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NEW LAB-ON-A-CHIP SYSTEM FOR INFECTIOUS DISEASE ANALYSIS

Marion Ritzi-Lehnert, Jan Claussen, Eva Schaeffer, Ole Wiborg, Isabell Wick, Klaus S. Drese
Institut fuer Mikrotechnik Mainz GmbH
Mainz, Germany

Ralf Himmelreich, Hans Attig, Rainer Dahlke, Gerd Grosshauser, Markus Jeziorski, Andy Wende, Thomas Rothmann
Qiagen GmbH
Hilden, Germany

ABSTRACT

Early diagnosis followed by personalised efficient therapy of infectious diseases (e.g. respiratory diseases, meningitis, sepsis) can lead to considerable reduction of costs in health care. Point-of-care testing (POCT) can provide early detection since this kind of decentralised analysis can be done by unskilled personnel at any time. Other advantages of automated miniaturised Lab-on-a-Chip systems (LoC) are reduction of time and reagents, elimination of cross-contamination and enhanced reproducibility due to enhanced process control. Such Lab-on-a-Chip systems will establish themselves on market only when sensitivity and specificity meet clinical requirements.

An integrated cost-efficient lab-on-a-chip system is presented which allows performing all diagnostic process steps for pathogen analysis of respiratory viruses from nasopharyngeal samples. The microfluidic disposable chip comprises structures for lysis of nasopharyngeal swab samples, preparation of total nucleic acids using magnetic silica beads, reverse transcription followed by QIAplex PCR technology and labelling of the nucleic acids by hybridisation with LiquiChip Beads and streptavidin-R-phycoerythrin. Labelled target sequences are transferred for analysis into a QIAGEN LiquiChip 200 workstation. The core of the instrument is a construction based on rotating heating bars allowing for fast cycling. All chemicals needed for performing of 24 analyses are either stored freeze-dried on the single-use disposable microfluidic chip (processing cartridge) or as liquids in a separate reagent cartridge.

After introducing the sample into the lysis chamber of the microfluidic chip and inserting the chip into the device all steps are done automatically. To realise these steps, fluidic control in terms of light barriers and turning valves are integrated into the injection moulded disposable chip. This includes metering structures as well as magnetic stir bars for mixing.

The functionality was proven by direct comparison of samples processed manually vs. automatically using the "ResPlex Panel II" for detection of respiratory viruses from nasopharyngeal samples. The efficiency of the automated LoC system yields at about 30-60% as compared to the manually performed reference experiments. Comparing the performance of the instrument with commercially available kits and nucleic acid preparation devices showed slightly weaker but clearly positive final signal intensities obtained from the prototype device even without protocol optimization.

Key words: Lab-on-a-chip, infectious disease analysis, on-chip nested PCR, cost-efficient integrated system.

INTRODUCTION

Huge effort was done during the last decades to establish fully automated miniaturized micro total analysis systems for diverse applications. Especially diagnostic applications are of interest since they can lead to better, faster, more specific and more cost-efficient medical treatment in terms of appropriate diagnosis, medication and therapy monitoring. Although a lot of microfluidic-based concepts and technologies were developed and published, only few succeeded to enter the market [1,2,3].

One of the major drivers of such developments is costs per analysis and per single test result. Production costs in combination with significance and reliability of the result decide on the marketability of new systems. And in addition, only when sensitivity and specificity meet clinical requirements these systems will establish themselves on the market.

Established in 1983 by Kary Mullis PCR found its way into modern molecular diagnosis and is nowadays a common method for nucleic acid based diagnosis of a huge variety of diseases like infectious diseases and cancer or predispositions

and genetic disorders. Since this method gains more and more attention for diagnosis, consequently, it is transferred to microfluidic lab-on-a-chip systems[4]. Nevertheless, most lab-on-a-chip systems still lack the sample preparation since this step often is complex and needs a reduction of initial milliliter sample volumes to the microliter range. This is a prerequisite for the progression within the microfluidic chip environment.

Here, an integrated lab-on-a-chip system is presented which allows performing all diagnostic process steps - preparation, amplification, and detection - for pathogen analysis of respiratory viruses from nasopharyngeal samples. The new device combines several unique solutions. Among them are a combined lysis and nucleic acid binding step, a novel magnetic separation principle, the "on-chip" nested multiplex amplification, and the internal processing and amplification controls. Parallel development of disposable analysis chip and corresponding instrument led to a cost-efficient LoC-system. The at present estimated costs per analysis will be in the range of 5-15$. This system can be adapted to identify various infectious diseases and therefore will allow opening completely new markets for in-vitro diagnostics.

FLUIDIC CONCEPT

Starting point were several basic assumptions with the final goal of a robust microfluidic system. Assay steps to be realized were lysis, nucleic acid separation, amplification and detection of amplicons. Since these steps have to be performed without any cross-contamination number of inlets into the cartridge have to be as minimal as possible and T-junctions as well as overlapping structures have to be avoided. A very simple valve concept had to be developed in combination with a (under or over) pressure driven actuation.

According to the demand for a cost-efficient system there should be just one valve design which can be opened or closed. All connections should be on one side of the chip. To ensure precise pumping and positioning of the fluids the gas volume should be as small as possible to avoid uncontrollable movements due to the compressibility of gas.

A new simple open/closed valve concept was developed (Fig. 1A). A pentagonal valve body contains two channels in two layers. In position 1 an inlet channel is connected to an outlet channel. By pushing the valve body in position 2 the inlet channel is connected to another outlet channel. According to this valve design a fluid plan was established as can be seen in Fig. 2A. To avoid high gas volumes silicone oil was intended to be used as pumping mediator. Unfortunately, these new valves were not suitable for easy, cost-efficient production. Therefore, the concept was changed to the usage of turning valves. To increase sensitivity it was also decided to implement a nested PCR. Consequently, a second PCR chamber was integrated as well as metering structures to separate an aliquot of the first PCR for progression in the second amplification step and to separate an aliquot of the second PCR for the following hybridization step.

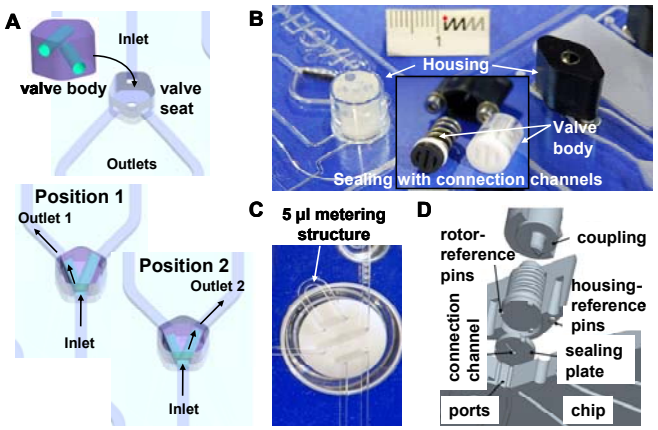

Fig. 1 Different valve concepts. (A): Schematic drawing of the simple open/close switch within the chip. Position 1: inlet channel connected to left outlet channel. Position 2: inlet channel connected to right outlet channel. (B) Rapid prototyping multi component turning valve version (black) and made by two component injection molding (white). (C) Top view of a turning valve comprising the sealing plate with connecting channels and a 5 µl metering structure. (D) Schematic drawing of rapid prototyping valve assembly.

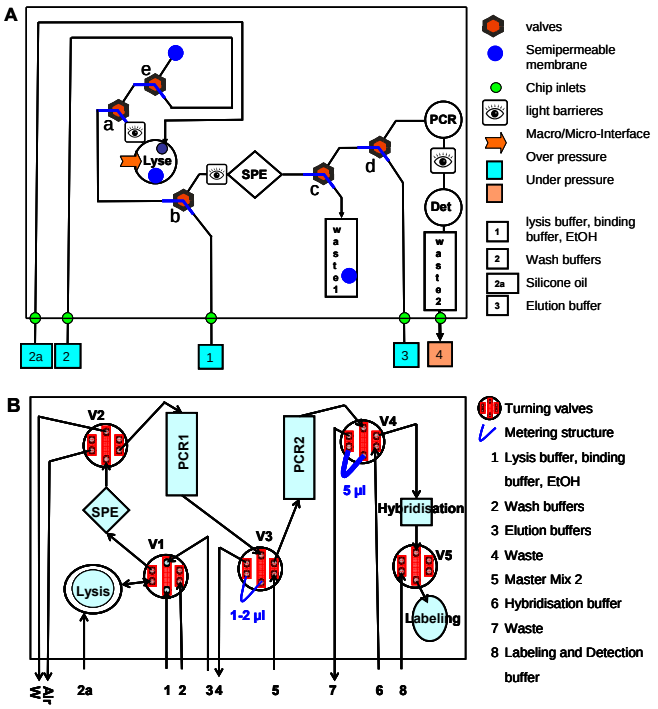

Fig. 2 Fluid plan (A) First fluid plan using the new valve concept. (B) Optimized fluid plan using turning valves and nested PCR. (SPE = solid phase extraction, v = valve)

According to these changes a new fluid plan was established as shown in Fig. 2B. The originally used turning valve concept, which was complex in manufacturing and

assembly, was transferred to a two-component injection moulding part (Fig. 1B) where the housing now is part of the chip. The valve core is made from PC combined with a soft thermoplastic elastomer (TPE) for the structured sealing plate. The pressure for tight connection is applied by the instrument. Fluid movement is driven by syringe pumps.

PROCESSING AND REAGENT CARTRIDGES

The microfluidic disposable processing cartridge (chip, Fig. 3 top) is injection molded from Polycarbonate (PC) while the slip-on lysis chamber is made from Polyethylene (PP). The size is comparable to a micro titer plate. The lysis chamber has a total volume of 3 ml. Channel dimensions are 0.6x0.6 mm^2, PCR reservoirs have a height of 1 mm and a volume of 120 µl.

These are rather high volumes and not really in accordance with microfluidic systems. Nevertheless, a reduction in volumes would be easily achievable with standard microfabrication techniques. Obviously, lower volumes have advantages for a microsystem but are often not in accordance with the real world outside of pure analytical testing. In addition, lower volumes do not consequently lead to better results in terms of sensitivity due to limited target concentration. Here, up to 1 ml sample volume (standard volume in clinical molecular diagnostics) can be processed since binding of nucleic acids under chaotropic conditions requests the addition of one volume of lysis buffer and one volume of binding additives.

For the PCR 120 µl volume was chosen which is in accordance with e.g. the Roche Blood Bank PCRs which run at 100 µl for good reasons. Even the best sample preparation technologies do not remove all PCR inhibitors present in a sample. These inhibitors are less relevant if a large PCR volume is processed. Since the PCR reagents are stored dried within the PCR chambers the complete eluated DNA/RNA solution is used for rehydration to have as much target sequences for amplification as possible. This enhances the sensitivity of the system. Due to less complexity the second PCR was set to the same volume although this could have been reduced.

At the transition from the lysis chamber into the microsystem a polypropylene frit (~150 µm pore size) is placed to retain particles. All reagents needed for the assay are either stored freeze-dried (beads, enzymes, primers) in the single use disposable processing cartridge (Fig. 3 top) or as liquids in a separate reagent storage cartridge (buffers) allowing the performance of 24 analyses (Fig. 3 bottom).

After introduction of the nasopharyngeal swab sample extract (500 µl) into the lysis chamber the microfluidic chip is inserted into the device and the automatic analysis is started. Within the lysis chamber the sample gets mixed with 250 µl high molar chaotropic salt containing lysis buffer and magnetic silica beads (MagAttract Suspension G; QIAGEN) for solid phase extraction of nucleic acids. Beads are stored as dried pellet within the lysis chamber together with a magnetic stir bar. This pellet additionally contains dried Proteinase K as well as the internal "Processing and Amplification Controls" (iPAC) containing the RNA bacteriophage fr. The chamber gets heated up by a resistive heating structure to support lysis and homogenization is assisted by magnetic stirring at 1000 rpm.

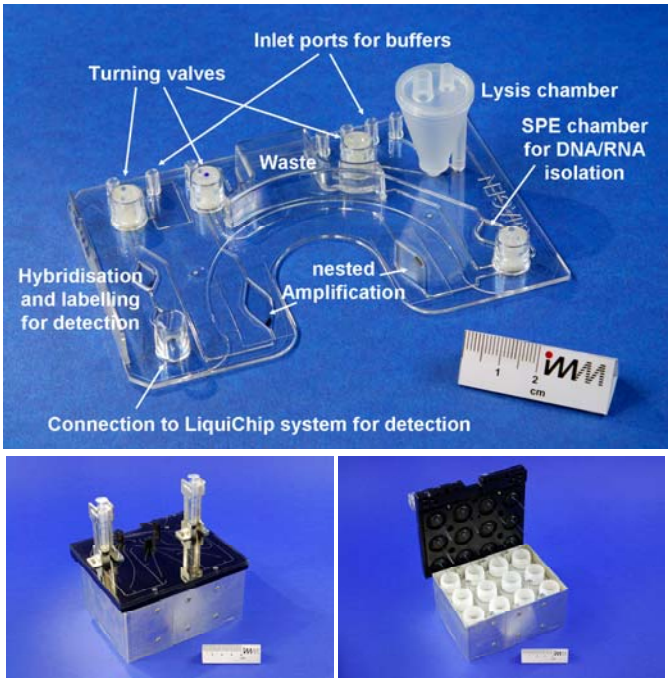

Fig. 3 Disposable process cartridge (top) and reagent cartridge (bottom). The process cartridge contains various chambers for lysis, SPE (solid phase extraction), nested PCR, hybridisation and waste, several buffer inlets and turning valves. The reagent cartridge bears 12 bottles with buffers needed and a distributing cover plate.

After heat supported lysis at 56°C for 15 minutes and addition of further 250 µl of lysis buffer in between, 500 µl isopropanol are added to adjust proper binding conditions. Magnetic beads bind nucleic acids within one minute and solution is slowly moved (0.6 ml/min) to the SPE chamber where magnetic beads are retained by a magnetic stirrer. Washing efficiency of serial flushes of 0.8 ml wash-buffers 1 and 2 (QIAGEN) is increased by homogenizing the beads by magnetic stirring. To remove residual ethanol beads are rinsed with water (1 ml) without mixing. Elution of the bound nucleic acids is performed by mixing the beads with the remaining water volume of the water rinse for 40 seconds. The entire volume of eluted nucleic acids is pumped into PCR chamber 1 for rehydration of the dried amplification reagents. Both amplification steps of the nested PCR show a volume of 120 µl, respectively. Depleted sample fluid and all buffers used are collected within a waste reservoir.

Purified viral RNA is amplified by reverse transcription followed by QIAplex PCR technology[5]. For the second PCR reaction 1.6 µl of the first PCR reaction is metered by a small

interconnecting channel within the turning valve. For subsequent detection 5 µl of the final amplified nucleic acids are again aliquoted by a metering loop (Fig. 1C) and diluted with 50 µl hybridisation buffer. Hybridisation with colour-coded LiquiChip Beads which are individually coupled to target specific reverse complement detection oligonucleotides is performed at 52°C. Following quantitative labelling with streptavidin-R-phycoerythrin via the 5'-biotin PCR-primers leads to dual-labelled target sequences. These are finally transferred for analysis into a QIAGEN LiquiChip 200 workstation via tube connection. The amount of phycoerythrin (secondary fluorescence) considering the respective bead code (primary fluorescence) is used for determination of the presence of each amplified target sequences. Using primers and detection oligo sequences adopted form the QIAGEN ResPlex II Panel v2.0 several viruses can be detected[6].

The reagent cartridge is covered by a plate guaranteeing tight sealing of the reagent bottles (Fig. 3 bottom). This cover also possesses channels and valves for metering and distribution of the buffers. Two syringes keep buffers coming at the right point in time. By controlling these syringes in combination with the integrated turning valves exact metering and positioning of fluids is realised. The originally planned usage of silicone oil as pumping mediator could be avoided by realising a clever valve control in combination with small air cushions within the syringe pumps. This eliminated overtravel caused by bigger air cushions within the chip system.

INSTRUMENT SET-UP

The operational device (Fig. 4) comprises an elaborated mechanical system harbouring mechano-electronic features like heating elements, syringe pumps, valve-driving motors and magnetic stirrers. The electronic components are controlled by a main board, which itself is controlled by a computer running a complex custom-made LabView (National Instrument Corp.) software protocol.

Chip and reagent cartridge have to be inserted into the loading drawer of the system by the user after introducing up to 1 ml sample to the lysis chamber on the chip. The loaded drawer is pushed into the system and chip, reagent cartridge and instrument get connected. The processing cartridge gets connected to the operating device via the couplings of the turning valve driving motors. Additional connections to the reagent cartridge by Luer adaptors allow providing the liquid buffers. As described in the previous paragraph the reagent cartridge (Fig. 3) contains two syringe pumps which drive the fluids within the cartridges. By pushing the loaded drawer into the instrument the syringes get connected to linear drives within the instrument. The minimal pumping volume is 0.12 µl which can be dispensed precisely into the chip. The clever fluid transport concept is based on minimal dead volumes. Compressible air plugs within the addressed channels are reduced to a minimum. All fluids are guided to the addressed reservoirs via the on-chip integrated turning valves.

The developed device is designed to carry out a very fast PCR reaction. To achieve this it is necessary to heat and cool the PCR reactions with high rates. Therefore, the core of the instrument is represented by the PCR carrousel (Fig. 4) where preheated clamps mounted on a rotation axis allow for fast switching between the temperature zones and thus fast cycling times. Three of the four heating clamps are set constantly to the PCR cycling temperatures needed (95°C, 72°C, 52°C). The forth clamp has room temperature and is used to support cooling. The carrousel positions the clamps on the PCR chamber. By closing the clamp the reservoir is heated/cooled from both sides of the chip for the time needed. Clamps get attached one after the other to the PCR chamber to perform the PCR cycles. Fig. 5 shows a standard measurement for the determination of the temperature profile within a PCR chamber.

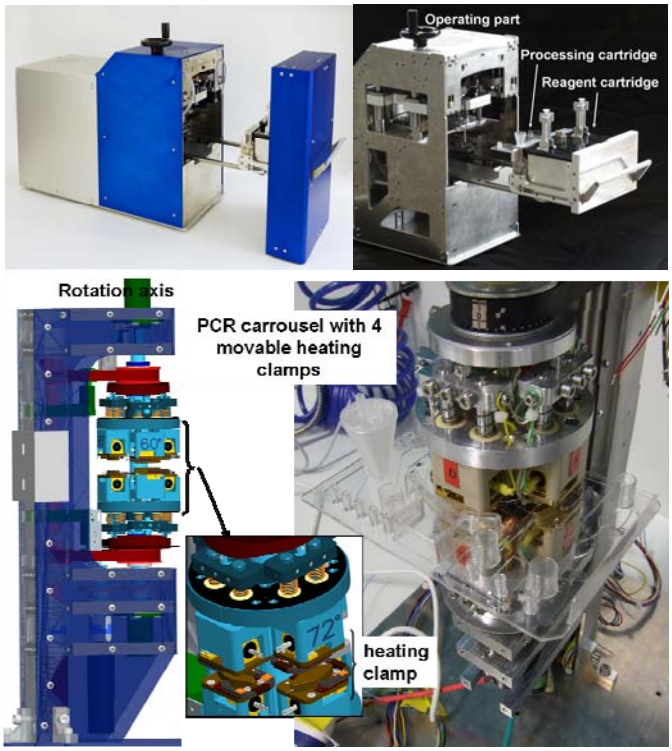

Fig. 4 Operating device for the automated analysis of nasopharyngeal swab samples. The device is composed of the electronic unit, the operating part and a drawer for processing and reagent cartridge. Core of the operating part is the PCR carrousel comprising four heating clamps, each with constant temperature arranged on a rotating axis (top). PCR chambers on chip are clamped between two heating elements showing the same temperature (bottom).

The final PCR reaction needs the following temperature cycle to work properly: 24s at 95°C, 40s at 56°C, 30s at 72°C. Each step includes heating/cooling and hold time. Cooling from 95°C to 52°C needs about 8s therefore the cooling rate is 5.4°C/s. Heating from 52°C to 72°C takes about 17s which equals a heating rate of 1.2°C/s. Heating from 72°C to 95°C is

done in about 12s which corresponds to a rate of 1.9°C/s. Differences in heating rates can be explained by differences in the contact pressure of the clamps. Allowing for a PCR time of around one hour for 35 cycles at a volume of 120 µl makes this system the fastest high-volume PCR-chip at the moment[4]. To establish fast heat transfer the reactions are stirred by small magnetic foil stirrers within the reaction reservoirs. This concept was also applied to achieve homogeneous mixing.

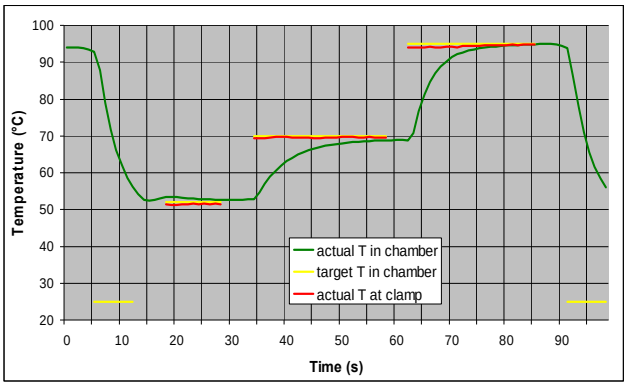

Fig. 5 Determination of the temperature profile within the PCR chamber. The blue line represents the actual temperature of the fluid. The red line stands for the actual temperature of the heating clamps.

EXPERIMENTAL RESULTS

First, performances of individual assay steps were compared to the standard assay. Experiments showed that the standard assay needs adaptation to the microfluidic environment to reach sufficient efficiency. To investigate the lysis and extraction performance swab sample extracts were spiked with bacteriophage fr. Lysis was done either conventionally using the Qiagen EZ1 Advanced system or in the lysis and SPE chambers of the processing cartridge. For quantitation conventional qPCR assays against human and bacteriophage targets were done. Isolation with the microsystem yielded in slightly lower RNA and slightly higher DNA amounts compared to the conventional tube method (Fig. 6 top left).

Efficiency of the nested RT-PCR reaction in tubes and on microchip is comparable (Fig. 6 top right). Here, one microliter of the first PCR reaction was transferred to the second PCR reaction by syringe. Five microliters of the second PCR reaction were finally analyzed in Qiagens LiquiChip system. Surprisingly, it turned out that blocking reagents restored efficiency of the RT-PCR reaction but are unfavorable for the second PCR reaction. As can be seen in Fig. 6 (bottom) efficiency of the nested RT-PCR dropped when done on chip having no blocking reagent in both reactions (on-chip V1). It even decreased more with blocking reagent in both PCR reactions (on-chip V2) compared to the conventional RT-PCR. By adding blocking reagent only to the RT-PCR (on-chip V3) efficiency is comparable to the conventional tube performance.

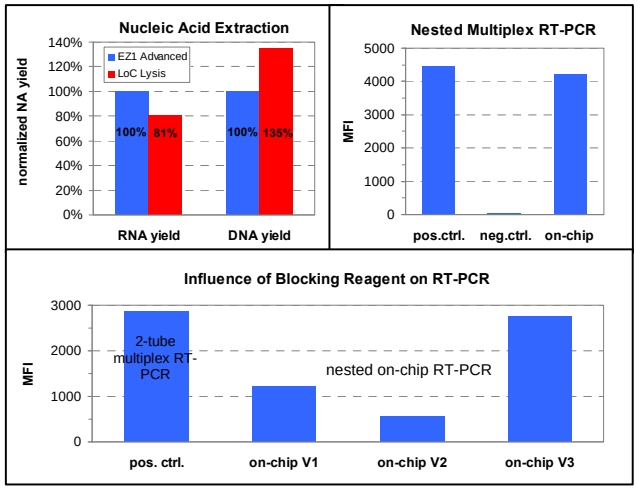

Fig. 6 Efficiencies of module performance. Top left: swab sample extracts spiked with $4x10^6$ fr- bacteriophages were lysed, RNA/DNA isolated and quantitated in qPCR against human and bacteriophage targets. Top right: 10^3 copies RsvB in vitro transcript were amplified in a nested RT-PCR. Bottom: Influence of blocking reagents on the PCR on chip was investigated using no blocking reagent (on-chip V1), in both PCR reactions (on-chip V2) or only in the RT-PCR reaction (on-chip V3). (MFI = Median fluorescence intensities values measured by Qiagen LiquiChip 200 workstation). (Per graph one representative experiment is shown.)

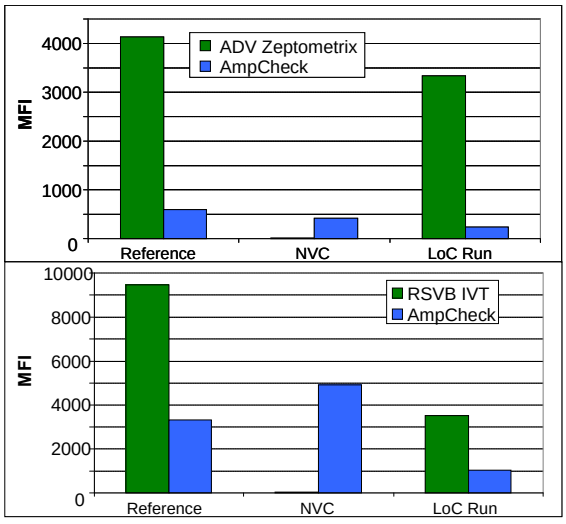

Fig. 7 Median fluorescence intensities values (MFI) measured by QIAGEN LiquiChip 200 workstation. Top: MFI of Adenovirus Standard from ZeptoMetrix Corp. and the internal controls ("Amp Check"). "Reference" samples were prepared using the BioRobot EZ1 for automated sample preparation and the QIAGEN ResPlex II Panel v2.0 for amplification and detection. Bottom: MFI of the RSVB in vitro transcripts and the internal controls ("Amp Check"). "Reference" samples were prepared manually. "LoC Run" represents the fully integrated microsystem procedure. Non-virus-control (NVC) (prepared on operating device) is without virus standard added to the sample. (Per graph one representative experiment is shown.)

To investigate the performance of the complete assay using the developed LoC system, *in-vitro* transcribed RNA or commercially available standards from the ZeptoMetrix Corp. "NATrol Respiratory Validation Panel" were spiked into Copan Universal Transfer Medium (UTM) containing cell material derived from a nasopharyngeal swab. Each half of the sample was processed with the LoC device and processed manually according to reference protocols, respectively. Due to the complexity of the processing protocol, the efficiency of the automated LoC system yields at about 30-60% as compared to the manually performed reference experiments (Fig. 7). Detection was performed in the LiquiChip 200 workstation by measuring the median fluorescence intensity values.

Comparisons with commercially available kits and nucleic acid preparation devices again showed weaker but clearly positive final signal intensities obtained from the prototype device even without protocol optimization. For nucleic acid preparation the BioRobot EZ1 Workstation was used with EZ1 Virus Mini Kit extraction protocol. Amplification and detection were done using QIAGEN ResPlex II (Panel v2.0). The final signal intensities obtained by using the prototype device were weaker when compared to the commercial systems but clearly positive even without protocol optimization. Since the experimental results already vary drastically for the references (see for example MFIs in Fig. 7) signal ratios have to be observed instead of the absolute values. Main differences of LoC-assay and standard assay are summarized in Table 1.

	Stormbreaker	ResPlexII Kit
RT-PCR input volume	■ entire eluate (120µl)	■ 10µl eluate
Amplification / Cycling	■ 2tube format (1µl of RT-PCR1 as template in PCR2)	■ 1tube format (50µl Rxn)
Reagents	■ lyophilized	■ liquid
Hybridization Buffer	■ 6x Hyb Buffer PT	■ Detection Buffer

Table 1 Main differences of LoC- and standard assay

SUMMARY

An integrated lab on a chip system was developed which allows performing all diagnostic process steps for pathogen analysis of respiratory viruses from nasopharyngeal samples. Parallel development of disposable analysis chip and corresponding instrument led to a cost-efficient LOC-system being suitable for serial production. Adabtability to identify various infectious diseases will be given and therefore will opening completely new markets for in-vitro diagnostics will be allowed. One major bottleneck for LoC systems still is robustness at affordable costs. To achieve this demand, simplicity of assay and instrument has to be the key strategy for future integrated molecular diagnostic LoC devices[7].

Regarding new construction principles in the field of LoC systems the chosen valve design as well as the simple touch-free stirring process provides easy production as well as robust and reliable performance. Additionally, applying a PCR carrousel strategy the cycling rates for 120 µl solution are outstanding as compared to commercial end-point thermal cyclers. Although this system represents the fastest high-volume PCR-chip at the moment there are other chip developments showing noticeable shorter cycling times. Nevertheless, these reactions are either based on pre-amplified DNA or have small reaction volumes of less then 20 µl[8].

First tests with the realised functional model revealed that the overall performance of the chip system is comparable with standard lab procedures whereas there are differences in the efficiency of the individual steps. The functionality was proven by the direct comparison of samples processed manually vs. automatically using the "ResPlex Panel II" for detection of respiratory viruses from nasopharyngeal samples. First results were promising and show an excellent basis for further developments.

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