

Convenient detection of HPV virus in clinical sample using concurrent rolling circle and junction probe amplifications

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Experimental Protocols:

1. Ligation

Padlock probe ligation reactions contained 1 μM probe, various concentrations of templates, 10 mM MgCl_2 , 50 mM Tris-HCl (pH 7.5 @ 25 °C), 1 mM ATP, 10 mM DTT, and 10 U/ μl T4 DNA ligase (NEB) in a volume of 20 μl . The reactions were heated to 95 °C and cooled to room temperature before addition of ligase, followed by incubation at 37 °C for 2 h. Ligation was terminated by incubation at 65 °C for 10 min.

Padlock probe:

5'-p-TGCCTGCAGGTCGACTTTTTATGTTAAGTGACCGGCAGCATTTTTTTAAG TGGTTTCCCCAGCAAATACCTTTTTAGTGCCAAGCTTGCA-3' ("p" means phosphorylation).

dsDNA template, which was used for optimization of the platform:

5'-TTTTTGTCGACCTGCAGGCATGCAAGCTTGGCACTTTTTT-3'

2. Rolling circle amplification (RCA)

Aliquots of 5 μl of ligation reactions were used to template RCA reactions in a final volume of 20 μl of 2.5 μM primer, 50 mM Tris-HCl (pH 7.5 @ 25 °C), 10 mM MgCl_2 , 10 mM $(\text{NH}_4)_2\text{SO}_4$, 4 mM dithiothreitol and 0.2 $\mu\text{g}/\mu\text{l}$ BSA, 2 μM dNTP, 0.25 U/ μl $\Phi 29$ DNA polymerase (NEB). The reactions were heated to 95 °C and cooled to room temperature prior to addition of polymerase, followed by incubation at 37 °C for the 19 hours. Reactions were terminated by incubation at 65 °C for 10 min, and some of the mixtures were purified on a Sephadex G-50 (GE Healthcare) spin column.

Primer: 5'-TGCTGCCGGTCACTTAACAT-3'

3. Junction probe (JP) detection

Aliquots of 9 μ l of ligation reactions were used in junction probe reactions in a final volume of 60 μ l of 200 nM probe A and probe B, respectively, 33 mM Tris-Ac (pH 7.9 @ 37 °C), 10 mM Mg(OAc)₂, 66 mM KOAc, 0.02% Triton X-100 and 0.1 μ g/ μ l BSA, 0.083 U/ μ l Bsp143I (Thermo Scientific). The reactions were heated to 95 °C and cooled to room temperature prior to addition of restriction endonuclease, followed by incubation at 37 °C for 20 minutes. Fluorescence difference was recorded (λ_{ex} =470 nm, λ_{em} =525 nm on an OP-161 Fluorescence series, Opulen Technologies Co., Ltd., Wuxi, Jiangsu, China).

Probe A: 5'-AAGTGGTTTCCT^{FAM}TTGATC(dabcyl)-3' ("T^{FAM}" means fluorescein labeled dT and "(dabcyl)-3'" means that 3'-end was labeled with dabcyl)

Probe B: 5'-AG(S)GATCGGAAAACC(S)GATCCT(S)GATCATACCAGCAAATACC-3' ("S" means phosphorothiolation)

Detection of HPV-16 using a step-by-step protocol (that is not a single tube protocol)

A: A human papilloma virus type 16 (HPV-16) positive clinical sample was detected using the step-by-step protocol. The sample was a gift from Changzhou Kelai Clinical Laboratory, Inc. (Changzhou, Jiangsu, China) and confirmed by the commercial diagnostic kit (HPV Typing PCR-RDB (reverse dot blot) Kit, Daan Gene Co., Ltd. of Sun Yat-sen University, Guangzhou, Guangdong, China). 10 μ L of sample solution, which was prepared by adding 1 mL saline to a tube containing a cervix swab and briefly vortexed, was incubated in 1X Takara L buffer (10 mM Tris-HCl, pH 7.5 @ 25 °C, 10 mM MgCl₂, 1 mM dithiothreitol) in a total volume of 20 μ L containing 0.5 U/ μ L restriction endonucleases Sac I (Takara) and BstX I (Takara) at 37 °C for 1 hour. And then 6 μ L of above solution was treated as the detection target by the same protocol as Figure 2.

Ai: 3 μ L of the saline solution of the clinical sample was treated as the detection

target by the step-by-step protocol without endonuclease digestion.

B: As a negative control, 10 μ L of ddH₂O was processed as the sample solution by the endonucleases and the step-by-step protocol.

Bi: As the other negative control, 3 μ L of ddH₂O was treated as the detection target by the step-by-step protocol without endonuclease digestion.

C: 100 nM synthetic template (5'-GGCATTGTGGGGTAACCAACTATTTGT-3') was detected by only junction probe reaction, without prior amplification.

Padlock probe (Sequence: 5'-p-ACCCCAACAAATGCCTTTTTATGTAAAGTGACCGGCAGCATTTCCTAATCTGAAGCTTGGTGGACTCTTTTTTACAAATAGTTGGTT-3') was designed to hybridize to a part of the L1 region of HPV-16 (5'...GGCATTGTGGGGTAACCAACTATTTGTT...3', position 6600 to 6629 according to the HPV-16 sequence PPH16, GenBank accession no. K02718). The sequences of probe A and B are 5'-AATCTGAAGCT^{FAM}TTGATC(dabcyl)-3' and 5'-AG(S)GATCGGAAAACC(S)GATCCT(S)GATCATATTGGTGGACTCT-3', respectively.

A single tube detection of HPV, without isolation or separate steps.

One-pot protocol: 500 nM ligation probe, 1 μ M primer, 600 nM junction probe A and B, respectively, various concentrations of the synthetic template, 50 mM Tris-HCl (pH 7.5 @ 25 °C), 10 mM MgCl₂, 5 mM (NH₄)₂SO₄, 7 mM DTT; 0.1 μ g/ μ l BSA, 1 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.5 mM TTP, 10 U/ μ L T4 DNA ligase, 0.167 U/ μ l Φ 29 DNA polymerase, 0.083 U/ μ l Bsp143I in a total volume of 60 μ l. The mixtures were incubated at 37 °C for 21 hours.

Padlock probe: 5'-p-TCCCCAGCAAATACCTTTTTATGTAAAGTGACCGGCAGCATTTCCTAATCTGAAGCTTGGTGGACTCTTTTTTGCAAATAGTTGGTT-3'

Synthetic template: 5'-GGTATTTGCTGGGGAATTTATCTTTTTGC-3'

The primer and junction probes are the same as in the step-by-step protocol.

Note: The HPV55 and negative clinical samples were also from Changzhou Kelai Clinical Laboratory, Inc. and tested by the commercial diagnostic kit. The HPV negative clinical sample was negative for HPV6, 11, 16, 18, 31, 33, 35, 39, 43, 45, 51, 52, 53, 56, 58, 59, 66, 68 and CP8304 tested by the commercial kit. The HPV16 plasmid (about 10^5 copies/ μ L) containing the L1 region of HPV16 was purchased from the National Institute of Food and Drug Control, China. For the single tube detection, all of the samples were treated with endonucleases.

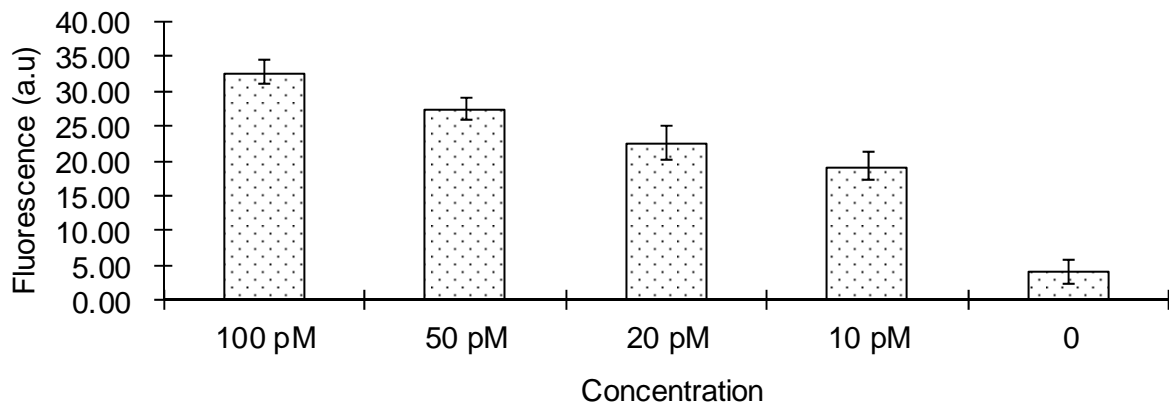


Figure S1: Single tube detection of various concentrations of 5'-GGCATTGTGGGGTAACCAACTATTTGT-3', a fragment of the HPV16 virus, using the concurrent protocol.