Hands-free sample preparation platform for nucleic acid analysis

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A Lab-On-Chip system with an instrument is presented which is capable of performing total sample preparation and automated extraction of nucleic acid from human cell samples fixed in a methanol based solution. The target application is extraction of mRNA from cervical liquid based cytology specimens for detection of transformed HPV-infections. The device accepts 3 ml of sample and performs the extraction in a disposable polymer chip of credit card size. All necessary reagents for cell lysis, washing, and elution are stored on-chip and the extraction is performed in two filter stages; one for cell pre-concentration and the other for nucleic acid capture. Tests performed using cancer cell lines and cervical liquid based cytology specimens confirm the extraction of HPV-mRNA by the system.

Introduction

From its early days the ability to perform analytical tasks away from a dedicated laboratory has been one of the main driving forces for development within the Lab-On-A-Chip area. The main goal is to develop a more widespread range of tests with significantly higher analytical sensitivity and clinical specificity and to reduce the time to a valid test result. This is especially crucial in medical diagnosis, in particular when a whole population should preferably be negative with the test. An early detection of diseases such as pre-cancer can save precious time before treatment is initiated, which is especially critical in an emergency ward or in intensive care, for example for the diagnosis of sepsis.

A good example for a diagnosis platform that is able to combine speed and decentralisation is lateral flow bioassays.[1,2] These rely on capillary forces for fluid transport and usually feature dried biomarkers that trigger a colour reaction in the presence of the desired target proteins, but have a limited ability in terms of the sample preparation and assay sophistication that can be performed. Nevertheless, they combine the salient features of being easy to use even by a non-trained operator, inexpensive and allowing a short time to result. These systems have been around since the 1960s and a host of different diagnostic applications have been developed with more emerging on the market every year.

The Lab-On-Chip community has strived towards the implementation of more complex procedures into the Point-Of-Care (POC) setting. Most prominent is the desire to open the field of nucleic acid diagnosis (NAD) and in particular RNomics for rapid hands-free testing. This involves complex operations including total sample preparation processes (sample collection, cell lysis, nucleic acid extraction), amplification of one or more target sequences and finally detection. Discrete examples of these sub-functions have been adapted successfully to a chip format for some time now. However, serious attempts have only been made very recently to integrate all necessary steps onto a chip platform in a way that is user friendly even to a non-scientific operator.[3–8] However, these systems still need a substantial part of operator interaction and also require the external supply of reagents to the system in order to run the assay.

Within the EU funded project MicroActive an automated platform for chip-based sample pre-concentration, nucleic acid extraction, amplification, and fluorescent detection has been developed. The idea is to develop a desktop system that is able to perform a complex mRNA detection protocol with no user intervention. In this way a procedure currently carried out in selected clinical laboratories by highly trained personnel can be accomplished at the bed-side or in a doctor's office by non scientific personnel. All reagents necessary for this analytical task are stored on the chips and a liquid-free instrument performs the necessary operation. Two modular prototypes for sample preparation and detection have been set up and tested individually making it very easy to integrate them later into one single setup. This presentation focuses on the sample preparation chip and its instrument. As an exemplary target application, screening for cervical pre-cancer by detection of oncogenic human papillomavirus (HPV) gene expression was chosen. However, the system is general enough that its applicability does not limit itself to this particular target.

Cervical cancer is the second most predominant form of cancer among women in developed countries.[9] Nearly all cases of this cancer are directly linked to previous infections with one or more of cancer-inducing types of human papillomaviruses.[10] Detection of persistent HPV infections is thus desirable in order to initiate treatment before the disease can develop. Moreover, opposed to

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HPV-DNA detection, the detection of HPV E6/E7 mRNA expression indicates a transforming infection and higher oncogenic potential.[11]

For the developed device a standard laboratory assay was adapted to the chip format. Briefly, the cervix sample was collected and concentrated on a filter where it was subsequently chemically lysed. The released nucleic acid was then captured further downstream onto a silica membrane in the presence of a chaotropic salt solution and extracted by solid phase extraction (SPE) using a variant of Boom's method.[12] Several washing steps were performed to carry away cell debris from the SPE matrix and after air-drying the purified nucleic acid was eluted. We present results from the successful extraction of HPV mRNA from several different cell lines and at various amounts of cell material present in the sample. Furthermore, results from successful extractions of HPV mRNA from patient based liquid samples are shown and demonstrate that the system indeed can deal with complex genuine cervical samples.

The eluate from this chip can be processed further for the actual detection of pathogenous mRNA present in the sample. As has already been shown, a parallel amplification by NASBA (Nucleic Acid Sequence Based Amplification) and subsequent fluorescence detection is readily integrated into a chip format.[13,14] Also such a system has been successfully automated and tested within the MicroActive project, as will be presented in a separate publication, demonstrating that a hands-free POC-NAD™ system is finally within reach of the Lab-On-A-Chip technology.

On-Chip sample preparation

The sample preparation system described in this paper consists of a disposable chip shown in figure 1 and its instrument shown in figure 2. Due to the risk of cross contamination between different samples, the chip and all other parts that come in contact with the sample, such as valves and connectors, need to be disposable. Therefore a polymer device is advantageous because mass production of polymer-based disposable products can be realized at low costs by injection moulding.

The assay that is implemented on the chip is shown schematically on figure 3. The sample is a cervical cytology specimen

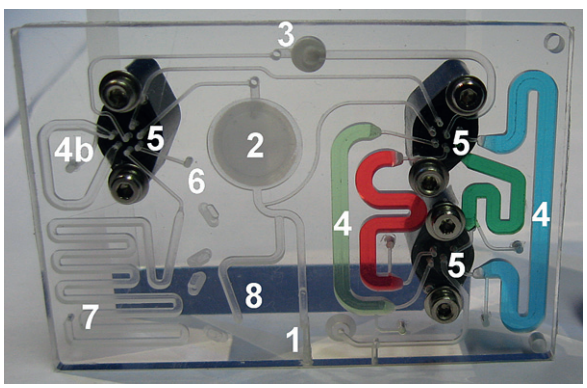

Fig. 1 Pre-filled sample preparation chip showing (1) sample inlet, (2) cell filter, (3) SPE chamber, (4) reagent storage, (4b) storage DMSO/sorbitol, (5) turning valves, (6) waste outlet, (7) sample outlet, (8) pressure sensor. For demonstration reasons only, the fluids have been dyed in this photo.

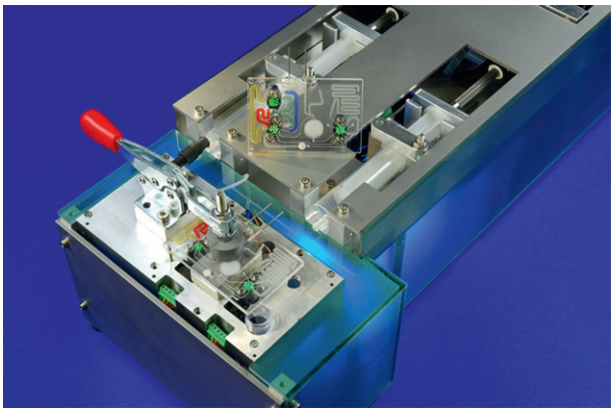

Fig. 2 Instrument for the sample preparation chip.

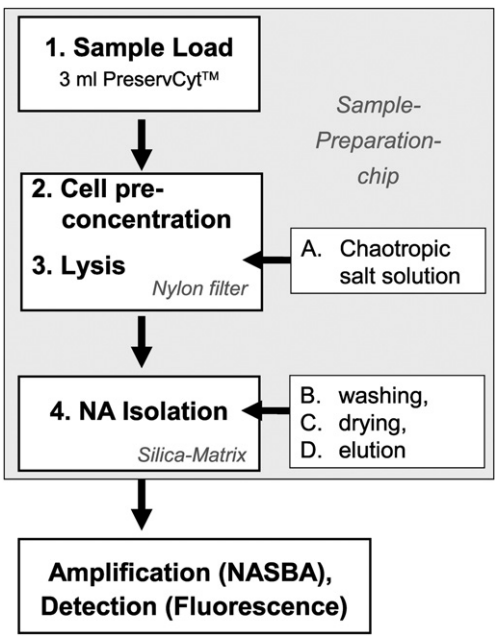

Fig. 3 Sample preparation assay implemented on chip.

stored in the liquid based cytology medium, PreservCyt™ (Cytyc Corporation), a methanol based solution that inactivates and fixates the epithelial cells including RNA and DNA at room temperature for at least one month. A convenient way to introduce the sample to the system on a prototype level is to use a conventional disposable syringe since a doctor or technical assistant performing the analysis is accustomed to its use. Before inserting both chip and syringe into the instrument, the syringe is connected to the chip.

After insertion of chip and sample and starting the instrument, the extraction procedure proceeds further without any user intervention. In the first step the cells are captured from the sample on a nylon filter. This first step is essential in order to reduce the sample volume of several ml to a manageable size for chip operation. Subsequently, the cells are chemically lysed and the lysate is guided through a solid phase extraction (SPE) chamber, where the nucleic acid is retained. Two washing steps remove cell debris, proteins, and other cellular components from

the extraction matrix. Before elution, the SPE chamber has to be dried. This is done by continuously pumping air through it. During the whole process of lysis and nucleic acid extraction the region of the chip containing the cell filter and SPE chamber is heated to approximately 50 °C in order to increase the lysis efficiency and to speed up the drying procedure.

The chip material is COC (cyclic olefin copolymer; Ticona, COC-5013) sealed with COP (cyclic olefin polymer; Zeon, Zeonor 1420R). All chips were fabricated by milling[15] into blank injection moulded chips with a size of 64 × 43 × 3 mm^3.

The pre-concentration filter used was obtained from Bückmann (Bückmann GmbH & Co. KG, Mönchengladbach, Germany; Nylon, mesh width 10 μm). The filters are soldered into the chip material. For this the filter chamber carried a step from 11 mm in diameter to 9 mm, which defines the free filter surface. Using a hot soldering iron with a custom made tip the filters are soldered onto the step. In order to keep the dead volume small a shallow chamber design was chosen. After soldering of the filter material the chips are treated for several hours with an aqueous 3% H_2O_2 solution (Fluka Chemie AG, Buchs, Switzerland) in order to destroy RNAses.

The silica filter material for solid phase extraction was supplied by Genomed (Genomed GmbH, Löhne, Germany) and has a fleece-like fabric. Three layers of this material are placed into the SPE chamber which has a volume of approximately 30 μl. Experiments with dyed fluids indicate that the filter chamber as implemented into the chip is indeed adequately filled and rinsed by the successive fluids, since even a dye of darker colour is completely replaced by a fluid with a light colour. After mounting of the filters the chips are solvent bonded with a 100 μm COP foil. Subsequently three turning valves are mounted onto the chip.

Besides pumping for fluid actuation, valves are the second most important entity for fluid control on the chip. For the present chip system turning valves have been chosen as shown in figure 4. These valves consist of housing and valve body, both of which were injection moulded in PEEK (poly ether ether ketone). The sealant is a Viton disk (DuPont Performance Elastomers) glued with instant adhesive onto the valve body. Channels are carved into the Viton by laser ablation with a frequency doubled Nd:YAG laser. These channels connect different ports on the chip, in this way connecting different channels. A Teflon ring inserted into the housing serves as guiding structure for the valve body.

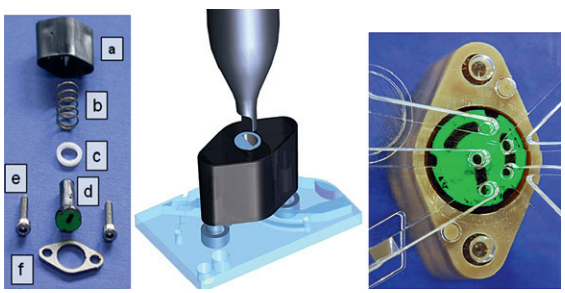

Fig. 4 Turning valves used for fluid control and sealing of the storage chambers. Left: valve components (a) valve housing, (b) spring, (c) guide ring, (d) valve body with sealant disc, (e) screws for on-chip mounting, (f) spacer (optional). Middle: mounted valve with connector to the motor. Right: bottom view through a chip showing sealant disc and channels.

As evident from figure 1, the valves have been fastened to the chips by screws. However, other more cost effective joining techniques for a commercial system such as clamping, gluing, and soldering thermally or by ultrasonic welding have been tested successfully already. The fluidics relying on on-chip turning valves in combination with syringe pumps for actuation are very robust and have been shown to readily integrate with other actuation principles such as capillary filling downstream. A leakage rate for liquids of only 0.5 μl/min (at 1 bar applied pressure) is more than sufficient for lab-on-a-chip applications. The valves are even suitable for higher pressures up to 3 bar, if they are fitted out with stronger springs and therefore increasing the surface pressure of the sealant.

Besides serving as guiding structures for the fluids during operation the valves are also implemented for sealing several storage chambers during storage of the chips. On-chip fluid storage is convenient since this alleviates the need for fluidic operation in the instrument itself, checking sufficient external supply or pipetting of reagents prior to use, as is done in almost all devices presented so far. The reservoirs sealed in this way are able to store the fluids for up to several weeks without significant fluid loss.

Two storage sections are implemented into the chip. The first consists of four chambers and holds the lysate buffer as well as two washing and one elution buffer (denoted 4 on figure 1). The second structure is located near the outlet of the chip and holds reagents necessary for the amplification reaction on the eluate (denoted 4b on figure 1). Since in this study only the sample preparation is considered, this second reservoir is currently not used as the amplification was performed off-chip.

In the experiments the reservoirs were manually filled between a day and a few minutes before the experiments. Filling is done *via* a side hole and across the first valve, using the second valve as air outlet. In order not to contaminate the buffers during this procedure the filling order was elute, wash 2, wash 1, lysis buffer. The reagents and amounts that were used during the experiments are summarised in table 1. For the extractions from cell lines the Guanidine thiocyanate was obtained from Sigma Aldrich and the ethanol from Arcus, Norway. During extraction from cervical liquid based cytology specimens Guanidine isothiocyanate from Fluka-Biochemica and ethanol from Sigma Aldrich was used. Also, a custom made lysis buffer was used during the extraction from cervical specimens (5M Guanidinium isothiocyanate (Fluka Chemie AG, Buchs, Switzerland), 50 mM Tris HCl (Carl Roth GmbH + Co. KG Karlsruhe, Germany), 0.1% Triton X-100 (Fluka Chemie AG, Buchs, Switzerland)).

Table 1 Reagents used for the extraction experiments[a]

Operation	Reagents	Amount
Lysis	BioMerieux NucliSens® easyMAG™ Lysis Buffer	110 μl
Wash 1	75% Ethanol + 25% 3M GIT (v/v)	230 μl
Wash 2	96% Ethanol	120 μl
Elute	DEPC water	50 μl

[a] GIT: Guanidine thiocyanate, DEPC water: water treated with Diethylpyrocarbonate.

Pressure sensor

Since the cell capture filter may clog depending on the cell and mucus content of the sample it is desirable to include a means for pressure measurement in the sample preparation device. On the other hand, it is advantageous to have as few connections to the outside in order to reduce the risk of contamination. Therefore a dead end channel was included into the chip as it is possible to measure the pressure by inspecting the partial liquid filling of a gas-filled dead-end channel in contact with the pressurised liquid (similar to a Boyle-Mariotte depth gauge used by divers).

This pressure measurement method was demonstrated for different channel geometries and fluids (methanol and water). Due to creeping of methanol, leading to low reproducibility of the filling, a special channel cross section as shown in figure 5 was preferred. At the channel bottom, corner creeping is prevented by a radius. However, due to manufacturing constraints this is not possible at the channel top where it is sealed. Therefore, a sharp corner was realised here in order to facilitate creeping between sealing foil and chip material. In this way the fluid reproducibly wets the wedge channel first before the fluid meniscus is pushed into the channel by pressure. This can be seen as a light shade around the channel on figure 5 in the case of methanol, while the wedge remains empty in the case of the non-wetting fluid water. Fig. 6 shows the fraction of the dead-end channel that is not filled with fluid when it is in contact with pressurised methanol. A sensitivity of approximately 100 mbar is obtainable with this geometry. This is sufficient for the present purpose of detecting filter clogging. However, it should be easy to adjust the geometry in order to reach different sensitivities. Similar results are obtained with watery solutions.

According to the Boyle–Mariotte law the product of pressure and volume occupied by a gas is a constant at fixed temperature. As can be seen on figure 6, the filling length seems to deviate somewhat from this ideal case, since the channel tends to fill further than expected. For example the channel is filled more than halfway at 1 bar applied pressure. This is simply caused by the flexibility of the sealing foil that at higher pressures tends to bulge outward, in this way not keeping the channel cross section fixed.

The instrument

In order to perform the tasks with as little user intervention as possible, an instrument for the chip has been built that performs

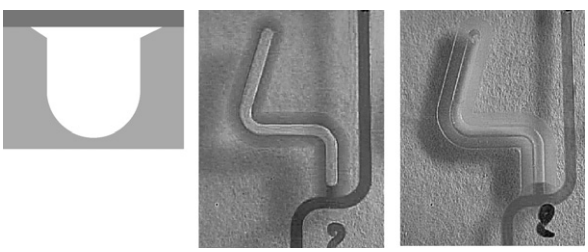

Fig. 5 A special cross section shape was chosen for the channels in the pressure measurement channels (left), leading to reproducible capillary filling of the edge region for wetting fluids (middle, methanol) while not filling by capillary forces for non-wetting fluids (right, water).

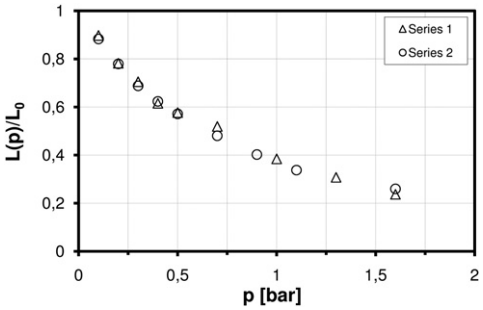

Fig. 6 Fraction of the pressure measurement channel that is not filled with liquid, $L(p)/L_0$, as a function of the applied overpressure p. L_0 is the total length of the channel, L(p) the length of the gas column at pressure p. Two separate tests series are shown, both with 95% methanol as liquid.

the different tasks automatically (figure 2). For performing the sample preparation the user draws 3 ml of sample (stored in a methanol based buffer) into a disposable syringe, connects the syringe to the chip, and inserts the two into the instrument. The extraction procedure is then started *via* a customised LabView program running on a laptop connected to the device controller. Since all fluids are stored on the chip no further user intervention is needed during the extraction procedure.

The fluid actuation is performed by two syringe pumps. The first one pumps the 3 ml sample through the cell capture filter. All other fluidic operations are actuated by pressurised air delivered from the second liquid free syringe pump. An external 3-port/2-way valve allows for reloading this syringe with ambient air, so that several piston strokes are possible without the need to draw air through the chip. In this way contamination of this syringe is impossible.

Fluid control is achieved *via* the three on-chip turning valves. The motors for their actuation are located below the chip holder table onto which the chip is clamped during operation. This table also holds the removable reservoirs for waste and eluate. Furthermore a heater below the lysis chamber and nucleic acid filter elevates the temperature during lysis and drying of the filter material.

Sensitivity study of on-chip nucleic acid extraction

An important parameter for assessing the adequacy of the sample preparation system is the amount of material needed to obtain a sufficient amount of viral mRNA for the NASBA amplification and subsequent detection. To this end various amounts of cells have been used from three different cell lines of human cervical cancer origin that express different strains of HPV-mRNA. Specifically, the cell lines CaSki, MS751 and HeLa were used, each expressing one of the strains HPV16, HPV45 and HPV18, respectively.

In the study samples with 50 000, 5000, 500, 50 and 5 cells from the different cell lines were extracted in the sample preparation device and examined by running conventional NASBA in three dilutions (1 : 1, 1 : 5 and 1 : 10, the latter two each being prepared by mixing 5 µl of the extract with a corresponding amount of water), to detect the HPV type of interest and the internal control U1A (human U1 small nuclear ribonucleoprotein (snRNP) specific protein A). For lysis the bioMérieux NucliSens® easyMAG™ Lysis Buffer (ref. 280134) was used. Due to the constraint on the

amount of chips manufactured only one extraction experiment was performed for each cell type and dilution.

All samples with 50 000 cells down to 5 cells were made by diluting the original sample with a corresponding volume of PreservCyt™ fixation buffer. In this way the presented cell numbers are to be understood in a statistical sense, including a particular spread notably for the small cell numbers. The dilution procedure was to consecutively dilute by 1 : 10 until the respective cell concentration was reached.

In order to validate the successful extraction of viral RNA from the samples, the eluate was transferred for specific amplification and detection of the target HPV-mRNA to the PreTect™ HPV-Proofer analysis platform (NorChip AS, Norway), using primers/probes included in the PreTect HPV-Proofer kit.[16–18]

Table 2 shows the NASBA results of the extracts. The data displays the positive dilutions of each concentration and cell line, together with the mRNA and U1A. All negatives are excluded from the table, except in cases where none of the three dilutions gave a positive result.

As a simplified assumption, the three cell lines can be viewed upon as one type of cell, since all three lines are epithelial cells of similar size. In this way we showed that it is possible to detect mRNA extracted from a sample of cell lines containing as little as 5 HPV-mRNA expressing cells. However, for the lowest cell level of 5 cells in the sample only two out of three entries in the matrix show a detectable amount of HPV-mRNA, indicating that this might be close to the sensitivity limit of the procedure. Nevertheless, the failure to detect HPV45 at the five cell level and U1A in the three lowest concentrations of MS751 is most likely due to long storage time in PreservCyt. This cell line was stored for about three months. The user guide of the Pretect™ HPV-Proofer recommends a maximum storage of four weeks for clinical samples. Most likely the mRNA of the cell line was degraded to a certain degree, and applying the cell line within the preferred storage time would most likely improve the sensitivity. However, promising results were achieved even though the cell line exceeded the recommended storage time.

We would like to address the following points. In the samples containing 50 000 cells, only the HeLa cell line shows positive NASBA for both HPV and U1A in an undiluted extract. This suggests that the extracts include a large number of biomolecules that may inhibit enzymatic reaction. Another explanation that can not be ruled out is that the eluate contains contaminating GIT or ethanol from the extraction procedure.

Secondly, the extracts containing nucleic acids from 5 cells show negative NASBA for low dilutions. However, one has to keep in mind that the number of cells in the sample is to be understood in a statistical sense as the samples were obtained by dilution. Particularly, the cell content of the sample labelled "5 cells" may thus deviate substantially from this number. Another likely reason is that the extract is diluted too far before performing the NASBA. The output extracted from the sample preparation device is approximately 40 μl. In the 1 : 5 and 1 : 10 dilutions, two volumes of 5 μl are taken from the extract and mixed with water before amplification.

Third, the U1A amplification is less sensitive than the HPV amplification. Due to this the U1A is not detectable in all extracts with only 5 cells. This may be due either to a technical bias to HPV amplification or by the different steady state levels of HPV and U1A RNA molecules. Nevertheless, in the Pretect HPV Proofer assay a specimen that is U1A negative but HPV positive is still considered positive regardless of the U1A result.

Extraction of nucleic acid from cervical liquid based cytology specimens

In order to demonstrate that the system is capable to manage the extraction of HPV mRNA derived from cervical samples, several extractions from cytology based liquid samples tested positive for HPV16 were performed. These samples are more difficult to handle than the cell-line based samples since these express a large range of viscosities due to their different amount of cell material as well as mucus or blood in the sample. Indeed, it was necessary to adopt a discontinuous sample loading procedure due to the large pressure built up observed for some samples. Instead of a continuous flow, the loading was alternated by small intervals where the syringe pump was operated in the reverse direction, in this way reliably releasing the blockage of the filter.

In order to demonstrate the successful extraction, the eluate from the chip was again transferred to a NASBA amplification and fluorescence detection. Fig. 7 shows the amplification curves for on-line fluorescence detection during the NASBA procedure for several samples. In this case all amplifications were performed on the undiluted eluate extracted from the samples. The curves show the strong increase in fluorescence signal after a certain time delay and the final saturation characteristic for a successful NASBA reaction.[19] Although there is

Table 2 NASBA results for the sensitivity study of the sample preparation device. All extracts were tested with dilutions of 1 : 1, 1 : 5 and 1 : 10. Only results with positive amplification are shown. The cell count is to be understood in a statistical sense, since the numbers were obtained by dilution

Cell line/cell count	CaSki	MS751	HeLa
50 000	**HPV16**: 1 : 5, 1 : 10 **U1A**: 1 : 5, 1 : 10	**HPV45**: 1 : 5, 1 : 10 **U1A**: 1 : 5, 1 : 10	**HPV18**: 1 : 1, 1 : 5, 1 : 10 **U1A**: 1 : 1, 1 : 5, 1 : 10
5000	**HPV16**: 1 : 1, 1 : 5, 1 : 10 **U1A**: 1 : 1, 1 : 5, 1 : 10	**HPV45**: 1 : 5, 1 : 10 **U1A**: 1 : 5, 1 : 10	**HPV18**: 1 : 5, 1 : 10 **U1A**: 1 : 5, 1 : 10
500	**HPV16**: 1 : 1, 1 : 5, 1 : 10 **U1A**: 1 : 1, 1 : 10	**HPV45**: 1 : 5 **U1A**: not positive	**HPV18**: 1 : 1, 1 : 5, 1 : 10 **U1A**: 1 : 1, 1 : 5, 1 : 10
50	**HPV16**: 1 : 1, 1 : 5, 1 : 10 **U1A**: 1 : 1, 1 : 5, 1 : 10	**HPV45**: 1 : 1, 1 : 10 **U1A**: not positive	**HPV18**: 1 : 1, 1 : 5, 1 : 10 **U1A**: 1 : 10
5	**HPV16**: 1 : 1, 1 : 5 **U1A**: not positive	**HPV45**: not positive **U1A**: not positive	**HPV18**: 1 : 1 **U1A**: not positive

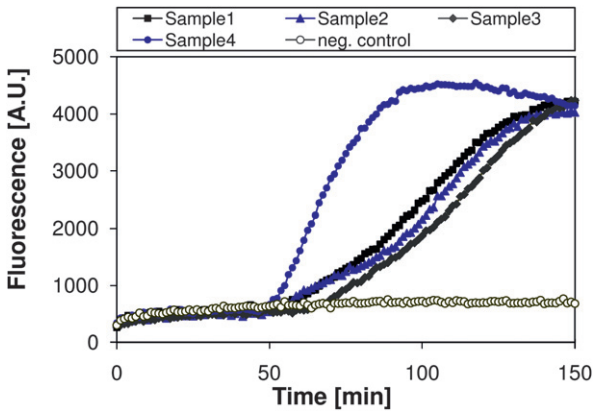

Fig. 7 Curves showing fluorescence intensity *vs.* time during NASBA amplification. The filled symbols represent amplification curves for eluate obtained by extraction from several HPV16 positive cytology based liquid samples, the open circles correspond to a negative control. The amplification was done on the undiluted eluate extracted from the samples. The fluorescence intensity for the samples follows the S-shape characteristic for a successful NASBA amplification.

a difference in signal form between the individual amplification curves, all curves are classified as belonging to an HPV16 positive specimen by the signal evaluation software of the Pretect HPV Proofer. Positive results are identified through their relative fluorescence signal increase compared to the stabilized background by a factor larger than 1.7 and an S-shaped amplification curve. The experiments thus indeed demonstrate that a sufficient amount of HPV-mRNA can be extracted by the automatically operated chip and that the device is able to handle relevant cytology samples.

Conclusions and outlook

We have demonstrated a lab-on-a-chip system capable of hands free automatic sample preparation and nucleic acid extraction from complex samples. The necessary steps of cell pre-concentration, cell lysis, and nucleic acid concentration by solid phase extraction are performed within a disposable chip. The procedure is controlled by an instrument into which the sample and chip are inserted and which autonomously controls the whole chip and thereby the whole sample preparation process. The chip also stores all reagents necessary for the on-chip sample preparation in order to keep the user intervention and device maintenance to a minimum. The viability of the automatic sample preparation by this system was demonstrated by extraction of HPV-mRNA from cell line samples as well as from fixated cells derived from cervical liquid based cytology specimens.

The assay that has been adapted to the chip format is directly derived from a standard laboratory assay for nucleic acid concentration and purification. In particular it relies on well established solid phase extraction filters integrated into the chip. The device has been designed for integration with a system for parallel and multiplex real-time NASBA amplification[13] of more than six transcripts which will be presented in a separate publication. However, the fluidics implemented into the chip do not rely on a peculiar property, trade name or brand name of the used membrane. Due to this, the assay can be readily adapted to different sample preparation applications, in particular also DNA preparation. Moreover, the system can be readily integrated with further downstream operations such as RT-PCR amplification instead of NASBA. As a consequence, it is possible to adapt the system to the most appropriate nucleic acid analysis for a targeted application. The range of possible applications for such a system could encompass foodstuff analysis, animal feed control, personalised medicine, Point-Of-Care, forensics, security application and others.

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