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PAPER IN FOREFRONT

Continuous-flow, microfluidic, qRT-PCR system for RNA virus detection

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Abstract One of the main challenges in the diagnosis of infectious diseases is the need for rapid and accurate detection of the causative pathogen in any setting. Rapid diagnosis is key to avoiding the spread of the disease, to allow proper clinical decisions to be made in terms of patient treatment, and to mitigate the rise of drug-resistant pathogens. In the last decade, significant interest has been devoted to the development of point-of-care reverse transcription polymerase chain reaction (PCR) platforms for the detection of RNA-based viral pathogens. We present the development of a microfluidic, real-time, fluorescence-based, continuous-flow reverse transcription PCR system. The system incorporates a disposable microfluidic chip designed to be produced industrially with cost-effective roll-to-roll embossing methods. The chip has a long microfluidic channel that directs the PCR solution through areas heated to different temperatures. The solution first travels through a reverse transcription zone where RNA is converted to complementary DNA, which is later amplified and detected in real time as it travels through the thermal cycling area. As a proof of concept, the system was tested for Ebola virus detection. Two different master mixes were tested, and the limit of detection of the system was determined, as was the maximum speed at which amplification occurred. Our results and the versatility of our system suggest its promise for the detection of other RNA-based viruses such as Zika virus or chikungunya virus, which constitute global health threats worldwide.

Keywords Lab on a chip · Quantitative reverse transcription polymerase chain reaction · RNA-based virus detection · Infectious diseases · Point of care · Ebola virus

Introduction

Infectious disease diagnosis is crucial to the delivery of proper healthcare and to improving the health of individuals and populations [1]. One of the main challenges in the diagnosis of infectious diseases is the need for rapid detection of the causative pathogen that allows proper and timely clinical decisions to be made for patient treatment in any setting [2]. Currently, most diagnostic testing is performed in centralized laboratories with accurate and automated equipment that allows analysis of a large number of samples at a relatively low cost [3]. Infectious pathogens are commonly identified through polymerase chain reaction (PCR) methods, which allow rapid and sensitive pathogen detection. Such a health-provider-centered model, also known as "conventional diagnostics," is dominant around the world and is well suited for populations with easy access to centralized hospitals and laboratories [3].

In remote and rural settings, however, this conventional diagnostic laboratory-based model presents a major

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drawback: long turnaround times of typically many days [4]. The long confirmatory diagnostic turnaround times and the fact that patients in rural settings, who live far from the clinic, are less likely to come back in a timely manner for a second medical appointment or confirmatory diagnosis, lead many physicians to manage diseases during the first clinical encounter without waiting for confirmatory diagnosis [5, 6]. However, the differential diagnosis of many febrile infectious diseases (e.g., Ebola, malaria, Lassa fever) based on early signs and symptoms is not possible because of their similar presentations. Because of this, very frequently, febrile illnesses are treated with antibiotics regardless of their cause [7–9]. This extensive practice not only contributes to worse patient outcomes due to inappropriate treatment but also leads to antibiotic overuse and thus contributes to the rise of antibiotic-resistant microbes [10–12]. Because precautions are often not taken before diagnosis, delayed diagnosis of infectious diseases contributes to the spread of infections, especially viral ones [13]. Furthermore, co-infection with more than one pathogen is frequent, and differentiating between them is essential for successful disease management.

In this context, point-of-care (POC) testing has arisen as a promising, rapid, and accurate diagnostic approach in the global health field [1]. These tests have the potential to combine the accuracy and sensitivity of centralized laboratory equipment while being performed close to the patient site to allow more rapid management of clinical decisions [3, 4]. The need for POC tests for infectious disease detection in global health is well illustrated if we look back to the management of the 2014–2015 West African Ebola epidemic. Because of the nonspecific early symptoms of Ebola virus infection (e.g., fever), confirmatory diagnosis was a key epidemic control element [14–16]. The World Health Organization guidelines recommended conventional reverse transcription PCR (RT-PCR) testing for case diagnosis [14, 17]. However, the poor medical and transportation infrastructures in most Ebola-affected countries resulted in long diagnosis delays [14]. Overwhelmed by the epidemic, in November 2014, the World Health Organization announced an urgent call for rapid, sensitive, safe, and simple POC Ebola diagnostic tests.

Therefore, in the last few years, efforts have been intensified to develop POC PCR and RT-PCR devices for the identification of DNA and RNA pathogens, respectively. We recently described the development of a POC PCR system, incorporating a microfluidic chip, that allows real-time detection of pathogen DNA via fluorescence measurements [18]. This platform is based on continuous-flow PCR, passing the PCR solution cyclically between fixed-temperature zones. This design has several advantages over conventional stationary PCR. It allows faster heat transfer and, as a result, faster PCR. It also reduces the risks of cross contamination and simplifies incorporation into other systems [18–20]. Considerable attention was given to the manufacturing aspects of the system, such as the design and selection of materials, to ensure rapid and easy translation to the market. The biology was then optimized within the constraints of the initial design and materials, making this a "holistic approach" to POC device design [18].

Here, we report the development of a low-cost POC RT-PCR platform that accomplishes the detection of RNA pathogens (including emerging viral pathogens such as Zika virus and chikungunya virus). Similarl to the previously described PCR platform [18], this system is continuous flow based, allows real-time detection, and uses a novel disposable chip designed to be manufactured by large-scale roll-to-roll production methods. As a proof of concept, the system was tested for Ebola virus Zaire detection. The corresponding amplification results, as well as the system adaptations and the novel chip design, are presented.

Materials and methods

Chip design, chip manufacture, and preparation

Chips were made from Zeonex® 690R film, a thermoplastic cycloolefin polymer, purchased from Plitek (Des Plaines, IL, USA). The outside dimensions of the RT-PCR chip are 50.0 mm × 41.0 mm × 0.4 mm. Two inlet ports and one outlet port are situated in the top-middle area of the chip (Fig. 1a). Both inlet ports are connected to the outlet port through a 1952-mm-long and 50-μm-deep microfluidic channel with a volume of 25 μl. The channel is subdivided into four sections: mixing, reverse transcription, activation, and thermal cycling. The mixing section is 95 mm long and 180 μm wide, and has sharp zigzag turns to facilitate mixing of the fluids from both inlet ports. The reverse transcription section is 372 mm long and 260 μm wide, and is heated at 52 °C. The activation area is 354 mm long and 260 μm wide, and is kept at 98 °C to activate the DNA polymerase. The serpentine thermal cycling channel is 1131 mm long and 260 μm wide, and passes 40 times through heated areas at 97 °C and 62 °C (Fig. 1b).

With use of a CNC-milled aluminum mold, channel features were hot embossed into Zeonex® films (0.25-mm thick) at 170 °C and 15.73 MPa for 2 min with use of a hydraulic hot press (Fraunhofer CMI). Next, the microstructured films were chemically bonded to Zeonex® Z14 coverslips (0.185-mm thick, Zeon Chemicals, Louisville, KY, USA) by a thermally assisted solvent bonding method to seal the microfluidic channels [21]. Briefly, microstructured films and coverslips were immersed for 30 s in a solution of decahydronaphthalene–denatured ethanol (35:65 vol%). These were then rinsed in denatured ethanol (Sigma Aldrich, St Louis, MO, USA) for 2 min, and dried with pressurized air. Microstructured films and coverslips were then bonded together at 130 °C and 3.93 MPa for 2 min with use of the Fraunhofer CMI press.

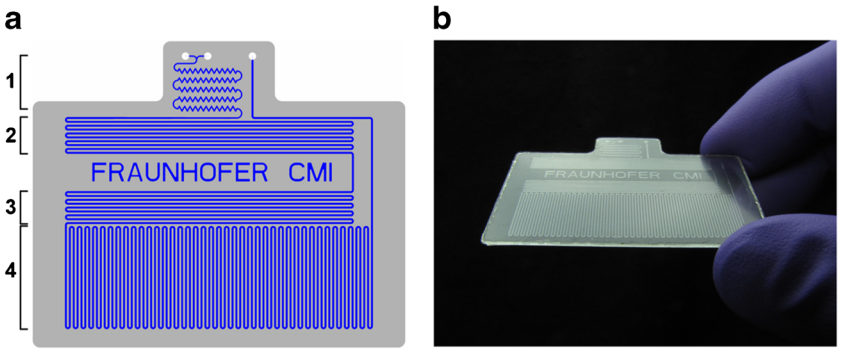

Fig. 1 **a** The reverse transcription polymerase chain reaction chip: 1 mixing zone, 2 reverse transcription zone, 3 polymerase activation zone, 4 thermal cycling zone. **b** Photograph of the thermoplastic chip

The outside border of the completed chips was then cut with a Zing 16 laser (Epilog Laser, Golden, CO, USA).

System description

The instrument prototype was based on a previously designed system for continuous-flow real-time PCR [18], but several key modifications were incorporated to allow on-chip reverse transcription. Briefly, the system includes a microfluidic pump, heaters, and an optical detection system. The microfluidic pump is used to introduce the sample along with PCR reagents into the chip through a microfluidic connector, which facilitates chip loading. Heaters below the chip, thermocouples, and programmable temperature controllers are also integrated into the system to control the chip temperature in three differently heated areas. The optical system allows detection in real time of nucleic acid amplification via fluorescence measurements. The optical system has an LED that excites the sample in the fluorescein isothiocyanate (FITC) region. Fluorescence from the sample is then collected and split into two different paths in a 30:70 ratio. In the first detection path, a camera is located for focusing purposes. In the second detection path, there is a photodetector connected to an optical power meter, which measures changes in the photodetector current. A computer connected to the power meter allows real-time fluorescence intensity data readout and collection.

Off-chip and on-chip PCR reagents and protocol

A pcDNA3.1(+) plasmid vector containing a target region of the Ebola virus L gene was designed in-house from Makona variant Ebola virus sequences (2014 Ebola outbreak) and was purchased from Genscript. The L gene was selected as the target since it codes for the well-conserved RNA-dependent RNA polymerase [22, 23]. NEB 5-alpha competent *Escherichia coli* cells purchased from New England Biolabs (Ipswich, MA, USA) were transformed with the plasmid of interest for plasmid banking and replication purposes. Bacteria containing the target plasmid were selected by exposure to the antibiotic ampicillin. The cloned L gene fragment was digested and gel purified to provide a clean template for RNA in vitro transcription using a MEGAscript in vitro transcription kit (catalog number AM1333) and a MEGAclear transcription cleanup kit (catalog number AM1908), respectively (Thermo Fisher Scientific, Waltham, MA, USA). The primers/probes were designed to amplify a 120-bp-long segment of the L RNA gene and were custom-made by Integrated DNA Technologies (Coralville, IA, USA). The forward primer was 5'-GTCCGTCGTTCCAGTCATTT-3' and the reverse primer was 5'-CCCTCTTGGATGCTGAGTTA TG-3'. The fluorogenic probe (5'-TAAGTGACTCTGCT TGCGGTACAGC-3') was labeled at the 5' end with the reporter dye 5(6)-carboxyfluorescein (FAM), with an internal ZEN™ quencher, and with an Iowa Black FQ quencher at the 3' end.

Two one-step quantitative reverse transcription PCR (qRT-PCR) master mixes were tested. First, the commercial VeriQuest™ probe one-step qRT-PCR master mix (product number 75700) was used (Affymetrix, Santa Clara, CA, USA). It is composed of a reverse transcriptase and a standard DNA polymerase, and is referred to here as the "commercial" master mix. Second, a custom-made one-step master mix that combines the reverse transcriptase from the Affymetrix VeriQuest™ probe one-step qRT-PCR master mix and the fast DNA polymerase included in the Affymetrix USB® VeriQuest™ fast quantitative PCR (qPCR) probe master mix (product number 75680) was kindly provided by Affymetrix (Santa Clara, CA, USA), and is called the "custom" master mix. Independent of the master mix used, bovine serum albumin (Thermo Fisher Scientific), primers, and probe were added to the master mix such that their final concentrations, following addition of RNA, were 0.05% (*w*/*v*), 500 nM, and 250 nM, respectively.

Off-chip PCR was conducted before on-chip PCR to verify reagent performance. An Applied Biosystems 7500 PCR system was used for off-chip real-time qRT-PCR (Thermo Fisher Scientific). Different off-chip thermal profile protocols were followed, and they are summarized in Table 1. For both master mixes, the slow protocol corresponds to the protocol recommended by the manufacturer.

The operational protocol of the system for on-chip RT-PCR is similar to our previously described method [18]. Briefly, on-chip RT-PCR was conducted with 70 μl of RNA sample and PCR reagents, which were mixed, introduced into the inlet

Table 1 Thermal profile protocol for off-chip reverse transcription (RT) polymerase chain reaction (PCR)

	RT	Polymerase activation	Thermal cycling	
Commercial 1-step RT-PCR mix				
Slow	15 min, 50 °C	10 min, 95 °C	15 s, 95 °C	30 s, 60 °C
Mid	5 min, 50 °C	10 min, 95 °C	15 s, 95 °C	30 s, 60 °C
Rapid	5 min, 50 °C	5 min, 95 °C	3 s, 95 °C	30 s, 60 °C
Custom 1-step RT-PCR mix				
Slow	15 min, 50 °C	5 min, 95 °C	3 s, 95 °C	30 s, 60 °C
Rapid	5 min, 50 °C	5 min, 95 °C	3 s, 95 °C	30 s, 60 °C

tubing, and pumped into the chip. During the course of the RT-PCR, the flow velocity was regularly determined by measuring the time it takes for an air bubble or fluid front to travel a known distance. The residence time inside the chip was calculated from flow velocities. Optical measurements were taken for each even-numbered channel. The camera was positioned over the desired channel and focused, and 50 fluorescence measurements were taken in a 5-s time frame. Fluorescence data were then adjusted to a sigmoidal dose response (variable slope). Error bars in all graphs show the standard deviation of 50 measurements taken during a single experiment. The intersection between each curve fit and a threshold line (established as the average baseline signal of channels 4–16 plus two times its standard deviation) was used to determine the corresponding cycle thresholds (C_T).

The on-chip and off-chip RT-PCR amplicons were analyzed by gel electrophoresis, with use of 6% tris(hydroxymethyl)aminomethane–boric acid–EDTA (TBE) gels and a low molecular weight DNA ladder (New England Biolabs). Nucleic acids were visualized with SYBR® Green I nucleic acid stain (Lonza Group, Basel, Switzerland).

Results and discussion

System design for industrial-scale manufacturing

We present the development of a low-cost POC qRT-PCR system for the detection of RNA viruses. The system was upgraded from a qPCR platform for DNA pathogen detection that we recently described [18]. The main components of the qPCR and qRT-PCR systems include a disposable chip and the instrumentation that surrounds it. The surrounding instrumentation was further improved, as described in "Materials and methods," and a novel RT-PCR chip was designed.

The RT-PCR chip incorporates four areas with different functionalities. First, the RT-PCR master mix and the RNA sample traverse a zigzag mixer. The reagents then travel through a 50–57 °C heated area, where reverse transcription occurs. Next, the newly formed complementary DNA (cDNA) and the reagents travel through a 95 °C zone, where the DNA polymerase is activated (i.e., the DNA polymerase's active site becomes unblocked) and DNA double strands are melted. Afterward, the DNA is amplified as the reagents go through 40 repetitive cycles between areas heated to 95 and 62 °C. Annealing, DNA synthesis, and denaturation occur during each cycle. The percentages of the length of the denaturation, DNA synthesis, and annealing zones in each cycle are 43%, 22%, and 35%, respectively. As the velocity of the fluid in each zone is different, the real time the liquid spends in each zone cannot be estimated with certainty from these ratios.

The total chip channel volume is merely 25 μl, which allows low reagent consumption and consequently reduced consumable costs. In the same vein, the fact that no static passivation but only dynamic passivation with bovine serum albumin is required to avoid nonspecific binding of the PCR reagents to inner channel walls also aids to reduce the production costs and time, and facilitates the overall chip manufacturing process.

The chips were made of Zeonex®, a thermoplastic material with low autofluorescence and high glass transition temperature, transparency, biocompatibility, and flexibility. Additionally, an advantage of thermoplastic materials is the low fabrication costs compared with other materials commonly used for chip production, such as silicone glass, elastomers, hydrogels, or thermosets [24]. The chips were designed to be produced by roll-to-roll UV embossing, an economic production method that can be easily implemented industrially on a large scale. To be compatible with roll-to-roll UV embossing, the depth of the chip features was a mere 50 μm, ensuring high replicability and imprinting accuracy when translated to industrial-scale production [25, 26].

Pathogen detection

The function of the chip was assessed with Ebola virus. An in vitro transcribed fragment of the L gene, one of the best conserved and most genetically stable Ebola virus genes, was targeted [22, 23]. The L gene codes for the RNA-dependent RNA polymerase, which is highly conserved in RNA viruses with no DNA stage [23]. Assays targeting well-conserved genomic regions are more likely to be valid for different outbreaks with evolved Ebola virus variants. As previously stated, because of the nonspecific early symptoms of Ebola, which may be common to diseases endemic in the same areas

affected by the outbreak (e.g., malaria, Lassa fever, typhus), rapid confirmatory diagnosis of Ebola virus infection is crucial to minimize spread of the infection [27].

Two one-step master mixes, a commercial mix and a custom mix, were tested at various flow rates. Both contain the same reverse transcriptase but different DNA polymerases. To determine the sensitivity of each master mix and the influence of the flow rate on performance, off-chip and on-chip experiments were performed. In the case of the off-chip experiments, the thermal profile protocol recommended by the manufacturer was modified and performed more rapidly. For both master mixes, the protocol recommended by the manufacturer is referred to as "slow" (see Table 1). The "rapid" protocol was selected on the basis of the protocol recommended for the Affymetrix USB® VeriQuest™ fast qPCR probe master mix, which contains the DNA polymerase used in the custom mix (see Table 1). Additionally, for the commercial mix, a "mid"-speed protocol was performed where only the reverse transcription time was varied with respect to the slow protocol. Figure 2 shows the performance of each master mix both on-chip and off-chip with different thermal profile protocols for the same initial template concentrations (10^5 and 10^2 RNA copies per microliter).

From the results shown in Fig. 2a, the commercial master mix does not lose efficacy by reducing the revere transcription time from 15 to 5 min (from the recommended slow protocol to the mid-speed protocol). However, reducing the thermal cycling time increases C_T values from 24.7 and 33.2 (slow protocol) to 30.3 and undetermined (rapid protocol) for 10^5 and 10^2 RNA copies per microliter, respectively. This suggests that the standard DNA polymerase contained in the mix does not achieve full amplification of the cDNA target at such a high thermal cycling speed. In the case of the custom mix, the slow and rapid protocols are equally effective (Fig. 2b). The C_T values are the same as those obtained with the commercial mix with use of the slow and mid-speed protocols. These results indicate that the fast DNA polymerase functions well at rapid thermal cycling speeds. Overall, the custom mix allows faster RT-PCR runs than the commercial mix for the same sensitivity. Gel electrophoresis results (Fig. 2c) confirm the optical data shown in Fig. 2a and b.

Next, on-chip experiments were performed by varying the residence time inside the chip. The same initial template concentration, 10^6 RNA copies per microliter, was used for all the residence time experiments. The optical data for the commercial mix and the custom mix are presented in Fig. 3a and b, respectively. Figure 3c shows gel electrophoresis results for the on-chip qRT-PCR amplicons.

From comparison of the amplification curves for a residence time of 50 min with the two master mixes, the C_T values for the commercial and custom mixes are identical. When the residence time is decreased to 40 min, C_T for the custom mix remains the same; however, in the case of the commercial mix,

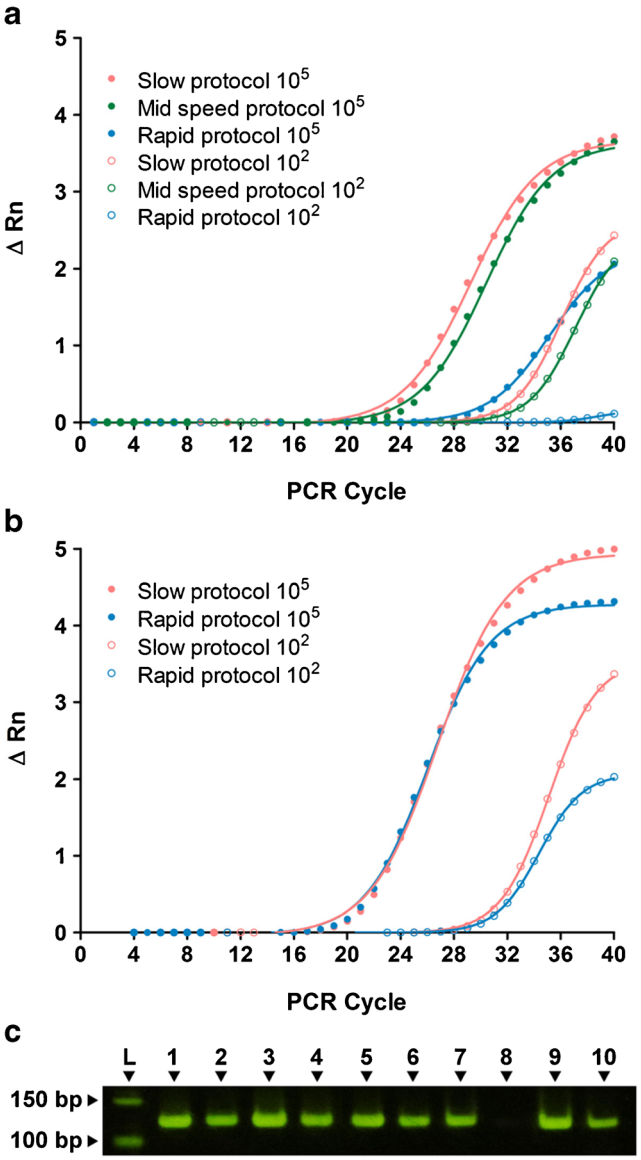

Fig. 2 Ebola virus off-chip quantitative reverse transcription polymerase chain reaction (qRT-PCR) results. **a** Off-chip qRT-PCR optical detection data obtained with the commercial one-step qRT-PCR master mix. Data were normalized by the internal thermocycler software. Template concentrations were 10^5 and 10^2 RNA copies per microliter. Slow, mid-speed, and rapid thermal profile protocols were performed (see Table 1 for information on the protocols). **b** Off-chip qRT-PCR optical detection data obtained with the custom one-step qRT-PCR master mix. Data were normalized by the internal thermocycler software. Template concentrations were 10^5 and 10^2 RNA copies per microliter. Slow and rapid thermal profile protocols were performed (see Table 1 for information on the protocols). **c** Gel electrophoresis of off-chip amplicons (120 bp). Amplicons in lane 1 (slow, 10^5 RNA copies per microliter), lane 2 (slow, 10^2 RNA copies per microliter), lane 5 (mid speed, 10^5 RNA copies per microliter), lane 6 (mid speed, 10^2 RNA copies per microliter), lane 7 (rapid, 10^5 RNA copies per microliter), and lane 8 (rapid, 10^2 RNA copies per microliter) correspond to off-chip qRT-PCR runs presented in **a** with the commercial mix. Amplicons in lane 3 (slow, 10^5 RNA copies per microliter), lane 4 (slow, 10^2 RNA copies per microliter), lane 9 (rapid, 10^5 RNA copies per microliter), and lane 10 (rapid, 10^2 RNA copies per microliter) correspond to off-chip qRT-PCR runs presented in **b** with the custom mix

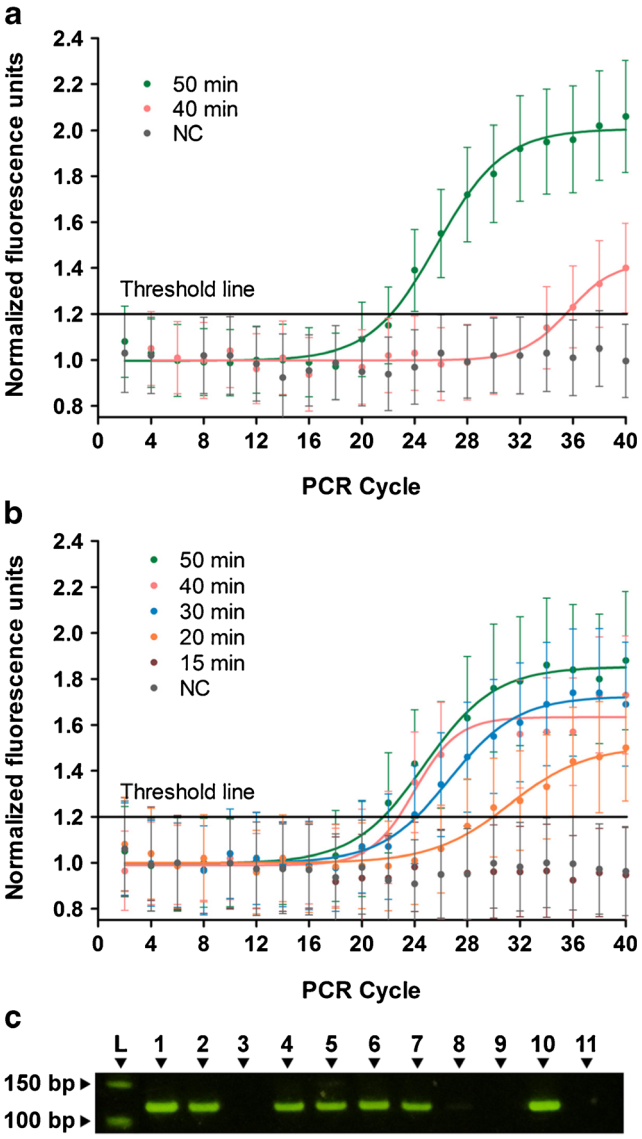

Fig. 3 Ebola virus on-chip quantitative reverse transcription polymerase chain reaction (qRT-PCR) results with an initial template concentration of 10^6 RNA copies per microliter. Each data curve was normalized with its average baseline from cycles 4–16. **a** On-chip qRT-PCR optical detection data for different on-chip residence times obtained with the commercial one-step qRT-PCR master mix. Independent experiments were run with total on-chip residence times of 40 and 50 min, as indicated. Additionally, a negative control (zero RNA copies per microliter) with a residence time of 50 min was run. **b** On-chip qRT-PCR optical detection data for different on-chip residence times obtained with the custom one-step qRT-PCR master mix. Independent experiments were run with total on-chip residence times of 15, 20, 30, 40, and 50 min, as indicated. Additionally, a negative control (zero RNA copies per microliter) with a residence time of 50 min was run. **c** Gel electrophoresis of on-chip amplicons (120 bp). Amplicons in lane 1 (50 min), lane 2 (40 min), and lane 3 (negative control) correspond to on-chip RT-PCR runs presented in **a** with the commercial master mix. Amplicons in lane 4 (50 min), lane 5 (40 min), lane 6 (30 min), lane 7 (20 min), lane 8 (15 min), and lane 9 (negative control) correspond to on-chip RT-PCR runs presented in **b** with the custom master mix. Off-chip amplicons from RT-PCR runs for 80 min with 10^4 RNA copies per microliter (lane 10) and zero RNA copies per microliter (lane 11) are shown for comparison. NC negative control

C_T increases considerably. For the custom mix at residence times of 50 and 40 min, the DNA polymerase performs a full amplification of the cDNA created by the reverse transcriptase. However, for the commercial mix, the C_T shift can be attributed to insufficient time for the DNA polymerase to achieve full amplification (given that the reverse transcriptase is the same in both mixes). Thus, for the commercial mix, probably the best tradeoff between rapidity and sensitivity is a residence time of 50 min. These results confirm our off-chip results: the commercial mix is unable to perform as well as the custom mix in the context of rapid thermal cycling.

From the off-chip and on-chip results, for our application the most promising enzymatic combination appeared to be the custom master mix. To further explore the maximum speed at which amplification occurs and to determine the optimal flow rate for the custom mix, additional experiments were performed with shorter residence times. The C_T values for the 50- to 30-min residence time curves are very similar (Fig. 3b); at a residence time of 20 min, C_T shifts to the right by approximately six cycles. With a residence time of only 15 min, no amplification was optically detected on-chip. Thus, in the case of the custom mix, a residence time of 30 min is probably the best compromise for sensitivity and speed. The gel electrophoresis results (Fig. 3c) confirm the optical data.

Although the custom mix is the most promising master mix, we performed tests to determine the level of detection of the assay using the commercial mix, which could be more quickly translated to the market. Limit of detection (LOD) experiments were conducted with a residence time of 50 min to achieve full polymerization of all targets. These experiments were done with Ebola virus RNA serially diluted by factors of 10 ranging from 10^6 RNA copies per microliter to one RNA copy per microliter. On-chip qRT-PCR results are presented in Fig. 4a.

The LOD for our on-chip qRT-PCR system obtained with the commercial mix and Ebola virus as the target is 10 RNA copies per microliter. For low template concentrations, such as 100 RNA copies per microliter and especially 10 RNA copies per microliter, fluctuations in the optical signal were observed because of stochastic sampling. The LOD for the 80-min off-chip qRT-PCR performed with a commercial thermal cycler was also 10 RNA copies per microliter. It is remarkable that our on-chip qRT-PCR was performed faster than off-chip qRT-PCR. Additionally, the optical measurements on-chip were taken from only approximately 50 nl—the volume of approximately one sixth of one channel loop—compared with the 20 μl used off-chip with commercial benchtop thermocyclers. The PCR efficiency is 99 ± 6% as shown in Fig. 4b. This PCR efficiency is comparable to the efficiencies obtained with off-chip commercial thermocyclers; a 3.3 threshold cycle shift is observed for 10-fold dilutions of the initial template. For comparison, off-chip LOD results with

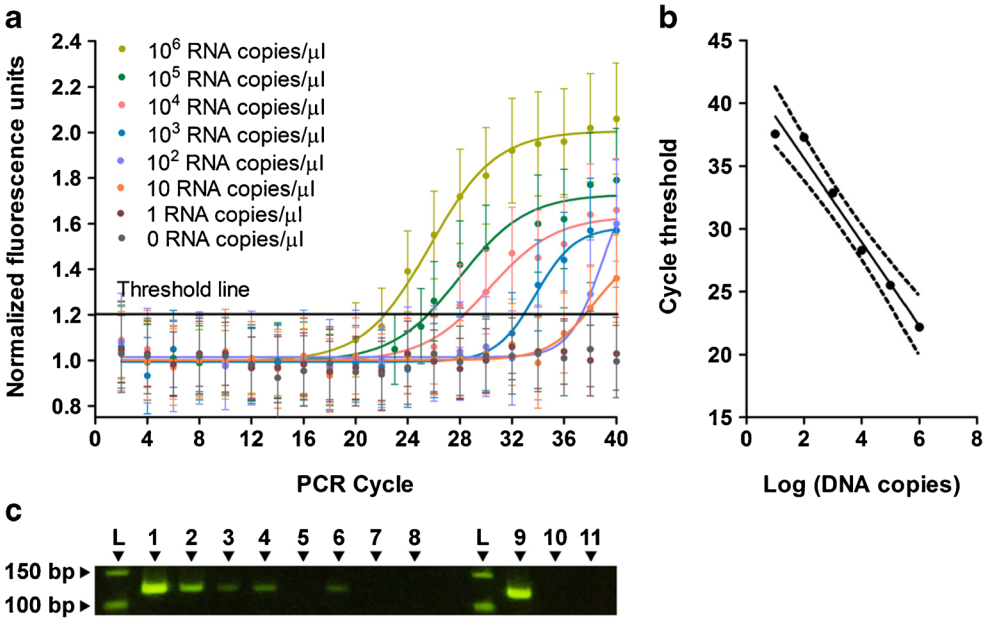

Fig. 4 On-chip quantitative reverse transcription polymerase chain reaction (qRT-PCR) of Ebola virus performed with the commercial one-step qRT-PCR master mix with a residence time of 50 min. **a** On-chip qRT-PCR optical detection data for the serially diluted RNA template. Each data curve was normalized with its average baseline from cycles 4–16. Template concentrations began at 10^6 RNA copies per microliter and were reduced by one order of magnitude until the final concentration of one RNA copy per microliter. Also included is the negative control (zero RNA copies per microliter). **b** On-chip qRT-PCR efficiency graph representing the cycle threshold versus the initial template concentrations on a logarithmic scale. Dashed lines represent the 95% confidence interval. The slope is 3.34 ± 0.28, which corresponds to a PCR efficiency of 99 ± 6%. **c** Gel electrophoresis of on-chip amplicons (120 bp) with various levels of the template as shown in **a**. Lane 1 has an initial template concentration of 10^6 RNA copies per microliter, and the template concentrations were reduced by one order of magnitude until the final concentration of 1 RNA copy per microliter in lane 7. The negative control (0 RNA copies per microliter) is shown in lane 8. Off-chip qRT-PCR results for a residence time of 80 min are shown in lane 9 (10^4 RNA copies per microliter), lane 10 (0 RNA copies per microliter), and lane 11 (10^4 RNA copies per microliter and master mix without reverse transcriptase) for comparison

the same master mix are presented in Fig. S1. Gel electrophoresis of on-chip amplicons confirms the results observed optically. For 100 RNA copies per microliter, no band can be distinguished by gel electrophoresis (Fig. 4c, lane 5), although amplification was optically measured; the amplicons obtained are probably under the LOD of the gel electrophoresis technique. For 10 RNA copies per microliter (Fig. 4c, lane 6), a faint band can be detected, confirming the LOD measured optically on-chip with a residence time of 50 min with the commercial master mix.

Given the equivalent sensitivity obtained for both the commercial mix and the custom mix with residence times of 50 and 30 min, respectively, we believe that a LOD of 10 RNA copies per microliter is likely to be achieved with a residence time of 30 min with the custom master mix.

The next steps in the development of our device will focus on sample pretreatment for RNA extraction from plasma. The extraction of high-quality RNA plays a critical role in obtaining successful RT-PCR results. Including a sample pretreatment step (either on-chip [28] or off-chip [29]) would facilitate our device's use at the POC. Additionally, we are working toward making the system more compact by reducing the instrumentation surrounding the chip.

Comparison with other RT-PCR devices

We have presented a POC real-time, continuous-flow RT-PCR system for RNA-based virus detection. Our system performs qRT-PCR faster than conventional benchtop thermocyclers (30 min vs 80 min), with the same sensitivity (10 RNA copies per microliter) and efficiencies between 90% and 110%.

To the best of our knowledge, no continuous-flow RT-PCR systems that use conventional microfluidics and that allow real-time detection have been reported in the literature. On the one hand, some reported real-time RT-PCR systems use digital microfluidic technology that, through modifications in the electrical field, transports droplets with PCR reagents through different stations of a chip [30, 31]. These assays work very similarly to traditional benchtop thermocyclers but with smaller volumes; they differ considerably from conventional microfluidics [31, 32]. On the other hand, several platforms were reported that rely on conventional microfluidics to perform RT-PCR but present end-point detection either online [33–35] or off-line [36, 37]. Few of these platforms report the sensitivity and maximum flow rate at which amplification is still observed. Li et al. [33] reported a sensitivity of 6.4×10^4 copies per microliter achieved in

approximately 1 h with RT-PCR; Yamanaka et al. [35] stated a LOD of 5.36×10^3 copies per microliter obtained in 15 min. Other reported RT-PCR speeds are 100–120 min [36] and 73 min [37] (calculated from the published data). To the best of our knowledge, the continuous-flow RT-PCR system based on conventional microfluidics reported by Yamanaka et al. [35] is the only one with faster amplification than ours; however, the sensitivity reported is lower and, importantly, only end-point detection was performed. Additionally, we believe that this is the first time that the PCR efficiency has been reported for continuous-flow RT-PCR.

It is also worth mentioning the emerging isothermal techniques for RNA amplification. In contrast to PCR, isothermal systems do not require thermal cycling, reducing the costs of their designs, usually based on a static chamber format [38]. There are a wide variety of isothermal techniques that differ in terms of complexity, reaction speed (usually from 30 to 50 min) [39], sensitivity, specificity, incubation temperatures, etc., making comparison with PCR challenging [40]. However, the main drawback of isothermal techniques compared with PCR is that they are not quantitative, which limits their use in applications that require quantification of the target such as in viral load determinations [41]. Additionally, isothermal techniques require complex assay optimization (difficult primer design and buffer optimization, requirement of multiple enzymes, etc.) [42].

With respect to chip characteristics, our chip has the lowest chip feature depth (50 μm) and thickness (410 μm) compared with other chip designs in the literature [34–37]. The depth of the chip was selected on the basis of the requirements for roll-to-roll UV embossing manufacturing to ensure easy translation to the industrial scale [18]. The low feature depth also means that there is flexibility in the design for alternative manufacturing methods. On the road to roll-to-roll processing, where production equipment can produce 17,000 chips per hour, it is beneficial to test smaller production volumes first. Injection molding of the RT-PCR chip can produce approximately 200 chips per hour, which easily provides enough cartridges for field and clinical testing. We generated milled injection molds (Fig. 5) and increased the thickness of the chip to 600 μm to reduce the risk of defects caused by injection forces. All other feature dimensions remained the same. A flexible holistic design throughout each chip optimization phase allows maximum portability between multiple manufacturing methods and therefore increases the probability of bringing the device to market.

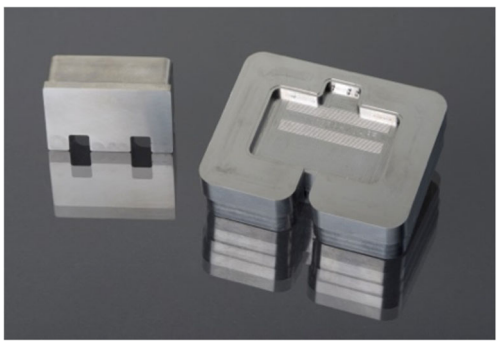

Fig. 5 Injection molds fabricated from steel for the high-volume manufacture of our reverse transcription polymerase chain reaction chips

With regard to the devices currently available on the market, the number of suppliers of portable, real-time RT-PCR devices is increasing. The features, prices (ranging from $3000 to $25,000), and time to result (most of the assays require 40–60 min) differ between suppliers. Some examples of these devices are cobas® Liat (Roche), genesig q16 (PrimerDesign), and GeneXpertOmni (Cepheid). To the best of our knowledge, all marketed qRT-PCR devices are based on a static chamber design. After the 2014–2015 Ebola outbreak, numerous suppliers (including the previously mentioned ones) developed assays for Ebola virus detection. Some examples of commercialized assays are FilmArray Biothreat-E (Biomerieux), RealStar® Filovirus Screen RT-PCR kit 1.0 (Altona Diagnostics), and DoD EZ1 real-time RT-PCR assay (US Department of Defense) [27]. The time to result for most of the commercialized Ebola virus assays is 40–60 min. The genes targeted in these assays encode RNA-dependent RNA polymerase (L), nucleoprotein (NP), glycoprotein (GP), and matrix protein (VP24), the L gene being one of the best genetically conserved and the most likely to be valid for future outbreaks [22, 23, 27, 43–45]. Blood plasma is the most common sample used. Typical Ebola viral loads in blood for acutely ill infected patients range between 10^3 and 10^6 RNA copies per microliter and then decrease during the recovery phase to approximately one RNA copy per microliter [46, 47], which is the lowest reported LOD of viral RNA in plasma on RT-PCR assays [47]. Our qRT-PCR device, with a LOD of 10 RNA copies per microliter, can effectively monitor nearly the entire course of infection. Most suppliers of Ebola virus assays reported sensitivities ranging from 10 to 10^3 copies per test.

We believe that our continuous-flow device, with its high sensitivity, PCR efficiency, low price (we anticipate a price of $2000 [18] for the instrument with a disposable chip cost of $0.5), portability, and rapidity is in a good position to enter this competitive market. It would allow not only diagnosis but also monitoring of the course of the disease. Additionally, the fact that our platform is versatile for other analytes would facilitate its adaptation to the diagnosis of new RNA viral threats such as Zika virus or chikungunya virus.

Conclusions

We have presented a quantitative and continuous-flow RT-PCR system for RNA-based virus detection. We believe this is the first reported quantitative and continuous-flow RT-PCR system that uses conventional microfluidics. The system was designed around a disposable and low-cost microfluidic chip designed to be compatible with roll-to-roll UV embossing appropriate for large-scale industrial production.

As a proof of concept, the performance of our system was tested with Ebola virus. Two one-step master mixes with different DNA polymerases were tested, and the LOD was determined. Our system performs qRT-PCR faster than conventional benchtop thermocyclers but with the same sensitivity (10 RNA copies per microliter) and efficiency (90–110%). Depending on the master mix used, amplification with high sensitivity was achieved in 30–50 min. Faster amplifications were possible (20 min), but sensitivity was reduced. Thus, our system achieves faster amplification than most commercial qRT-PCR platforms and commercialized Ebola virus detection assays, which typically require 40–60 min.

These successful results with Ebola virus make our system promising for use with other RNA viruses such as Zika virus or chikungunya virus. The portability, versatility, rapidity, sensitivity, and low cost of our POC diagnostic device make it ideal for remote settings with low medical infrastructure and for outbreak control in which rapid detection of the causative pathogen is key to avoiding spread of the infection.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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