Video Article

Microfluidic Chip Fabrication and Method to Detect Influenza

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Abstract

Fast and effective diagnostics play an important role in controlling infectious disease by enabling effective patient management and treatment. Here, we present an integrated microfluidic thermoplastic chip with the ability to amplify influenza A virus in patient nasopharyngeal (NP) swabs and aspirates. Upon loading the patient sample, the microfluidic device sequentially carries out on-chip cell lysis, RNA purification and concentration steps within the solid phase extraction (SPE), reverse transcription (RT) and polymerase chain reaction (PCR) in RT-PCR chambers, respectively. End-point detection is performed using an off-chip Bioanalyzer (Agilent Technologies, Santa Clara, CA). For peripherals, we used a single syringe pump to drive reagent and samples, while two thin film heaters were used as the heat sources for the RT and PCR chambers. The chip is designed to be single layer and suitable for high throughput manufacturing to reduce the fabrication time and cost. The microfluidic chip provides a platform to analyze a wide variety of virus and bacteria, limited only by changes in reagent design needed to detect new pathogens of interest.

Video Link

The video component of this article can be found at https://www.jove.com/video/50325/

Introduction

Millions of deaths have been reported during the three influenza pandemics of the 20th century[1]. Moreover, the most recent influenza pandemic was declared by World Health Organization (WHO) [2] in 2009, and as of August 1, 2010, 18,449 deaths were reported by WHO[3]. This pandemic demonstrated again the high burden of infectious disease, and the need for rapid and accurate detection of influenza to enable fast disease confirmation, appropriate public health response and effective treatment[4].

There are several methods widely used for diagnosing influenza, these include rapid immunoassays, direct fluorescent antigen testing (DFA) and viral culture methods. Rapid immunoassays dramatically lack sensitivity[5-8], while the other two methods are labor-intensive and time consuming[9]. Molecular tests offer multiple advantages including a short turn-around time, high sensitivity, and higher specificity. Several commercial entities have been working towards fast molecular tests (also called nucleic acid tests or NATs) for infectious diseases, and several have influenza assays in their pipelines. However most of them require off-chip sample preparation. None of the Clinical Laboratory Improvement Amendments (CLIA) waived molecular tests incorporate sample preparation into the assay cartridge or module.

Lab-on-a-chip technology plays an important role in the development of point-of-care testing devices. After the introduction of the first PCR chip in 1993[10], numerous efforts have been put into developing nucleic acid test chips. However, only a few of these have integrated crude sample preparation with downstream amplification.

We have previously demonstrated the miniaturization of a solid phase extraction column (SPE) into a plastic microfluidic chip[11] and the development and optimization of a continuous flow PCR chip[12]. Here, we extend the previous work to integrate the SPE with RT and PCR steps into a single chip for clinical diagnostics and demonstrate its capability to amplify nucleic acids from patient nasopharyngeal (NP) swabs and aspirates.

Protocol

1. Chip Fabrication[12]

1. Make two plaques from Zeonex 690R pellets: distribute 8-9 grams Zeonex pellets evenly in the center of a metal plate, preheat on the heated press at 198 °C for 5 min, and then apply pressure slowly to 2,500 psi for another 5 min. To complete this step, we used a Carver hot press.

2. Emboss the microfluidic channel in the plaque with an epoxy mold. Details on the mold fabrication are outlined elsewhere[12] (channel design in **Figure 2b**): put one plaque onto the epoxy mold; preheat them on the heated press at 157 °C for 10 min and then apply pressure slowly to 1,000 psi for another 10 min. Remove the plaque from the mold by hand or using a tongs before it cools down. (**Note:** wear thermal gloves during this step.)
3. Drill holes in the embossed chip at the inlet, waste port, and the outlet of the microfluidic channel. We used a 1.25 mm diameter drill bit to make the three holes.
4. Bond the embossed chip with another plaque (on top): Wash the chips with IPA, RNAse Away and deionized water in sequence; Air dry and preheat them on the heated press at 131 °C for 10 min and then press at 350 psi for another 10 min.
5. Attach Nanoports at the inlet, waste, and the outlet ports separately using J-B Weld Epoxy; cure for 15 to 24 hr as per the manufacturer's instructions.
6. Rinse the SPE channel with 50 µl RNAse Away, followed with 100 µl nuclease free water.
7. Load the SPE channel with 4 µl of the grafting solution (methyl methacrylate with 3% w/v benzophenone) and then crosslink the methacrylate by incubating for 10 min in a UV oven under 365 nm UV wavelength and 2,000 mJ/cm^2. Remove the residual grafting solution with vacuum. We used the wall vacuum. For the best results, a fresh solution should be used. It is usually acceptable, however, to store a prepared solution at 4 °C for up to one week.
8. Make the SPE column solution (all v/v %): 16% ethylene dimethacrylate, 24% butyl methacrylate, 42% 1-dodecanol, 18% cyclohexanol, and add 1% 2-dimethylamino-4-(methyl-phenylamino)-phenol as the photo-initiator.
9. Take out the same volume of silica microspheres solution as the SPE column solution (to make a 1:1 mixture), and completely dry it at 70 °C overnight in a vacuum oven. Break up the pellet by tapping on the tube with the lid closed. Add the SPE column solution into the dried silica microsphere tube and vortex to mix.
10. Load 4 µl of the SPE solution into the same channel and then crosslink it by UV irradiation for 2.5 min. Then, flip the chip over and irradiate for another 2.5 min. The polymerized SPE column should be an opaque white solid.
11. Wash the channel with 500 µl of 100% methanol to rinse away excess reactants. This wash also removes intra-polymer porogenic solvents, thereby creating the open pore structure of the SPE column when dried in ambient conditions.
12. Attach two thin-film heaters to the bottom of the chip with thermally conductive tape. The sides of the heaters need to be aligned with the edge of the wide channels for denaturation and annealing separately (see **Figure 1**).
13. Place five thermocouples into the chip through the dead ended open side channels of each chip (see **Figure 2b**). The thermocouples stay in these channels via press fit. Tape was used to further fix the location of thermocouples.

2. Solid Phase Extraction Method

1. Prepare 96 µl of 70% ethanol with DEPC-treated and autoclaved water (deionized or Millipore-filtered).
2. Add 4 µl of 25X RNA Secure to 96 µl of 70% ethanol in one tube, and duplicate the same mixture with 100% ethanol in a separate tube.
3. Prepare 300 µl of channel buffer by mixing the following: 75 µl of 6 M guanidine thiocyanate (GuSCN),150 µl of 2-propanol, 63 µl of nuclease free water, and 12 µl of 25X RNA Secure.
4. Prepare 312 µl of lysis buffer by mixing: 100 µl of 6 M GuSCN, 200 µl of 2-propanol, and 12 µl 25X RNA Secure.
5. Heat the 70% ethanol, 100% ethanol, channel buffer and lysis buffers at 60 °C for 10-20 min to activate the RNA Secure.
6. To equilibrate the microfluidic channel, run the channel buffer through the SPE channel at a flow rate of 0.8 ml/hr.
7. Quick-thaw the test sample in VTM at 37 °C in water bath, and remove the sample immediately from water bath as soon as it is thawed.
8. Centrifuge sample at 13,000 rpm for 10 min, and transfer 100 µl of the supernatant into 300 µl of the lysis buffer. Complete the mixture by adding 6 µl of 1 µg/µl carrier RNA.
9. Vortex to mix, and after spin for 5 sec, load the entire lysate into a Luer-lok 1 cc syringe.
10. Run lysate through the SPE channel at a flow rate of 0.8 ml/hr .
11. Wash the SPE channel with 100 µl of 70% ethanol , followed by 100 µl of100% ethanol at 1 ml/hr. (There is a 50 µl dead volume in the syringe tip that will not get pushed out, so actually only 50 µl is going through the channel.)
12. Position an empty syringe at the 0.5 ml mark and push air through the channel to dry it at 1 ml/hr.
13. Run 67.5 µl of nuclease-free water through the channel to elute the bound nucleic acids at 0.5 ml/hr, and collect 13.5 µl from the nanoport at the waste port. (The fluid loss is due to the 54 µl dead volume in the syringe tip and the SPE channel.)

3. RT-PCR

1. Prepare 36.5 µl of the RT-PCR master mix during the air dry step (2.12) using the reagents in a Qiagen OneStep RT-PCR kits.
2. Load RT-PCR reagent in the nanoport at the waste port, and mix with RNA to get the following 50 µl RT-PCR reaction: 13.5 µl eluted RNA in nuclease free water, 4 µl RT-PCR enzyme mix, 10 µl Q solution, 10 µl1X one step buffer, 2 µl 25 mM $MgCl_2$, 1 µl 50 µM forward primer 5'-GAC CRA TCC TGT CAC CTC TGA C-3', 1 µl 50 µM reverse primer 5'-AGG GCA TTY TGG ACA AAK CGT CTA-3', 2 µl dNTP, 0.75 µl 1.0% w/v BSA, 0.73 µl PEG8000 and 5.02 µl nuclease free water.
3. Load the RT-PCR mixture into the RT channel by gentle vacuum (we used the wall vacuum) and seal the Nanoport with a closed fitting.
4. Apply 35 V to heater 1. Keep the reagents in the RT channel for 30 min after the heater 1 equilibrates at 50 °C. Keep the reagents in the RT channel for 15 min after the heater 1 equilibrates at 95 °C.
5. Apply 27 V to heater 1 and 50 V to heater 2, wait about three minutes or until heater 1 equilibrates at 60 °C and heater 2 equilibrates at 95 °C.
6. Push the reagents into the PCR channel at 0.5 µl /min. It should take about 20 min for the reagents to flow through the serpentine channel.
7. As the sample makes it to the end of the serpentine channel, collect the PCR products at the outlet using an appropriately sized pipettor and tip.

4. PCR Products Detection

1. We use an Agilent High Sensitivity DNA test to detect the products. To run this test take out the Agilent High Sensitivity DNA Kit from refrigerator 30 min before testing.
2. Turn on the Bioanalyzer and open the 2100 Expert software.
3. Load the 1 µl of the PCR products into the testing and follow the Agilent protocol (http://gcf.pbrc.edu/docs/Agilent/Agilent%20Manual.pdf).
4. Analyze and record the data.

Representative Results

A typical result is shown in **Figure 3** for an influenza A infected nasopharyngeal wash specimen. Due to the different amounts of influenza virus in each patient specimen, the final concentration of PCR product will vary. A good result should have low noise, two clear ladder peaks (35 and 10380 bp) and a single product peak at the designed product size (107 bp) for the positive sample. While the product peak should theoretically be absent for negative controls, we did observe spurious PCR peaks near the flu-specific locus from primer-dimers formation in some samples. Multiple product peaks reflect potential contamination of the test. If this occurs, another aliquot of the sample should be retested. Gel electrophoresis can be used for further verification of the test results[13].

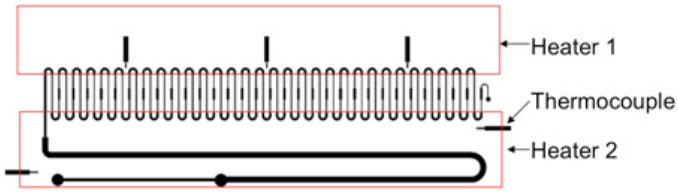

Figure 1. Simplified top down chip schematic. The red outlines show the placement of the contact heaters on the bottom of the chip.

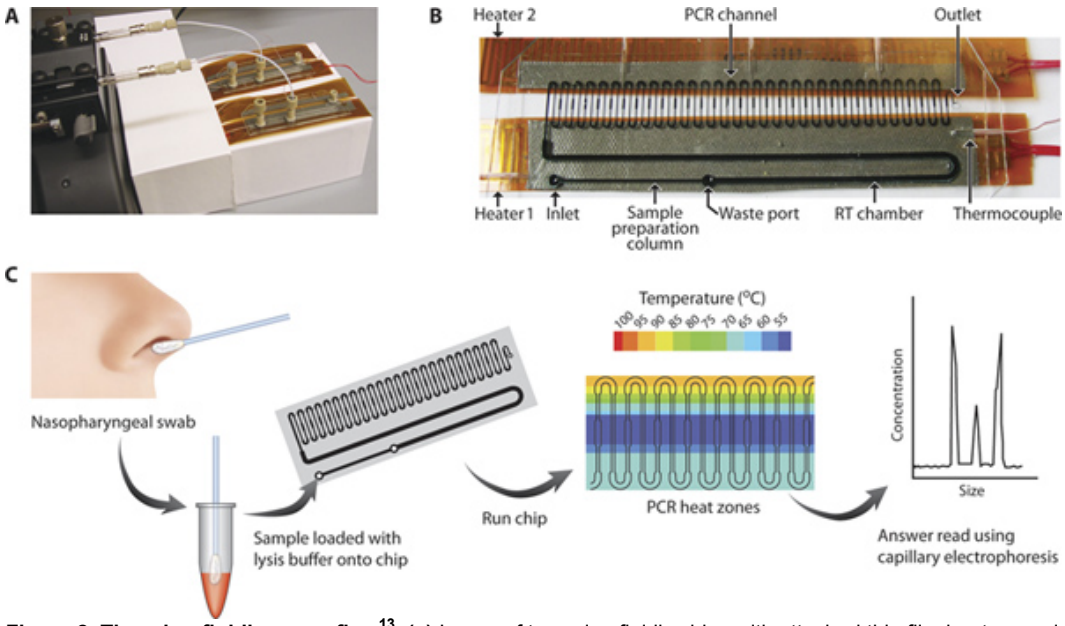

Figure 2. The microfluidic assay flow[13]. (a) Image of two microfluidic chips with attached thin film heaters and two-barrel syringe pump. Glass syringes were connected to each chip with flexible tubing to load reagents and samples. Three ports were glued at the inlets of SPE channel and the waste port, and the outlet of the PCR channel. **(b)** Channel design with three sections: sample preparation (SPE), RT chamber and continuous flow PCR channel. Two fixed resistance heaters are attached via thermal tape to the bottom of the chip. Fluid flow between the channels was laminar, and changes in fluid resistance allowed for valve-less operation. The depth is 500 µm for SPE and RT channels, and the PCR channel is 100 µm deep. The widths are 500 µm for the SPE column, 1 mm for the RT chamber, and vary from 200 to 400 µm for the wide and narrow sections of the continuous flow PCR channel. The chip is 70 mm in length, 25 mm in width and 1.4 mm in height. **(c)** Microfluidic assay process flow. The nasopharyngeal sample is mixed with lysis buffer, applied to the chip, the chip is run, and the PCR products are read using a commercial capillary electrophoresis chip.[13] Click here to view larger figure.

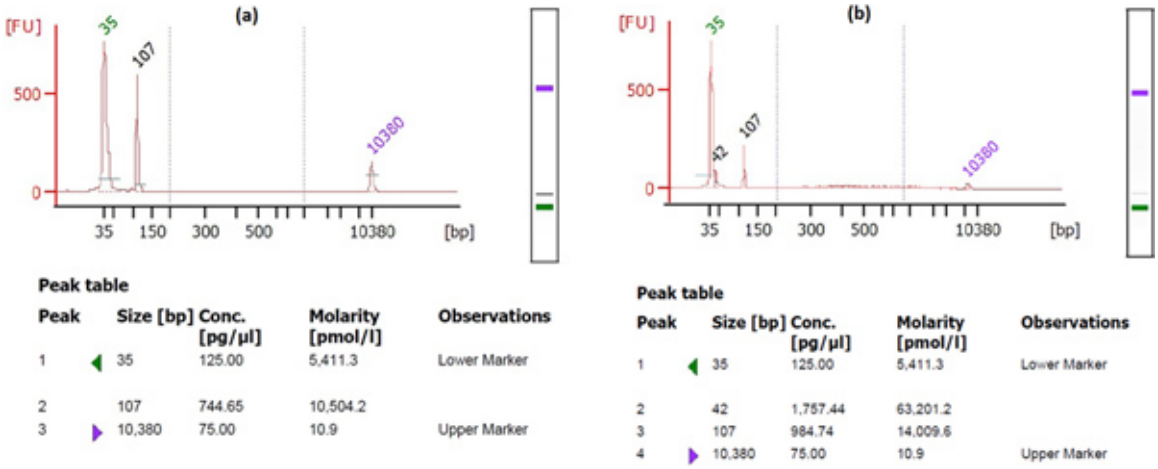

Figure 3. On chip end point diagnostic result. The PCR product peak is the middle peak at the size of 107 bp. Left (35 bp) and right peaks(10380 bp) are the control ladder peaks. The concentration of the products of each peak is the area under the curve. These are tabulated. **(a)** positive result without any noise peak. **(b)** positive result with primer-dimers at 42 bp. Click here to view larger figure.

Discussion

The diagnostic method presented here demonstrated the ability of an integrated microfluidic plastic chip to amplify influenza A RNA from patient specimens with high specificity and a low detection limit.[13] We designed this chip for potential point of care testing: (a) the temperature and fluidic control were simplified, (b) the chip is low cost and suitable for high throughput fabrication using injection molding, and (c) the chip is disposable and intended for one time use, thus reducing the concern of specimen cross contamination.

Although our current on-chip runtime of 3 hr, our extraction platform contains ample room for process optimization. For instance, the wash and dry times right before elution could be reduced by a combination of increasing the flow rate and reducing the wash volumes. Furthermore, cost can be trimmed by streamlining the chip fabrication process and replacing expensive or cumbersome peripherals, such as the syringe pumps, power supply, with alternatives like disposable syringe infusion pumps and small battery packs Opening up the solid phase extraction column geometry and porosity will allow for significant reductions in the pump requirements. Also, once the chip design is standardized for high volume manufacturing, many of the thermocouples used in device development will be eliminated.

More so than other factors, the robustness of our on-chip influenza detection module depends on both good temperature control and appropriate sample handing. Slight offset from the desired on-chip temperature, as well as unintentional multiple freeze-thaw cycles of the influenza specimen, could both reduce the final PCR yield. Aside from these factors, the variations in our on-chip PCR results[13] from different patient specimens could also be attributed to: Variation in viral load, different levels of contaminating human cells and blood; the presence of PCR inhibitors after nucleic acid extraction,[14,15] and unanticipated chemical and/or physical reactions between the specimens and the test reagents or the device itself[16].

In summary, we have demonstrated the feasibility of our microfluidic chip to amplify RNA from the influenza A virus in medically-relevant nasopharyngeal samples. The chip has the potential to be applied to other DNA or RNA targets.

Disclosures

The authors have declared no competing financial interests.

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References

1. Nicholls, H. Pandemic Influenza: The Inside Story. *PLoS Biol.* **4**, e50, doi:10.1371/journal.pbio.0040050 (2006).
2. Chan, Margaret. World now at the start of 2009 influenza pandemic [Internet]. Geneva (Switzerland): World Health Organization [cited 2009 Jun 11]. Available from http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/ (2009).
3. Global Alert and Response (GAR). Pandemic (H1N1) 2009 - update 112[Internet]. Geneva (Switzerland): World Health Organization [cited 2009 Aug 6]. Available from http://www.who.int/csr/don/2010_08_06/en/ (2009).
4. Wenzel, J.J., *et al.* Analytical performance determination and clinical validation of the novel roche realtime ready influenza A/H1N1 detection set. *Journal of Clinical Microbiology.* **48**, 3088-3094, doi:10.1128/jcm.00785-10 (2010).
5. Chan, K.H., *et al.* Analytical sensitivity of rapid influenza antigen detection tests for swine-origin influenza virus (H1N1). *Journal of Clinical Virology: the official publication of the Pan American Society for Clinical Virology.* **45**, 205-207 (2009).

6. Ginocchio, C.C., *et al.* Evaluation of multiple test methods for the detection of the novel 2009 influenza A (H1N1) during the New York City outbreak. *Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology.* **45**, 191-195 (2009).
7. Aeron, C.H., *et al.* Performance of influenza rapid point-of-care tests in the detection of swine lineage A(H1N1) influenza viruses. *Influenza and Other Respiratory Viruses.* **3**, 171-176 (2009).
8. Takahashi, H., Otsuka, Y., & Patterson, B. Diagnostic tests for influenza and other respiratory viruses: determining performance specifications based on clinical setting. *Journal of Infection and Chemotherapy.* **16**, 155-161, doi:10.1007/s10156-010-0035-y (2010).
9. Selvaraju, S.B. & Selvarangan, R. Evaluation of Three Influenza A and B Real-Time Reverse Transcription-PCR Assays and a New 2009 H1N1 Assay for Detection of Influenza Viruses. *Journal of Clinical Microbiology.* **48**, 3870-3875, doi:10.1128/jcm.02464-09 (2009).
10. Northrup, M.A., Ching, M.T., White, R.M., & Watson, R.T. In: *Transducer'93, seventh international conference on solid state sensors and actuators.*, 924-926 (1993).
11. Bhattacharyya, A. & Klapperich, C.M. Thermoplastic microfluidic device for on-chip purification of nucleic acids for disposable diagnostics. *Analytical Chemistry.* **78**, 788-792, doi:doi:10.1021/ac051449j (2006).
12. Cao, Q., Kim, M.-C., & Klapperich, C. Plastic microfluidic chip for continuous-flow polymerase chain reaction: Simulations and experiments. *Biotechnology Journal.* **6**, 177-184, doi:10.1002/biot.201000100 (2011).
13. Cao, Q., *et al.* Microfluidic Chip for Molecular Amplification of Influenza A RNA in Human Respiratory Specimens. *PLoS ONE.* **7**, e33176, doi:10.1371/journal.pone.0033176 (2012).
14. Rådström*, W.A. A.-S. a. P. Purification and Characterization of PCR-Inhibitory Components in Blood Cells. *J. Clin. Microbiol.* **39**, 485-493 (2001).
15. Boddinghaus, B., Wichelhaus, T.A., Brade, V., & Bittner, T. Removal of PCR Inhibitors by Silica Membranes: Evaluating the Amplicor Mycobacterium tuberculosis Kit. *J. Clin. Microbiol.* **39**, 3750-3752, doi:10.1128/jcm.39.10.3750-3752.2001 (2001).
16. Andreasen, D., *et al.* Improved microRNA quantification in total RNA from clinical samples. *Methods.* **50**, S6-S9 (2010).

