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# Reverse transcription-polymerase chain reaction (RT-PCR) in flow-through micro-reactors: Thermal and fluidic concepts

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**Abstract**

This paper presents a novel flow-through reverse transcription-polymerase chain reaction (RT-PCR) micro-reactor with optimized thermal and fluidic characteristics. We have designed a micro-reactor comprising a heating plate for different temperature zones and a fluidic chip with serpentine micro-channels. One feature of this chip system is the generation of a segmented flow for high-throughput analysis of PCR samples. We show the first successful reactions on the chip with different flow regimes and variations of PCR methods from a standard PCR to one-step RT-PCR reactions. The aim of the experiments was the detection of the HPV 16 DNA genome and of viral oncogene transcripts, respectively. Both markers are of importance in medical diagnostics.


*Keywords:* Flow-through PCR; Miniaturized PCR; PCR-chip; Lab-on-a-chip

## 1. Introduction

The polymerase chain reaction (PCR) is an enzymatic, thermal controlled process which specifically amplifies nucleic acids (DNA) with a high yield [1,2]. The application of PCR technology ranges from medical diagnostics to forensic and food industry. The reverse transcription-PCR (RT-PCR) is a modification of PCR in which the mRNA strand is first reverse transcribed into its DNA complement followed by amplification of the resulting cDNA using PCR. The exponential amplification via RT-PCR provides a highly sensitive technique for the detection of very low copy number of mRNAs. RT-PCR is widely established as diagnositic tool for the detection of genetic diseases. It can be used to quantify amounts of specific RNA molecules within tissues to measure the specific expression of genes (quantitative RT-PCR).

Because of the mechanistic simplicity of the PCR, this process was an ideal candidate for miniaturization during the last decade [3–5]. Low power consumption, fast reaction time and reduced amount of sample and reagents are the potential merits of miniaturized PCR-chip devices compared to conventional thermocycler.

Within the project various strategies of realizing PCR microchips have been put into practice. Of particular importance were stationary and continuous flow PCR chips. Silicon, glass, silicon-glass hybrids, plastics and even ceramics can be used as chip materials. Especially silicon has the great advantage of good thermal conductivity. The described PCR chips work either with integrated resistance heating or with an external heating element. Independent of the strategy of PCR chips, most chips are fabricated using micro-system technology permitting the production of small structures. Importantly, the micro-fabrication technique also provides a possibility for integrating various functional components on the same chip. The ultimate goal is to integrate all steps from sample preparation to the final results on the same microchip, resulting in a micro-total analysis system (μTAS) [6–8].

In this work we present our recent realisation of flow-through micro-reactors for special RT-PCR application in medical diagnostics. Thermal and fluidic concepts and the first successful PCR and RT-PCR results will be shown.

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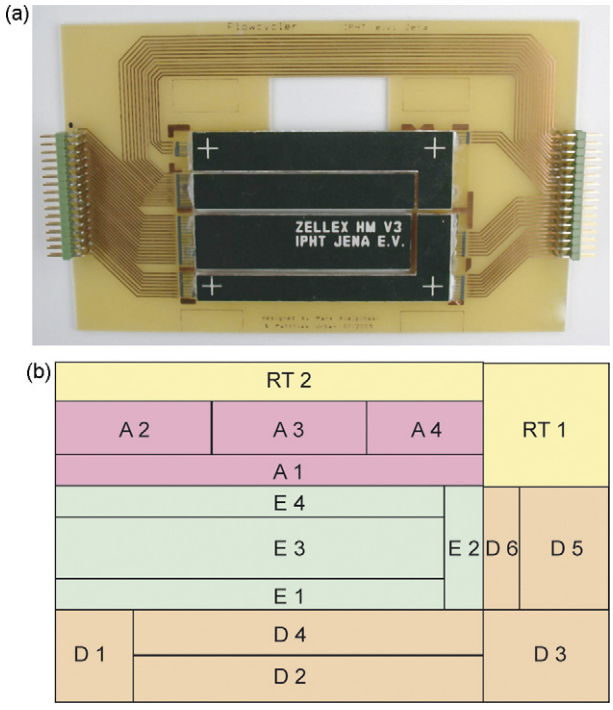


Fig. 1. (a) Photo of the heating plate on the circuit board. (b) Schematic drawing of the separated heating structures on the chip: RT = reverse transcription; D 6, 5, 3 = hot start activation; D 1, 2, 4 = denaturation; A = annealing; E = elongation.

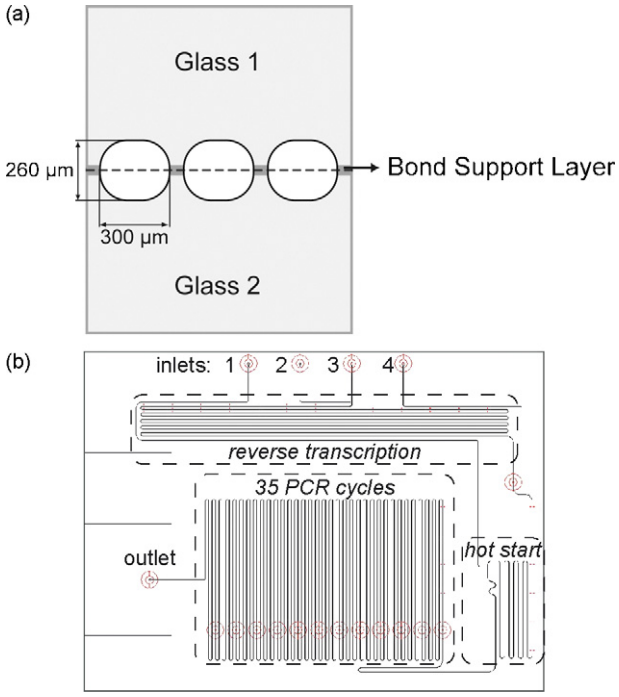


Fig. 2. (a) Schematic drawing of the profile from the micro-channels on the fluidic chip. (b) Schematic drawing of the fluidic chip with different RT-PCR reaction zones.

## 2. Experimental

### 2.1. Thermal concept

In contrast to the first published concept of micro-reactors for flow-through PCR [9–11] we used separate chips for thermal and fluidic management. The heating plate (Fig. 1a) generates five different temperature zones for reverse transcription, activation of modified DNA polymerase (hot start, HS) and the three thermal steps of the PCR process (denaturation, annealing, elongation). It consists of three layers in vertical direction: On the lower side there are 16 thin film platinum heater and sensor structures (Fig. 1b) on a glass substrate in order to generate and control the necessary temperature zones for the RT-PCR reaction. In the middle we used copper stripes to obtain a homogenous temperature disposition in the different temperature zones. The upper side of the heating plate comprises a structured glass cover plate permitting a uniform thermal connection to the fluidic chip.

Thermal gaps with a width of 2 mm between the heating chips of the several temperature zones guarantee the defined temperature gradients. The whole chip package is glued on a circuit board for electronic connection to the controller. To stabilize the several temperatures we developed a special electronic controller and programmed it according to user software.

### 2.2. Fluidic concept

For the fluidic management all fluidic modules were designed in glass to achieve an optical transparency. Half channels were etched with hydrofluoric acid into glass and bonded together face to face by an anodic bonding process. Anodic bonding was enabled for two glass substrates by application of a thin silicon bond support layer, which was deposited on one of the both glass substrates (Fig. 2a).

The entire micro-channel has a geometry of 300 µm (width) × 260 µm (height) × 2.8 m (length). The chip has four different inlets. One inlet is for the supporting medium (inlet 3, mineral oil) whereas the three other inlets can be used for different types of PCR reaction. Using one of the specific inlets (1, 4 or 2) results in a specific sequence of zones permitting the execution of standard PCR, HotStart-PCR or RT-PCR, respectively (Fig. 2b). After the 35 PCR cycles there is one outlet for the cycled PCR sample. In the fluidic chip special injection structures are integrated to facilitate the generation of a segmented sample flow for each of the specific PCR methods. The fluidic chip is applied on top of the heating plate and is exactly aligned over the temperature zones. The fluidic flow on the chip system was controlled by a four-channel syringe pumping system of cetoni (cetoni GmbH, Korbußen).

### 2.3. Surface modification

First, the bond support was removed out of the micro-channels with 5 M sodium hydroxide solution (NaOH). Therefore the micro-channels were filled with NaOH and incubated for 10 min at 80 °C. During following stringent washing steps 1 ml of 1 M NaOH or water were slowly pumped through the chip. Afterwards the chip was dried under a flow of nitrogen. Prior to surface modification the micro-channels were additionally washed with 1 ml of 2 M hydrochloric acid (HCl) and water.

The dry micro-channels were modified with a solution of 10 mM perfluorodecyl-trichlorosilane ($C_{10}H_4Cl_3F_{17}Si$) in perfluorooctane at room temperature. Finally the channels were stringently washed with 1 ml perfluorooctane, isopropanol and water.

### 2.4. RT protocol

For separate RT reactions the SuperScript Trade Mark II reverse transcriptase kit (Invitrogen, Karlsruhe) was used. Purified RNA from the HPV 16 positive cell line SiHa was used as template. One microgram of total RNA was reverse transcribed according to the manufacturer's instructions using poly-dT primers (Thermohybaid, Ulm).

### 2.5. PCR protocol

For all PCR reactions the SuperHot *Taq*-DNA-Polymerase Kit (Genaxxon, Biberach) was used. The cloned genome of human papilloma virus type 16 (HPV 16) was served as template. Using one forward primer (HPV16-F102) with different reverse primers we amplified fragments with 740 bp (HPV16-R720) and 124 bp (HPV16-R226) of length. The primer sequences (MWG-Biotech, Ebersberg) were: primer HPV16-F102 (5′-AAT GTT TCA GGA CCC ACA GG-3′), primer HPV16-R226 (5′-CTC ACG TCG CAG TAA CTG TTG-3′) and primer HPV16-R720 (5′-ATG GTT TCT GAG AAC AGA TGG GGC-3′). The PCR reaction mixture consisted of: 22 μl of water, 10 μl of Q-solution (Qiagen, Hilden), 5 μl of 5 × PCR buffer, 5 μl of 10 μM BSA, 1 μl of 10 mM dNTPs, 2.5 μl of 10 μM of HPV16-F102 and HPV16-R226 or -R720, 1 μl plasmid DNA (100 pg/μl) and 1 μl of 5 u/μl *Taq* DNA polymerase resulting in a total volume of 50 μl. For the flow-through PCR reactions 10 μl of this PCR reaction mixture was used. For off-chip PCR a commercial benchtop thermocycler (mastercycler gradient: Eppendorf, Wesseling-Berzdorf) with 200 μl polypropylene reaction tubes was used. Both the on- and off-chip PCR reactions were run in parallel using aliquots of the same reaction mixture under identical reaction conditions. The reaction mixture was initially heated to 94 °C for 15 min and then amplified for 35 cycles: 94 °C for 20 s, 60 °C for 20 s and 70 °C for 40 s. In continuous flow the flow rate was set on 0.75 μl/min for the short (124 bp) and 0.5 μl/min for the longer fragment (740 bp). In segmented flow the ratio of support medium flow to sample flow was 4:1. After completion of the reaction the PCR products were analyzed by gel electrophoresis. For gel electrophoresis a 3% agarose gel was prepared in 1 × TAE buffer, pH 8.0, which was pre-stained with 1.25 μM ethidium bromide. ØX174 RT DNA *Hinc* II [Advanced Biotechnologies] was used as size marker. The electrophoresis was performed at 100 V for 20 min.

### 2.6. RT-PCR protocol

To avoid the amplification of residual genomic DNA an E6*I splice product specific reverse primer (HPV16-R-E6*I 5′-CTT TTG ACA GTT AAT ACA CCT CAC G-3′) was used for RT-PCR. Successful amplification with HPV16-F102 and R-E6*I resulted in a PCR fragment of 143 bp. For two-step RT-PCR already prepared cDNA (2 μl) was put into the standard PCR protocol as aforementioned.

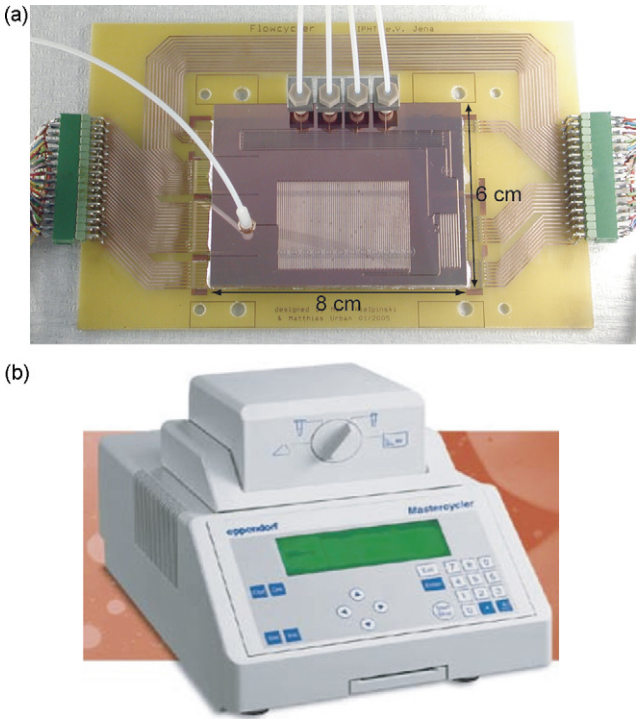


Fig. 3. (a) Photo of the complete chip system, that was used for the described experiments. (b) Photo of the reference thermocycler (Eppendorf, mastercycler gradient).

For one-step RT-PCR reactions the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase Kit (Invitrogen, Karlsruhe) was used. Purified RNA from the HPV 16 positive cell line SiHa was used as template. The reaction mixture consisted of: 25 μl of reaction mix, 10 μl of Q-solution (Qiagen, Hilden), 5 μl of 10 μM BSA, 2.5 μl of 10 μM of HPV16-F102 and HPV16-R-E6*I, 1 μl total RNA (0.3 μg/μl) and 2 μl of enzyme mix and additionally 2 μl SuperHot Taq DNA polymerase (Geaxxon, Biberach) resulting in a total volume of 50 μl. The reaction mixture was heated to 60 °C for 30 min followed by the hotstart to 94 °C for 15 min and then amplified for 35 cycles: 94 °C for 20 s, 60 °C for 20 s and 70 °C for 40 s. The flow rate for flow-through RT-PCR was set on 0.75 μl/min. The product analysis was accomplished as described already.

## 3. Results and discussion

With the conditions described above, we obtained successful flow-through PCR runs. The results will show that the used concept was applicable to perform PCR in micro-channels with segmented flow (Fig. 3a), comparable to conventional thermocyclers (Fig. 3b).

### 3.1. Thermal and fluidic profile

A very good thermal characteristic was obtained with the specified chip system. Using the presented heating plate all

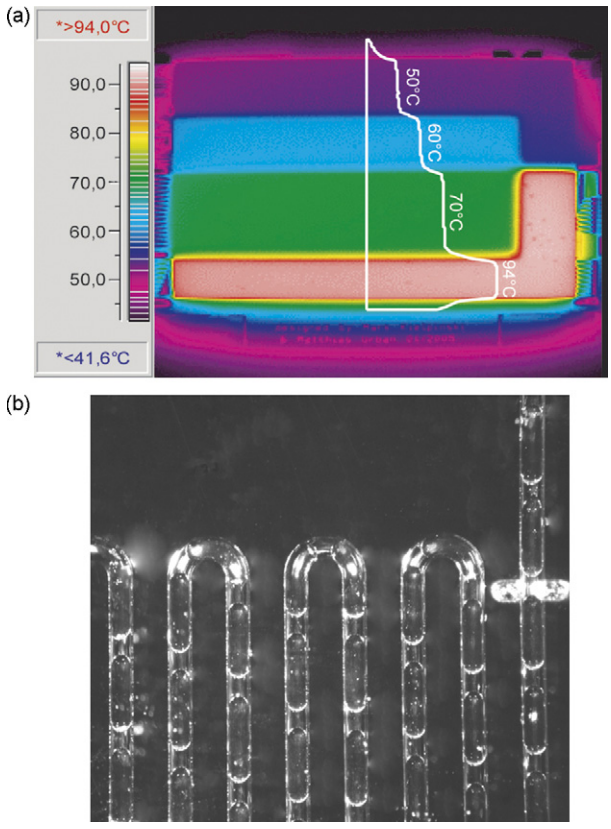


Fig. 4. (a) Photo of the infrared camera, that shows the exactly thermal profile of the heating plate. (b) Segmented flow with PCR solution on the chip (interface between alternating mineral oil and PCR sample appears as a light meniscus).

four different temperature zones are exactly separated from each other. At the same time we have a homogenous temperature distribution in each temperature zones (Fig. 4a). The temperature stability in each heating zone was ±0.1 K of the PCR temperature set point (the temperature measurements were external accomplished by an infrared camera and were additionally controlled by the integrated platinum sensors on the heating plate).

In combination with a special surface modification, that created large contact angles (approx. 100°) between the PCR solution and the micro-channel surface, it was possible to generate a stable segmented flow on the flow-through micro-reactor (Fig. 4b). By the indicated flow rates we generated sample compartments with a volume of 100 nl in a continued droplet sequence.

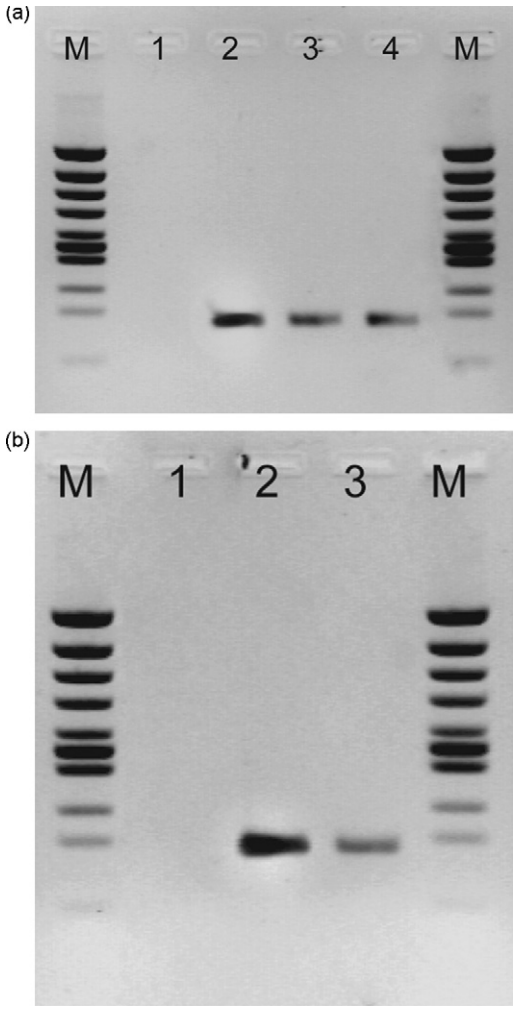


Fig. 6. (a) Photo of gel electrophoretic analysis of two-step RT-PCR's in flow-through micro-reactors with different flow regime in comparison to a commercial thermocycler: M = DNA marker; lane 1 = NTC; lane 2 = reference cycler; lane 3 = continuous flow; lane 4 = segmented flow. (b) Photo of gel electrophoretic analysis of one-step RT-PCR's in flow-through micro-reactors with different flow regime in comparison to a commercial thermocycler: M = DNA marker; lane 1 = NTC; lane 2 = reference cycler; lane 3 = continuous flow.

### 3.2. PCR and RT-PCR on chip

Possible areas of application of the PCR reactions on chip are clinical diagnostics. Therefore, we established a PCR system to detect the oncogene region of the HPV 16 genome. We accomplished PCR reactions on the flow-through micro-reactor in segmented sample flow with two fragment length

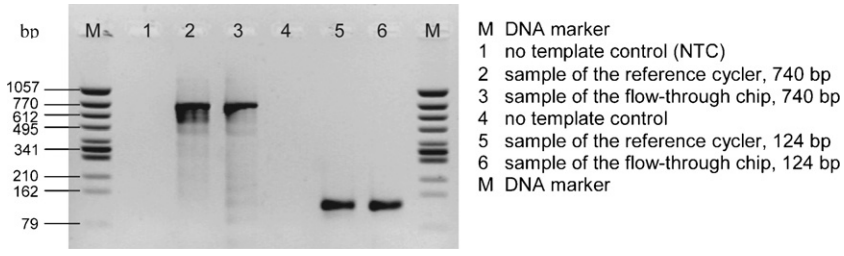


Fig. 5. Photo of gel electrophoretic analysis of PCR's in flow-through micro-reactors with different product length in comparison to a commercial thermocycler.

(740, 124 bp) using plasmid cloned HPV 16 DNA. In Fig. 5 we show the successful PCR results. There are no differences between the amount of PCR product of the chip PCR (lanes 3 and 6) and the PCR in a commercial benchtop cycler (lanes 2 and 5).

Moreover we wanted to transfer a two-step RT-PCR protocol from the commercial cycler to the flow-through PCR chip. The RT step was performed in a reaction tube with a standard protocol. The second step, the amplification of the cDNA, was parallel accomplished in the micro-reactor and in a conventional block cycler.

Fig. 5a shows a successful RT-PCR reaction in the flow-through micro-reactor both in continuous flow (Fig. 6a: lane3) and in segmented flow (Fig. 6a: lane4). The amount of the PCR product was again comparable to the commercial block cycler. The necessary final step was the performance of a one-step RT-PCR reaction based on RNA samples in the flow-through micro-reactor.

Fig. 6b shows the first experiments with one-step RT-PCR reactions on the flow-through micro-rector in continuous flow (lane 3). We amplified a specific product comparable to the commercial thermocycler (lane 2).

## 4. Conclusion

A micro-fluidic chip system for flow-through PCR reactions with an optimal thermal profile was presented. For a segmented flow PCR and RT-PCR to have a high throughput of samples in a small volume (10–100 nl) a fluidic chip device was designed. To achieve stable fluidic conditions the surface of the micro-channels was modified. The successful flow-through RT-PCR reaction in the micro-reactor to detect mRNA of HPV 16 for clinical diagnostics was shown.

Further work will be focused on *in situ* RT-PCR. *In situ* RT-PCR is a method for amplification of target sequences within intact cells [12]. It permits the identification of cells with specific gene expression signatures as well as the identification and classification of tumour cells on the basis of genetic markers. For the establishment of an *in situ* RT-PCR system we will detect disseminated tumour cells in the blood of patients with cervical carcinoma. A special characteristic of these tumour cells is the expression of viral oncogenes E6 and E7 [13] which can be specifically amplified by this procedure.

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## References

[1] R.K. Sakai, et al., Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sick cell anemia, Science 230 (1985) 1350–1354.
[2] Mullis, K.B., et al., The Polymerase Chain Reaction, Birkhäuser, Boston, 1994.
[3] D.T. Burke, et al., Microfabrication technologies for integrated nucleic acid analysis, Genome Res. 7 (3) (1997) 189–197.
[4] L.J. Kricka, P. Wilding, Microchip PCR, Anal. Bioanal. Chem. 377 (5) (2003) 820–825.
[5] P.A. Auroux, et al., Miniaturised nucleic acid analysis, Lab Chip 4 (6) (2004) 534–546.
[6] T. Vilkner, et al., Micro total analysis systems. Recent developments, Anal. Chem. 76 (12) (2004) 3373–3385.
[7] S.J. Lee, S.Y. Lee, Micro total analysis system (micro-TAS) in biotechnology, Appl. Microbiol. Biotechnol. 64 (3) (2004) 289–299.
[8] A. Manz, et al., Miniaturized total chemical analysis systems: a novel concept for chemical sensing, Sensors Actuators B: Chem. 1 (1–6) (1990) 244–248.
[9] Köhler, J.M., et al. Micro channel reactors for fast thermocycling, in: 2nd International Conference on Microreaction Technology, Springer, New Orleans, LA, USA, 1998.
[10] I. Schneegaß, et al., Miniaturized flow-through PCR with different template types in a silicon chip thermocycler, Lab Chip 1 (1) (2001) 42–49.
[11] I. Schneegaß, J.M. Köhler, Flow-through PCR in chip thermocyclers, Rev. Mol. Biotechnol. 82 (2) (2001) 101–121.
[12] A.K. Dixon, et al., Gene-expression analysis at the single-cell level, TiPS 21 (2000) 65–70.
[13] M. Dürst, et al., A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions, Proc. Natl. Acad. Sci. USA 80 (12) (1983) 3812–3815.