viral DNA detection has drawn increasing attention and been suggested as a supplement to the Pap test.^{19, 20} Several amplification-based technologies have been developed for viral nucleic acid screening. The most frequently used validated HPV testing methods are polymerase chain reaction (PCR) assay and the U.S. Food and Drug Administration (FDA)-approved hybridization capture assay (HC). PCR is a target-amplification-based method in which the viral DNA fragments can be replicated millions of times during thermal cycles.^{21–23} HC assay is a signal-amplification-based method involving enzymatic reaction. In this assay, cellular DNA is hybridized in a solution containing mixtures of RNA probes. Then, the hybridization complexes are captured to the tube wall coated with an antibody to the RNA-DNA hybrids. After binding to another antibody conjugated with alkaline phosphatase, the hybrids are detected by chemiluminescence reaction with a substrate.^{24, 25} Although both of these methods are highly sensitive,^{25–27} the process of amplification often leads to false positive due to contamination. PCR may not start at low copy levels. HC may not achieve 100% capture efficiency. Quantification is another major concern in clinical diagnosis especially since viral load is an important number. PCR is only semi-quantitative, since amplification is exponential and the gain depends critically on temperature, solution composition, enzyme activity, etc.²⁸ On the other hand, single-molecule detection,^{29, 30} which can count individual copies of DNA with 100% efficiency down to 10^{-17} M, can be more reliable than amplification-based schemes. In addition, the elimination of amplification simplifies the operation and the analysis time.

Here, we demonstrate a new quantitative single-molecule method for detecting HPV DNA without amplification. This approach avoids problems with contamination and/or enzyme inhibition. The assay is based on the hybridization between HPV DNA and fluorescently labeled complementary DNA probes. Among more than 100 types of HPV, HPV type 16, which alone accounts for ~50% of the cancer cases,³¹ was the target DNA. The length of HPV-16 DNA is about 8000 base pairs (bp), and its oncogene E6-E7 that is 800 bp is expressed in all HPV-containing tumors and derived cell lines. E6-E7 gene is not only required for initiation but also the maintenance of a proliferative and malignant phenotype in host cells.³² This gene can be found both in episomal and integrated forms in human chromosomes of the cells it infects,³³ thereby making it suitable for recognition. We have previously shown that the smallest DNA molecule that is detectable with this apparatus is 120 bp.²⁹ Therefore, the viral DNA was probed by eight 100 nt complementary ss-DNA. The individual probe molecules were below the threshold for detection of individual hybrid molecules. This technique not only allows the detection and quantification of viral DNA with low detection limit, but also offers a universal approach to any other DNA sequence by simply changing the sequence-specific probes.

Experimental Section

Sample preparation

Eight 100 nt ss-DNA probes were designed according to the sequences of E6/E7 region of HPV-16 from Genbank (K02718), and synthesized by Integrated DNA Technologies (Coralville, IA). The probes were dissolved in pH 8.0 1× Tris-EDTA (TE) buffer (Ambion, Austin, TX) and labeled individually with Ulysis Alexa Fluor 532 nucleic acid labeling kit

(Molecular Probes, Eugene, OR) according to the manufacturer's instructions. After the staining reaction, tubes were snap-cooled on ice, the probes were subjected to a clean-up procedure with micro bio-spin columns (Bio-Rad Laboratories, Philadelphia, PA) to get rid of excess labeling reagents. All eight probes were mixed together for the subsequent hybridization step.

In hybridization samples, the total amount of HPV-16 DNA (Maxim Biotech, San Francisco, CA) and female human genomic DNA (Promega, Madison, IL) was kept at 1 μ g, while the ratio of them was varied to achieve a series of HPV-16 contents from 0 to 10%. The probe solution was mixed with the DNA so that the concentration of each probe was 8 times that of HPV-16 DNA. Finally, hybridization buffer (Maxim Biotech) was added to make a total volume of 30 μ L. The samples were kept at 95 °C for 10 min for denaturation and at 42 °C overnight for hybridization in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA). The hybridization samples were diluted with nuclease-free water (Ambion, Austin, TX) to proper concentrations right before imaging (see Table 1).

In dual-color detection, the samples were further labeled with YOYO-3 (Molecular Probes) at a ratio of one dye molecule per five bases. Restriction endonuclease reaction was carried out to reach low detection limit. HPV-16 DNA and human genomic DNA were mixed together and digested by 5 $\times$ Hind III (New England Biolabs, Ipswich, MA) at 37 °C for 1 h. The enzyme was deactivated at 65 °C for 20 min. Then, the probes were added into the digested DNA for hybridization in the same way as for single-color detection.

Cervical cancer-derived cell lines CaSki and SiHa were purchased from ATCC (Manassas, VA). Normal Pap test specimens were donated by a healthy adult female. DNA from these specimens were extracted by the DNeasy tissue kit (Qiagen, Valencia, CA) and used without further purification. The DNA extraction yield is 60–80% according to the manufacturer's handbook. The DNA concentration was calculated from UV absorption at 260 nm. The amount of extracted DNA from each Pap sample was 8 μ g.

Imaging system

The imaging system was similar to the previous apparatus used in our group,^{34, 35} with some modifications (Fig. 1). A 532 nm solid-state continuous wave laser (45 mW, μ -green Model 4611, Uniphase, San Jose, CA) was used as the excitation source. A Uniblitz mechanical shutter (model LS2Z2, Vincent Associates, Rochester, NY) and a driver (model T132, Vincent Associates) were synchronized to the Pentamax 512-EFT/1E1A ICCD camera (Princeton Instruments, Princeton, NJ). The shutter let the laser beam pass through only when the ICCD was on to reduce photobleaching. After the mechanical shutter, the laser beam was expanded by the first cylindrical lens (focal length, 200 mm; Thorlabs, Newton, NJ) in the Y-direction. The expanded beam was then focused into the capillary at normal angle as a thin sheet by the second cylindrical lens (focal length, 25 mm; Edmund Industrial Optics, Barrington, NJ). The image of the laser focusing plane was schematically shown in the enlarged part in Fig. 1. Only molecules inside the laser focusing plane could be excited and detected. The illuminated volume was about 0.2 nl, implying a detection efficiency of 13% considering the illuminated portion compared to the total thickness of the capillary bore. Hybridization samples were injected into a 30 cm long square capillary (75 μ m i.d. $\times$ 365 μ m o.d.; Polymicro Technologies, Phoenix, AZ, USA), and the flow was driven hydrodynamically. Fluorescence from single molecules was collected by a Zeiss 20 $\times$ 0.75 NA Plan-Apochromat microscope objective lens. A 532-nm long pass edge filter (Semrock, Rochester, NY) and a 532-nm holographic notch filter (OD>6, Kaiser Optical, Ann Arbor, MI) were placed between the objective lens and the ICCD to cut off scattering from the excitation beam. A transmission grating with 70 grooves/mm (Edmund) was mounted in front of the ICCD camera to disperse fluorescence collected from the molecules. The distance between the transmission grating and the ICCD was set to

be 6.5 cm so that the zeroth-order and first-order images of the 75 μm i.d. capillary would not overlap each other. The sampling frequency was 2 Hz, with the shutter driver set to 40 ms exposure and 460 ms delay. Images were obtained with the WinView software provided by Princeton Instruments.

Results and Discussion

Single-color detection

In the single-color detection system, only one fluorophor was utilized to visualize the target. Alexa Fluor 532 was selected since its absorption maximum matches the wavelength of a readily accessible excitation source. Sequence-specific Alexa Fluor 532-labeled oligonucleotide probes were hybridized to target HPV-16 DNA and the hybrids were visualized by a single-molecule imaging system (Fig. 2A). About one label was incorporated for every 20 nt. When light passes through a transmission grating, it is divided into a nondispersed zeroth-order image and higher-order spectrally dispersed images. The physical separation between the orders is a function of the distance between the grating and the ICCD and the wavelength of the light. As the grating is located further away from the ICCD camera, the separation between the orders increases and the fluorescence spectrum covers a greater number of pixels to provide higher resolution. A typical fluorescence image of single DNA molecules in a capillary dispersed with a transmission grating is shown in Fig. 2B. The left half is the zeroth-order image of molecules in the capillary, which is identical to that obtained without the grating. The right half is the first-order imaging from the transmission grating, where light was dispersed horizontally according to the wavelength. The dispersion distances in the images were measured between the highest intensity pixels in the zeroth-order and the first-order as shown in Fig. 2B. Since the dispersion distance is proportional to the fluorescence wavelength, impurities can be easily distinguished from hybrids based on wavelength differences. A histogram of the dispersion distances of pure Alexa Fluor 532-probes/HPV-16 DNA hybrids is plotted as Fig. 2C. With the grating positioned 6.5 cm from the ICCD and a 20 $\times$ objective, the average dispersion of the 550 nm emission was 106.0 ± 1.1 pixels. Note that Fig. 2B shows only 1 molecule for clarity. We have previously shown that up to 200 molecules can be imaged at a time in such an arrangement.³⁶

As shown in Figure 1, the sample volume of each frame is $75 \times 75 \times 300$ (μm)³, which is 1.7 nl. In order to make sure that the spots are molecules instead of random noise bursts of the ICCD, the molecules were tracked for 5 consecutive frames. So, 1.7 nl liquid flowed through the capillary every 5 frames. If the movie had 7500 frames, the total volume flowed through the detection area was 2.6 μl .

To evaluate quantification, HPV-16 DNA was added into commercial human genomic DNA matrix. The total amount of viral and human genomic DNA in a vial was kept constant, while the ratio between them was varied to achieve a series of HPV-16 percentages from 0 to 10% ($\sim 7 \times 10^5$ copy/cell). HPV-16 DNA percentage higher than 10% was not included as clinical specimens rarely reach such a high concentration. After hybridization, the samples were diluted to suitable concentrations for counting individual molecules. The dilution factor for each sample was designed (Table 1) to make sure that molecules did not overlap with each other in the high concentration samples, and the number of hybridized molecules was higher than three times that of false positive counts in the low concentration samples. A flow system was selected to allow screening of a much larger volume of sample than that within the detection window. Note that only a small fraction of the total sample passed through the observation region per count period. For a meaningful comparison, it was necessary to keep the probed volume constant from run to run. The volume probed per detection experiment (movie) was proportional to the flow rate. Since the flow was driven by hydrodynamic force, the heights of the sample reservoirs at the two ends of the capillary were fixed to ensure reproducibility. After

the movies were recorded, the molecules were counted manually while fast forwarding the movies. The numbers were further normalized to the same standard considering the movie length and dilution factor. The blue data points in Fig. 2D, corresponding to normalized data column in Table 1, showed that a good linear relationship between HPV-16 DNA percentage and the number of molecules detected was obtained over a range of six orders of magnitude. At low concentrations, the precision is limited by counting statistics. Furthermore, intensity variations among molecules relative to the preset threshold, sample manipulation and loss of material due to adsorption limit the precision to about $\pm 8\%$.

In order to test the compatibility of the current Pap test sampling method with this assay, we extracted DNA from normal Pap smear specimens. About 8 μg total DNA could be obtained from each specimen. Viral DNA was added to the extracted DNA matrix in the same way as in the commercial human genomic DNA experiments (Table 1). In Fig. 2D, the red data points for the extracted DNA matched well with the blue ones for the standard samples. The results also show that the background levels of the two matrices were similar, indicating that DNA extracts from Pap test specimens did not have biological impurities that would interfere with single-molecule detection.

Single-molecule screening was further tested for cell lines with known viral load. The HPV-16 viral loads in cervical cancer-derived cell lines CaSki and SiHa are ~ 600 copies/cell and 1-2 copies/cell, respectively, as specified by American Type Culture Collection (ATCC). DNA was extracted in the same way as in the Pap test samples. We detected an average of 8200 and 49 molecules per experiment for these two cell lines, respectively. The detected numbers were converted to the HPV-16 DNA percentages based on the flow rate and the sample volume. Then, the DNA content of each cell is calculated by dividing the extracted DNA amount by the cell population. The calculated viral loads were 540 and 2.4 copies/cell, respectively. The agreement with the numbers from ATCC means that this quantitative assay is well-behaved.

The number of molecules measured for the 0% HPV-16 DNA sample (inset of Fig. 2D) represents false positives. The three possible sources of false positives are: aggregation of unbound probes, non-sequence-specific hybridization with human genomic DNA and random noise of the ICCD camera. The first factor could be neglected as no counts were found in control experiments which contained probes only. Cross-hybridization could be minimized by optimization of hybridization conditions, such as temperature and ionic strength. When two random noise spikes accidentally showed up in the images with reasonable dispersion distance, they would be counted as false positives as well. These could be greatly reduced by the dual-color detection mode described below. The ratio of the number of detected HPV-16 DNA molecules to that of false positives is the signal-to-noise ratio (S/N). The detection limit of HPV-16 DNA is 0.0001% (~ 0.7 copy/cell) when S/N equals three. This sensitivity is extremely high among the quantitative assays of nucleic acids. Moreover, this was achieved without amplification.

Dual-color detection

To improve the selectivity in identifying the target, a second fluorophor was added to the single-color detection scheme. Dual-color detection can greatly reduce the possibility of false positives from ICCD noise because there will be three spots per image for each hybridized molecule rather than two. The chances of three random noise spikes accidentally appearing with the correct dispersion distance is negligible. FRET can be involved in the dual-color detection system in the vicinity of the probes. The intercalating dye YOYO-3 was added after hybridization of Alexa Fluor 532 labeled probes to the target so that it stains the whole genome. YOYO-3 was chosen as the second dye according to three criteria. First, it has emission maximum at 661 nm, which is easily distinguished from that of Alexa Fluor 532. Second, its absorption maximum (612nm) is far away from the excitation wavelength (532 nm) so that it

is very dim without energy transfer from Alexa Fluor 532. Third, its fluorescent intensity is comparable to the Alexa Fluor 532 donor when partial FRET occurs. These features allowed us to identify the dual-color molecules conveniently.

Figure 3A shows the hybridized molecules labeled with YOYO-3 as an enhancement to the single-color system. When excited by a 532-nm laser, fluorescence from both Alexa Fluor 532 and YOYO-3 were detected. A typical dual-color image of pure HPV-16 DNA hybridization sample is shown in Fig. 3B. The left peak in the first-order image corresponds to the emission of Alexa Fluor 532 at 550 nm while the right peak corresponds to the emission of YOYO-3 at 661 nm. The average dispersion of Alexa Fluor 532 was 106.6 ± 0.9 pixels and that of YOYO-3 was 121.8 ± 1.2 pixels. The difference between the two averages was 15.2 pixels, which was 14.4 times the average of the two standard deviations. That is, the two peaks are clearly separated from each other. The statistical information was collected in the same way as for single-color detection, and the histogram is shown in Fig. 3C. There were 90 molecules counted in the experiment, in which 77 molecules had two peaks and 13 molecules had one. All the single-color molecules were associated with the dispersion distance of YOYO-3, which represented the target DNA bound with too few number of probes. The hybrids with at least 5 probes clearly appeared as bright spots in our setup (data not shown).

When bulky human genomic DNA was added as the matrix for two-color DNA detection, YOYO-3 made them appear as bright single color molecules. The background level increased and it was not practical to seek out the dual-color hybridized molecules among the single-color matrix especially when the HPV-16 DNA content was low. This problem resulted in poor sensitivity, and the detection limit was only 1% ($\sim 7 \times 10^3$ copy/cell) HPV-16 DNA. To solve this problem, we used a restriction endonuclease to digest human genomic DNA into small pieces, with an average length of 4 kb. Hind III worked very well for cutting human genomic DNA while preserving the E6-E7 region of HPV-16 DNA. There was one order-of-magnitude improvement in detection limit, that is, down to 0.1% ($\sim 7 \times 10^2$ copy/cell) with this digestion step. In the negative control experiment, where the sample contained everything except the target DNA, no dual-color signal was detected. It is worth pointing out that although the sensitivity could not compete with single-color detection, the lack of false positives was a significant advantage for dual-color detection.

Conclusions

This viral DNA screening system is sensitive with a detection limit of 0.7 copy/cell and has a wide linear dynamic range over six orders of magnitude. It is reliable in quantification since no amplification is involved. Although PCR methods have enabled the detection of the level of 0.05 copies/cell,³⁷ they require many (> 10) copies for proper amplification. False positives, contamination, and enzyme inhibition are other concerns. The probe design is a key step in this method. The single unbound probes are invisible at the selected threshold but become visible when at least five of them hybridized to the target. So, the digital counting approach avoids the need to remove the unbound probes or add quenchers after hybridization to decrease background. The whole procedure could involve little more than cell lysis, DNA denaturation, hybridization and imaging. Dual-color detection could achieve zero false-positive count. Moreover, this technique can be directly coupled with current sampling methods such as Pap smears. Since this screening technology does not require other HPV-16 DNA specific features (e.g. antibodies) except for the probe sequence, it is readily applicable to any DNA detection and quantification by changing the probes. In comparison with photon-burst detection,³⁸ imaging allows substantially higher throughput in molecule counting. Up to 2,500 molecules can be screened in 25 ms.³⁵ The transmission grating offers simple, accurate and sensitive spectral analysis when compared to using multiple optical filters or multiple lasers.³⁸ The detection efficiency can be further increased to near 100% with radial focusing of molecules

inside the capillary when an electric field is applied across the capillary along with hydrodynamic flow,³⁰ while confocal systems³⁸ inherently miss the larger fraction of molecules that pass outside the laser focal point. That feature allowed us to count molecules down to 10^{-17} M compared to 5×10^{-13} M in confocal systems.

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Figure 1.

Schematic illustration of the experimental setup: Laser, 532 nm continuous-wave solid-state laser; MS, mechanical shutter; CL1, cylindrical lens, FL= 200 mm; CL2, cylindrical lens, FL= 25 mm; SC, square capillary; O, objective lens, 20× NA 0.75; F1, 532 nm long pass edge filter; F2, 532 nm holographic notch filter; TG, transmission grating; ICCD, intensified CCD camera; PG-200, function generator for controlling the ICCD; PC, personal computer for data acquisition and analysis. The inset shows the excitation and detection regions.

A

B **C**

Figure 2.

Single-molecule detection of HPV-16 DNA in single-color mode. (A) Schematic diagram of single-color mode. After heat denaturing, HPV-16 DNA was hybridized to the 8 complementary oligonucleotide probes labeled with Alexa Fluor 532. 550 nm fluorescence images of the hybrids were recorded by ICCD camera when excited by 532 nm laser. (B) Fluorescence images of a single Alexa Fluor 532 labeled probes/HPV-16 DNA hybrid molecule dispersed with a 70 grooves/mm transmission grating. The dispersion distance between the zeroth-order image and first-order image was proportional to the fluorescent wavelength. (C) Distribution of dispersion distances from Alexa Fluor 532-labeled probes/HPV-16 DNA hybridized molecules. Total sampling number = 100; average dispersion = 106.0 ± 1.1 pixels. (D) Standard curve of the quantification of HPV-16 DNA. Both axes are logarithmic scales. The blue points depict the averages over three experiments with commercial human genomic DNA and the error bars are the standard deviations (Table 1) of these three sets; the red points are data from one experiment with extracted DNA from Pap smear sample. The inset shows the number of counts in negative control and the low concentration region.

A

B **C** **Figure 3.**

Single-molecule detection of HPV-16 DNA in dual-color mode. (A) Schematic drawing of dual-color imaging. After heat denaturing, HPV-16 DNA was hybridized to the complementary oligonucleotide probes labeled with Alexa Fluor 532. The hybrids were then labeled with YOYO-3. Both 550 nm and 631 nm fluorescence images of the hybrids were recorded by the ICCD camera when excited by 532 nm laser. (B) Fluorescence images of two Alexa Fluor 532 + YOYO-3 co-labeled probes/HPV-16 DNA hybrid molecule. (C) Distribution of dispersion distances from Alexa Fluor 532 + YOYO-3 co-labeled probes/HPV-16 DNA hybridized molecules. For the left group of data, total sampling number = 77, average dispersion = 106.6 ± 0.9 pixels; for the right group of data, total sampling number = 90, average dispersion = 121.8

± 1.2 pixels. The difference between the populations of these two groups is the count of YOYO-3 stained molecules bound to fewer than 5 probes.

Table 1
Contents of hybridization samples and the corresponding dilution factors for imaging

HPV-16 DNA (μg)	HPV-16 DNA %	Human genomic DNA (μg)	Probes (μl) ^a	Volume (μl)	Dilution factor	Movie length (# of frames)	Observed number of molecules	Normalized number of molecules
0.1	10%	0.9	1	30	5000	1500	280±16	700000±39000
0.05	5%	0.95	5(10)	30	5000	1500	144±8	359000±29000
0.01	1%	0.99	1(10)	30	1000	1500	135±8	67700±4800
0.005	0.5%	0.995	5(100)	30	500	1500	154±10	38600±3000
0.001	0.1%	0.999	1(100)	30	100	1500	157±12	7830±600
0.0005	0.05%	0.9995	5(1000)	30	100	1500	73±10	3600±500
0.0001	0.01%	0.9999	1(1000)	30	50	1500	36±4	890±110
0.00005	0.005%	0.99995	5(10000)	30	20	1500	44±3	440±50
0.00001	0.001%	0.99999	1(10000)	30	20	1500	13±1	130±10
0.000005	0.0005%	0.999995	5(100000)	30	10	7500	67±4	67±4
0.000001	0.0001%	0.999999	1(100000)	30	10	7500	9±3	9±3
0	0	1	1(100000)	30	10	7500	3±2	3±2

^aThe values in brackets of this column are the dilution factors for the probes, e.g. for 5% HPV-16 DNA, the original probe solution was diluted 10 times and then μl of the diluted probe solution was added into the sample.