Electronic Supplementary Information for

A Comparative Study of Three Different Nucleic Acid Amplification Techniques Combined with Microchip Electrophoresis for HPV16 E6/E7 mRNA Detection

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1. Experimental details

The CaSki and Hela cell lines were used as positive controls, HUVEC cell line was used as a negative control. All cell lines were purchased from Cell Culture Center of Cancer Institute & Hospital Chinese Academy of Medical Science (Beijing, China). The CaSki cells were cultured in DMEM media (GIBCO, Grand Island, NY), Hela cells and HUVEC cells were grown in RPMI 1640 media (GIBCO, Grand Island, NY). Every cell media were supplemented with 10% fetal bovine serum, 100μg/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37^{o}C.

Three kinds of nucleic acid amplification techniques mentioned above were described in detail as following. In the procedure of NASBA, 2 μL RNA templates were added to 17μL of NASBA solution including 40 mM Tris-HCl pH 8.5, 12 mM $MgCl_2$, 70mM KCl, 5 mM dithiothreitol, 15% dimethyl sulphoxide, 1 mM of each dNTP, 2 mM of ATP, CTP, UTP and GTP, 10 pM of each primer, and then pre-incubated at 65 ^{o}C for 5 min. Enzyme mix (0.08 U RNase H, 50 U T7 RNA polymerase, 6 U avian myeloblastosis virus reverse transcriptase (AMV) was then added, and the reaction mix was incubated at 44 ^{o}C for 109 min. RSM was used to optimize four factors of NASBA system, that is, T7 RNA polymerase, AMV reverse transcriptase, incubate duration and incubate temperature. The HPV16 E6/E7 mRNA products from NASBA amplification were analyzed by MCE. Reverse transcription product of E6/E7 DNA was amplified by RT-PCR. The standard protocol of two-step RT-PCR in this work was listed as following. At the first step, 3μL of RNA template was added, and then reverse transcription was carried out according to the protocol of TIANScript RT Kit. At the second step, the reaction volume was fixed at 50μL. It contained 5μL of 5× PCR buffer including 2.5 mM $MgCl_2$, 200 μM dNTP, 200 nM of each primer, 2 μL of cDNA and 1.5 U TaqDNA polymerase. Subsequently, inactivation of reverse transcriptase and activation of Taq DNA polymerase were achieved by incubation at 95 ^{o}C for 5 min, followed by 35 cycles of 95 ^{o}C for 35 s, 57 ^{o}C (55 ^{o}C for HPV18) for 35 s and 72 ^{o}C for 35 s. For one-step RT-PCR, the reaction mix volume was 20 μL, including 2 μL of 5× RT buffer, 2 μL of 5× PCR buffer, 3 μL of 2.5 mM dNTP, 2 μL of 25 mM $MgCl_2$, 2 μL of $Oligo(dT)_{15,}$ 1 μL of 200U/μL TIANScript M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase, 0.3

μL of 5 U/μL Taq DNA polymerase, 3 μL of RNA template and 4.7 μL of ddH_2O. The reaction was incubated at 42 °C for 50 min at first. Then, inactivation of reverse transcriptase and activation of Taq DNA polymerase were accomplished by incubation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 35 s, 57 °C for 35 s and 72 °C for 35 s. For one-step RT-touchdown-PCR, the reaction was incubated at 42 °C for 50 min at first. Then, inactivation of reverse transcriptase and activation of Taq DNA polymerase were completed by incubation at 95 °C for 5 min. The first cycling phase begins with an annealing temperature of 72 °C (70 °C for HPV18) for 35 s, and then decreased annealing temperature by 1 °C per cycle until 57 °C (55 °C for HPV18) for 15 cycles in total, then followed by 20 cycles at 95 °C for 35 s, 57 °C (55 °C for HPV18) for 35 s and 72 °C for 35 s. The RT-PCR products were analyzed by MCE. The sequence of each primer was shown in Tab. S1.

DNA/RNA products were labeled by fluorescence dye SYBR Gold which can bind to both DNA and RNA. Different DNA or RNA fragments were separated in electrophoresis channel, and arrived in detection window based on their different migration rates. Discrimination among each injection was eliminated with the use of two DNA markers as internal standards. Before every analysis, the chip was washed by ultrapure water. Running buffer for electrophoresis separation was prepared in advance. Then, DNA or RNA fragments were introduced into the separation channel via double-T injection.

2. Sequences of RT-PCR and NASBA primers used in this study.

Tab. S1 Sequences of RT-PCR and NASBA primers used in this study.

Primer purpose	HPV genotype	Amplicon (bp)	Sequence (5'-3')	Position (bp)
PCR	16	168	ACCGGTCGATGTATGTCTTGTTGCA	500-524
			CCTCCTCCTCTGAGCTGTCATTTAA	667-643
	18	98	AGTGCCATTCGTGCTGCAAC	514-533
			ATGTTGCCTTAGGTCCATGCAT	611-590
	β-actin	205	CTGGAAGGTGGACAGCGAGG	939-958
			TGACGTGGACATCCGCAAAG	1143-1124
NASBA	16	168	ACCGGTCGATGTATGTCTTGTTGCA	500-524
			AATTCTAATACGACTCACTATAGGGCCTCCTCCTCTGAGCTGTCATTTAA	667-643
	β-actin	205	TGACGTGGACATCCGCAAAG	939-958
			AATTCTAATACGACTCACTATAGGG CTGGAAGGTGGACAGCGAGG	1143-1124

3. The setup and mechanism of MCE.

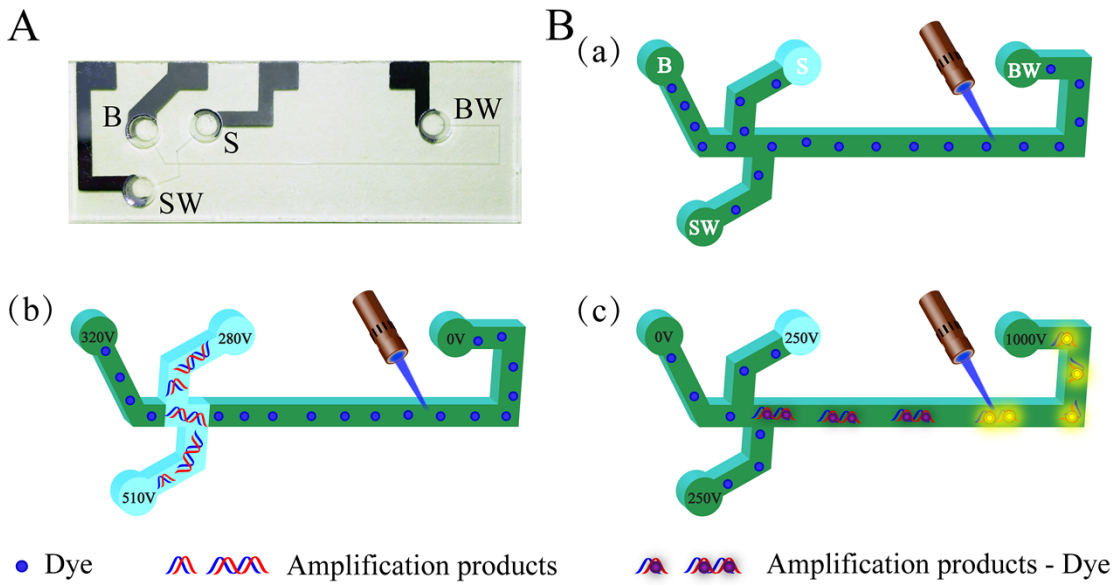

Fig. S1 The setup and mechanism of MCE. A. Pictures of device setup. B. The mechanism of MCE for DNA assay. (a) sample loading; (b) sample injection; (c) sample separation.

4. The electrophoretogram of DNA and RNA running buffer.

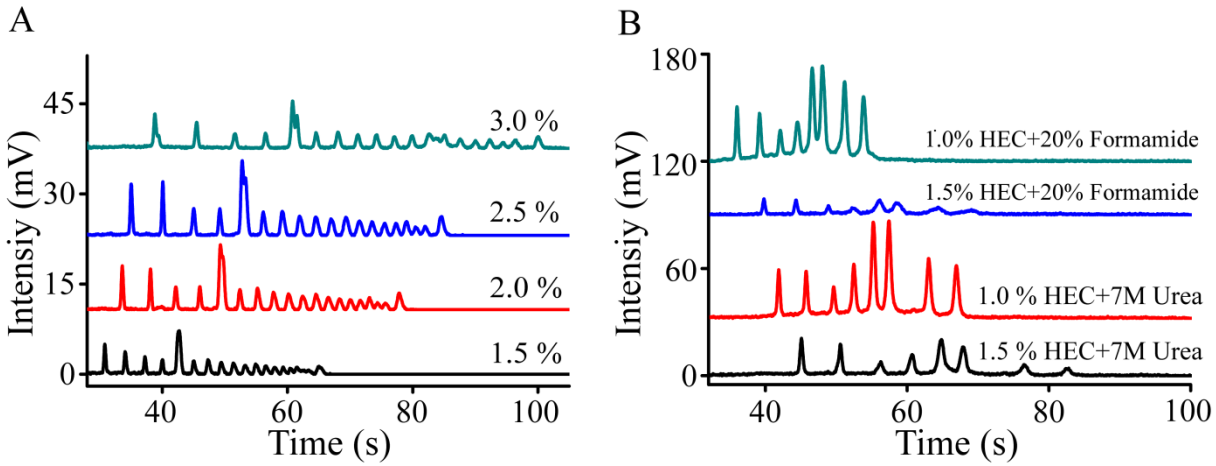

Fig. S2 The electrophoretogram of DNA and RNA running buffer. A. The electrophoretogram of DNA500 ladder separated by different concentrations of HEC solution (1.5%, 2.0%, 2.5%, 3.0% w/v, respectively). B. The electrophoretogram of RNA1000 ladder separated by different running buffer. Urea or formamide was used as denaturant.

5. The quality of extracted RNA.

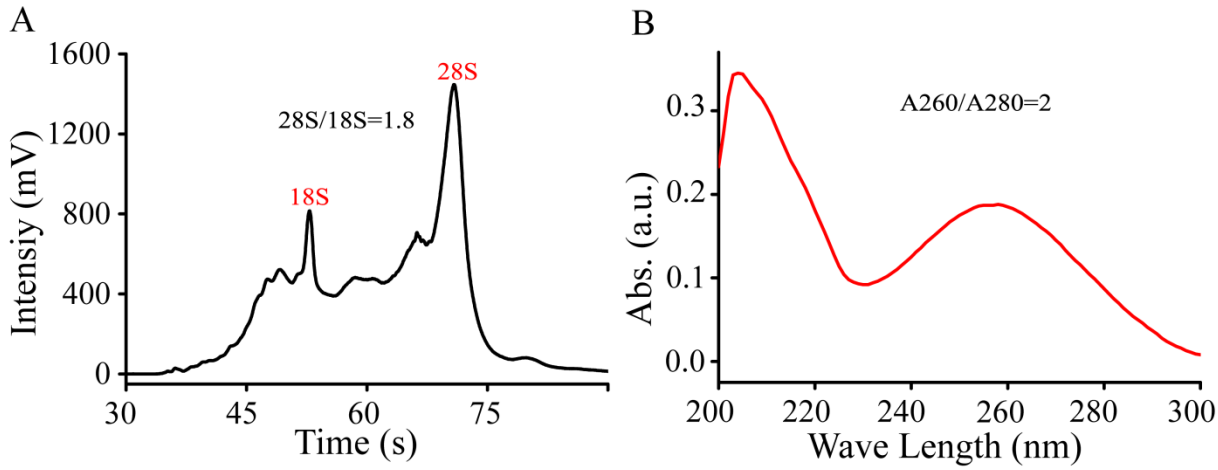

Fig. S3 The quality of extracted RNA. A. The electrophoretogram of total RNA. B. The ultraviolet absorption spectrum of total RNA.

6. Optimization of NASBA detecting conditions by response surface methodology (RSM).

Tab. S2 Response Surface Methodology (RSM) design in NASBA for HPV16 E6/E7 mRNA detection.

Run No.	Factor 1 Time (min)	Factor 2 Temp (°C)	Factor 3 T7 poly (U)	Factor 4 AMV RT (U)
1	120	38	40	6
2	60	41	40	10
3	120	38	30	8
4	120	41	40	8
5	120	44	40	6
6	120	41	40	8
7	120	41	30	6
8	120	44	50	8
9	120	41	40	8
10	120	38	40	10
11	120	44	40	10
12	60	38	40	8
13	180	41	30	8
14	120	41	50	6
15	120	41	40	8
16	180	41	40	6
17	120	44	30	8
18	120	41	50	10
19	180	41	50	8
20	60	41	50	8
21	120	38	50	8
22	180	38	40	8
23	60	41	30	8
24	180	44	40	8
25	120	41	30	10
26	120	41	40	8
27	60	44	40	8
28	60	41	40	6
29	180	41	40	10

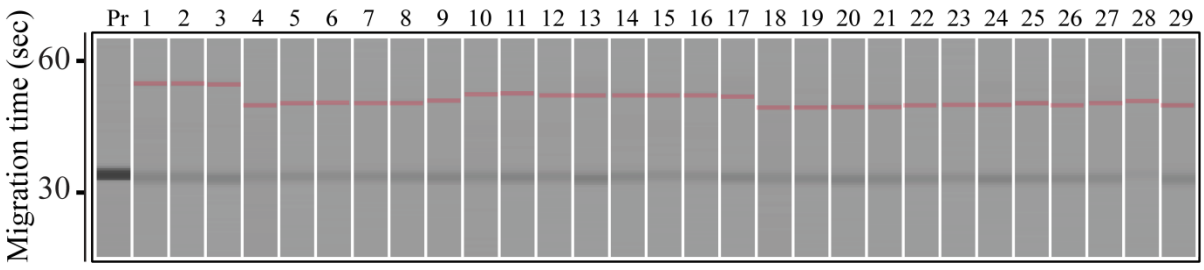

Fig. S4 The gel images of RSM projects in NASBA. Pr: negative control which contains primers only.

7. The specificity of NASBE-MCE for E6/E7 mRNA detection.

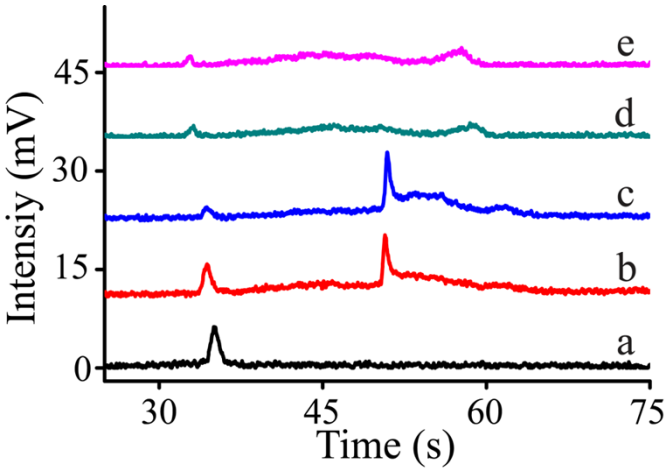

Fig. S5 The specificity of NASBE-MCE for E6/E7 mRNA detection. a, b, c, d, e were blank, beta-actin of Hela, beta-actin of HUVEC, negative control of Hela, negative control of HUVEC.

8. The reproducibility of NASBE-MCE and two step RT-PCR-MCE for E6/E7 mRNA detection.

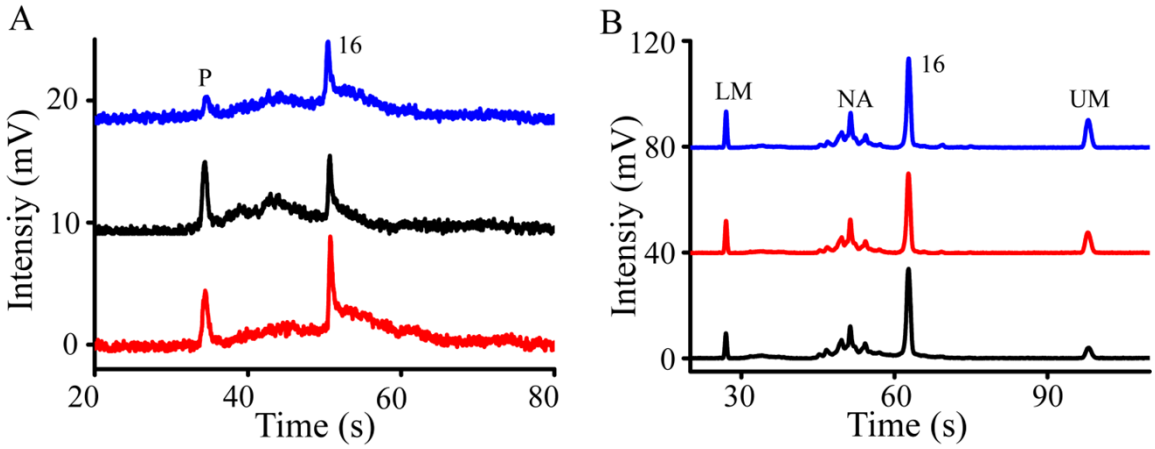

Fig. S6 The reproducibility of NASBE-MCE and two step RT-PCR-MCE for E6/E7 mRNA detection. A. The reproducibility of NASBE-MCE for E6/E7 mRNA detection. P: peaks of primers. 16: HPV16 E6/E7 mRNA amplification products. B. The reproducibility of two step RT-PCR-MCE for E6/E7 mRNA detection. LM: lower marker. UM: upper marker. NA: nonspecific amplification. 16: HPV16 E6/E7 mRNA amplification products.

9. The electrophoretogram of two-step RT-PCR DNA amplified products from different number of CaSki cells.

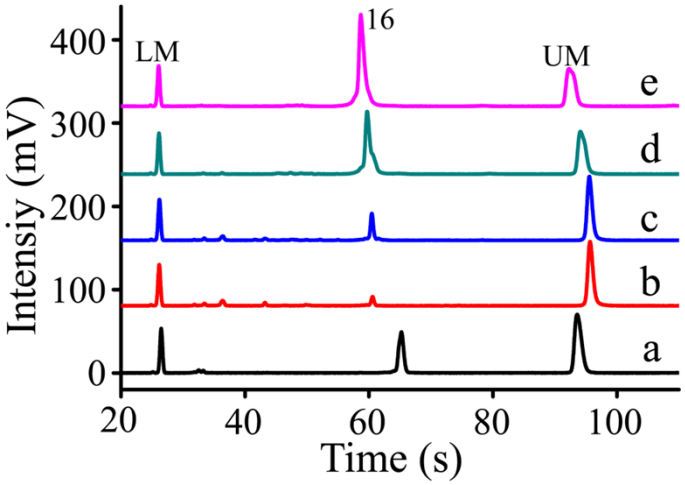

Fig. S7 The electrophoretogram of two-step RT-PCR DNA amplified products from different number of CaSki cells (from 10^0 to 10^3 of the CaSki cells number). a, b, c, d, e were beta-actin, 10^0, 10^1, 10^2, 10^3. LM: lower marker. UM: upper marker. 16: HPV16 E6/E7 mRNA amplification products.