

Supplementary Information

Influenza A Virus-Specific Aptamers Screened by Using an Integrated Microfluidic System

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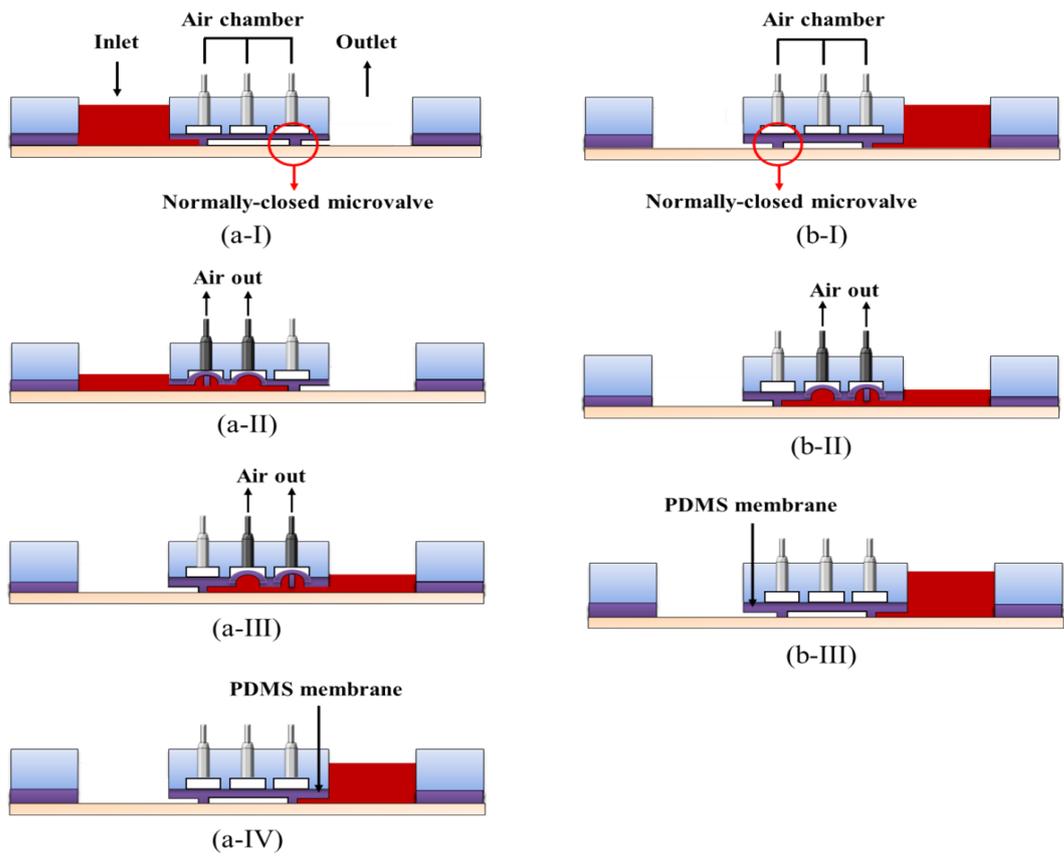
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Materials and Methods

The working principle for the micropump and micromixer

Detailed information about the operating principles of the micromixer and the micropump could be found in our previous work [1]. Briefly, Supplementary Figure 1(a-I) shows the initial state of the suction-type micropump. (a-II) shows the deflections of the PDMS membranes due to negative gauge pressure in the air chambers. Then, (a-III) shows the normally-closed microvalve on the left side that is released when the normally-closed microvalve on the right side is switched on. (a-IV) shows the floating-block structure in the left microchannel switched off to push the sample forward. Next, all the microfluidic pumps/valves are released to push the liquid forward from the fluidic reservoir into the reaction chamber (1). Similarly, Supplementary Figure 1(b) shows the mixing module when the PDMS membranes are deflected, which generates a mixing effect inside the mixing chamber by applying the negative gauge pressure.



Supplementary Figure 1: The working principle for the micropump and the micromixer.

The used specific primers in this study

Supplementary Table 1: The detail information of primers used in this study [2-5]

species	primer	Sequences (5'→3')	Annealing Temperature	Average amplicon size (bp)
Influenza A virus				
H1 subtype	forward	GTGTTTCATCACCCGCC	58°C	249
	reverse	AGCCTCTACTCAGTGC		
H3 subtype	forward	AATGCATCACTCCAAATGG	56°C	147
	reverse	TAGTTTGTCTCTGGTAC		
Influenza B virus	forward	AAATACGGTGGATTAAATAAAAGCAA	56°C	170
	reverse	CCAGCAATAGCTCCGAAGAAA		
Dengue virus type 2	forward	GAGTGGAGTGGGAAGGAGAAGGG	58°C	248
	reverse	CTTCTTGGTGTGGTCTTTGC		
Enterovirus 71	forward	AGTATGATTGAGACTCGGTG	58°C	232
	reverse	GCGACAAAAGTGA ACTCTGC		
Human chromosomaDNA (GAPDH)	forward	CCATGGAGAAGGCTGGGG	60°C	225
	reverse	CAAAGTTGTCATGGATGACC		

Preparation of the anti-influenza virus NP mAb conjugated magnetic beads

The primary mouse anti-influenza A virus nucleoprotein monoclonal antibodies (anti-NP-A mAb, H16L-10-4R5 cell line (HB-65), ATCC Co., USA) and primary mouse anti-influenza B virus nucleoprotein monoclonal antibodies (anti-NP-B mAb, influenza B nucleoprotein (B017), GeneTex Co., USA) were conjugated on epoxy-coated magnetic beads (diameter (\emptyset) of the beads = 4.5 μm , Dynabeads[®] M-450 Epoxy, Invitrogen Co., USA) [6]. The surface of the magnetic beads was coated with an epoxy amino group, thiol and hydroxyl functional groups, which were used to couple the specific anti-NP-A/ or B mAbs (Department of Microbiology and Immunology, National Cheng Kung University, Tainan, Taiwan). The detailed conjugation protocol is described as follows:

1. Add 25 μL of epoxy-coated magnetic beads (4×10^8 beads/mL) to a new eppendorf tube.
2. Place the eppendorf tube in a magnetic particle concentrator (DynaMag[™]-2, Invitrogen Co., USA) for 3 minutes and discard the supernatant.
3. Wash the collected magnetic beads twice with 1000 μL of $1\times$ phosphate buffered saline (PBS, pH = 7.4, Invitrogen Co., USA) and put in a magnetic particle concentrator for 3 minutes, then discard the supernatant.
4. Incubate 5 μg of anti-NP-A/ or B mAbs with 1000 μL of $1\times$ PBS buffer by using a wheeling rotator (INTELLI-MIXER RM-2L, ELMi Ltd, Latvia) for 25 rpm at 4°C for overnight.
5. Repeat steps 2 and 3 to remove un-conjugated anti-NP-A/ or B mAbs.
6. To prevent non-specific binding in the subsequent immunological steps, add 1000 μL of Tris buffer (10 mM Tris, 100 mM NaCl, 2 mM CaCl₂) containing 1% (w/v) bovine serum albumin (BSA, Sigma Co., USA) to the eppendorf tube to block the non-conjugated at 4°C overnight.

7. Repeat steps 2 and 3 to purify the anti-NP-A/ or B mAbs conjugated magnetic beads.
8. The anti-NP-A/ or B mAbs conjugated magnetic beads can be stored at 4°C in 1× PBS with 0.1% (w/v) BSA until used in the SELEX process.

Preparation of the influenza A/H1N1-specific aptamer conjugated magnetic beads

The InfA/H1N1-specific aptamer was surface-coated to the carboxylic acid coated magnetic beads (diameter (\emptyset) of the beads = 1 μm , Dynabeads[®] MyoneTM Carboxylic Acid, Invitrogen Co., USA). The surfaces of the magnetic beads were coated with carboxylic acid groups and could be used as bifunctional cross-linkers with amine groups. The detailed modified protocol is described as follows:

1. Re-suspend the solution of carboxylic acid coated magnetic beads solution using a vortex for 1 minute.
2. Add 100 μL of magnetic beads solution (4×10^8 beads/mL) to a new eppendorf tube.
3. Add 900 μL of double-distilled water (ddH₂O) and vortex for 5 sec.
4. Use a magnetic particle concentrator (DynaMagTM-2, Invitrogen Co., USA) to collect the magnetic beads for 3 minutes and discard the supernatant.
5. Repeat steps 3 and 4.
6. Mix 950 μL of carboxylic acid coated magnetic beads mixture and 30 μL of 100 μM InflA/H1N1-specific aptamers that were modified with amine groups at 5' end of oligonucleotide by using a vortex for 5 sec.
7. Next, add 20 μL of 120 mg/ml 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide (EDAC, Invitrogen, USA) and mix completely by using vortex for 5 sec.
8. Incubate the mixture with a wheeling rotator (INTELLI-MIXER, ELMI Ltd, Latvia) by 20 rpm for 18 hours at room temperature and avoid light.
9. Use a magnetic rack to collect the magnetic beads for 3 minutes and discard the supernatant.
10. Wash the magnetic beads twice with 1000 μL of 0.02% Tween 20 (Sigma Co., USA) solution and repeat step 9.

11. Wash the magnetic beads twice with 1000 μL of 0.1% sodium dodecyl sulfate (SDS, MERCK Co., Germany) solution and repeat step 9.
12. Incubate 1000 μL of 0.1 M ethanol amine (Sigma Co., USA) with the collected magnetic beads at room temperature for 1 hour. This serves to block the free uncoupling spaces on the surface of the magnetic beads.
13. Repeat step 9.
14. Suspend the coupled magnetic beads in 1000 μL of ddH₂O and store at 4°C.

Conversion of HAU to RNA copy number

Viral RNA was extracted from InfA/H1N1 virus sample using the QIAamp Viral RNA Mini kit (QIAGEN, Germany). The concentration of extracted RNA was measured by a spectrophotometer (Beckman, DU530 UV/Vis, USA). The viral RNA copy number was calculated based on a formula reported in a previous work [7]:

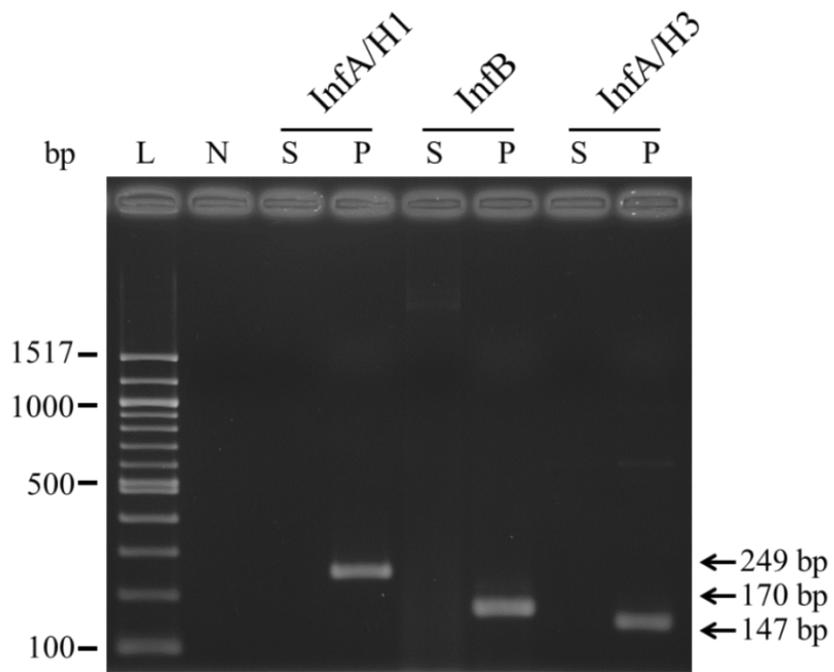
Number of RNA copy (copy / L) = [RNA concentration (g/ μ l) \times 6.02×10^{23}] / [Length of in vitro RNA (bp) \times 340].

From this process, the initial concentration of the InfA/H1N1 virus at 6.4×10^1 HAU was determined to contain $\sim 1.6 \times 10^8$ copies of viral RNA.

Results and discussion

The specificity test of SELEX primers

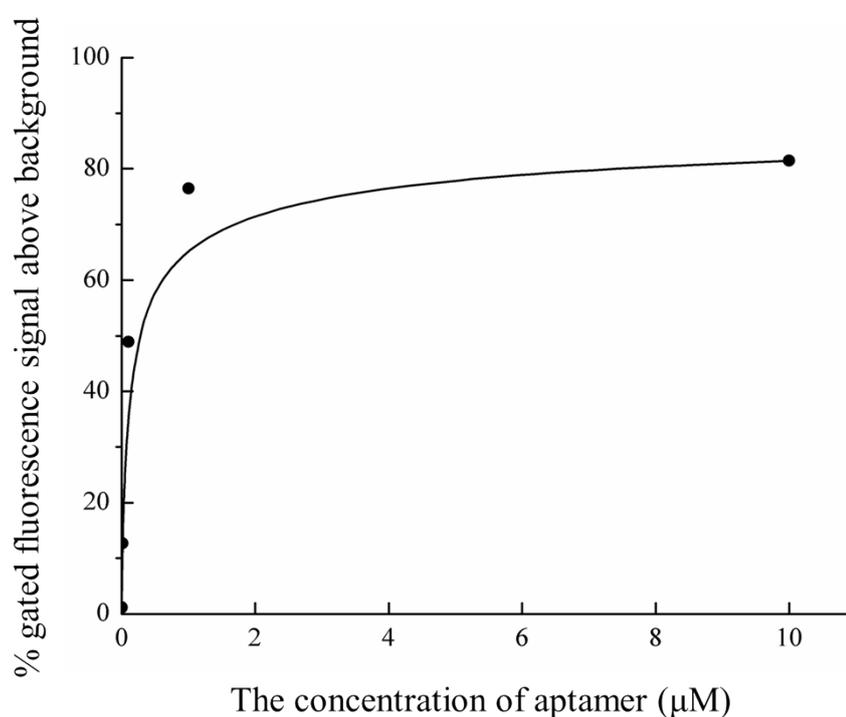
To test the specificity of SELEX primers, three types of influenza virus including InfA/H1N1, InfB, and InfA/H3N2 are tested using RT-PCR. Supplementary Figure 3 shows the electrophoretic results from the specificity test. The total viral RNAs of InfA/H1N1, InfB, and InfA/H3N2 were extracted and purified from the viruses, and tested with RT-PCR using SELEX primers. Lane S indicated the specific SELEX primer that was used with InfA/H1N1, InfB, and InfA/H3N2, respectively. Note that lane P indicated the positive control that used specific InfA/H1, InfB, and InfA/H3 primers with the tested samples. Lane N indicated the negative control and used only ddH₂O. Lane L was loaded with a 100-bp DNA ladder. The amplified size of the detected genes for InfA/H1, InfB, and InfA/H3 were 249, 170, and 147 base-pairs (bp), respectively. The experimental result showed that designed SELEX primers amplified a 72-bp PCR product successfully and demonstrated that these primers had a high specificity for the single stranded DNA (ssDNA) library. The designed SELEX primer had no observable recognition to the tested viral RNAs, and PCR products were amplified from ssDNA library. Therefore, the SELEX primer was specific for influenza viral aptamers within the ssDNA library when using the SELEX method.



Supplementary Figure 2: Specificity test of the SELEX primers against viral RNA from InfA/H1N1, InfB, and InfA/H3N2 by reverse transcription PCR (RT-PCR) and slab-gel electrophoresis analysis.

The binding affinity of the influenza A/H1N1-specific aptamer

The binding affinity of the InfA/H1N1-specific aptamer is shown in Supplementary Figure 5. Various concentrations of the FITC-labeled InfA/H1N1-specific aptamer were incubated with InfA/H1N1 virus, and then analyzed by using flow cytometry-based technique. In this study, four concentrations of the FITC-labeled aptamer (0.01, 0.1, 1, 10 μM) showed significantly higher fluorescence signal than the control sample.



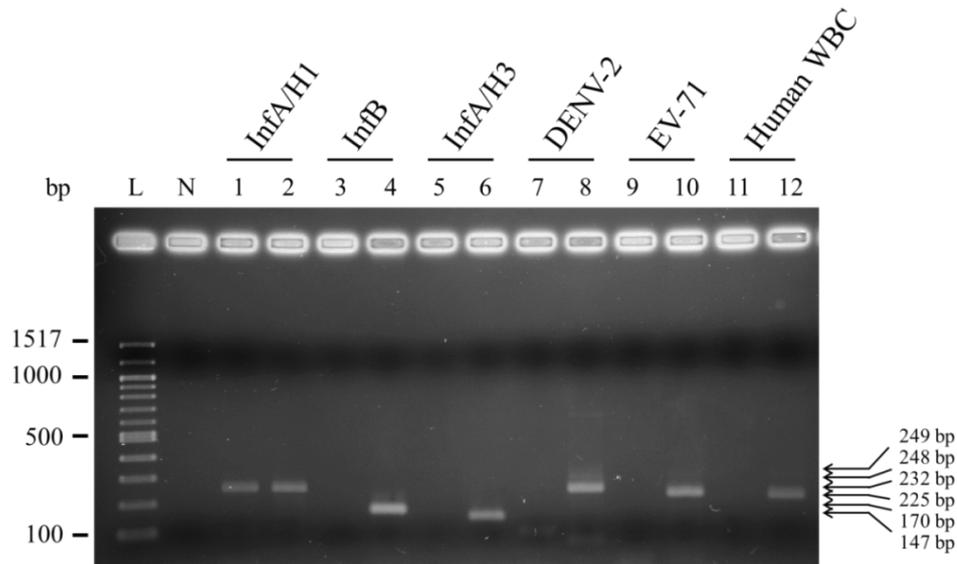
Supplementary Figure 4: The binding assay of FITC-labeled aptamer using flow cytometry. A nonlinear regression curve was fit to the data using GraphPad Prism 5.0. Consequently, the K_d of the InfA/H1-specific aptamer was analyzed to be 55.14 ± 22.40 nM by flow cytometry with GraphPad Prism software.

Specificity tests of the influenza A/H1N1-specific aptamer

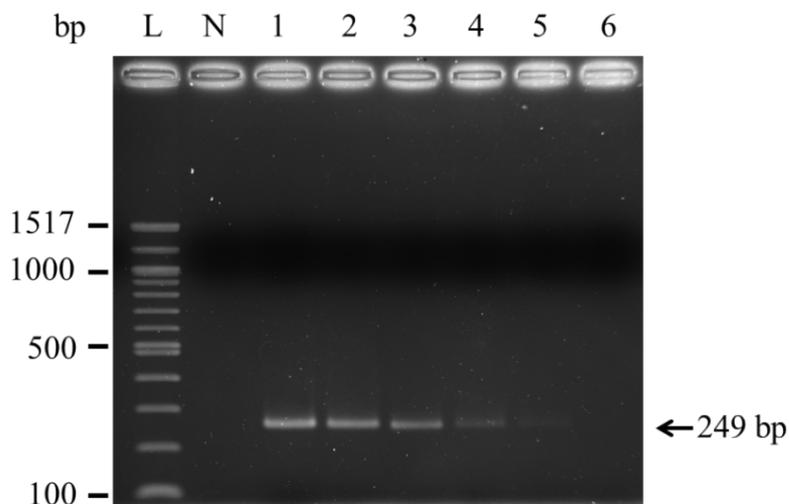
A test on the specificity of InfA/H1N1-specific aptamer conjugated magnetic beads was determined using RT-PCR and observed on a 2% agarose gel stained with ethidium bromide (EtBr). The electrophoretic results also verified the accuracy of the optical signals obtained from the specificity test. Supplementary Figure 6(a) shows the RT-PCR results of the InfA/H1N1-specific aptamer conjugated magnetic beads incubated with InfA/H1N1 (lane 1), InfB (lane 3), InfA/H3N2 (lane 5), DENV-2 (lane 7), EV-71 (lane 9), and human WBC (GAPDH, lane 11). The InfA/H1N1-specific aptamer conjugated magnetic beads successfully captured the viral particles of InfA/H1N1, which was loaded into lane 1. Furthermore, the viral RNA, including InfA/H1N1, InfB, InfA/H3N2, DENV-2, EV-71 and human chromosomal DNA used as positive controls were all amplified by their specific primers (lanes 2, 4, 6, 8, 10, and 12, respectively). The amplified sizes of InfA/H1N1, InfB, InfA/H3N2, DENV-2, EV-71, and human WBC (GAPDH) were 249, 170, 147, 248, 232, and 225 bp, respectively. Lane N contained the negative control with ddH₂O. Lane L contained the 100-bp DNA ladder. Moreover, the amplified PCR products from the viral RNA were clearly discriminated using gel electrophoresis (lane 1). The results showed that the expected InfA/H1N1 target was successfully amplified using the InfA/H1N1-specific aptamer conjugated magnetic beads. A comparison of the electrophoretic results with specificity tests showed that only the InfA/H1N1 sample was amplified successfully, with the high fluorescence signal of InfA/H1N1 corresponding to the high signals of electrophoretic tests. Note that there was no cross reactivity between the various types of tested samples using the two diagnostic methods. The tested specific aptamer demonstrated high specificity with InfA/H1N1 leading to differentiate various types of viruses, and can be used for detection of an influenza

infection.

(a)



(b)



Supplementary Figure 5: (a) Slab-gel electrophoresis showed specific capturing of InfA/H1N1 virus by InfA/H1N1-specific aptamer. Here, extracted RNA of InfA/H1N1, InfB, InfA/H3N2, DENV-2, EV-71, and human WBC (GAPDH) were used as positive controls to confirm RT-PCR. The analytic result showed that the InfA/H1N1-specific aptamer demonstrated high specificity with

InfA/H1N1 and was able to differentiate various types of virus. (b) Slab-gel electrophoresis revealed the sensitivity of the assay to be 6.4×10^{-3} HAU, as indicated by the 249-bp RT-PCR product band in the gel (lane 5). Note that Lane N contained the negative control using ddH₂O, and lane L was the 100-bp DNA ladder.

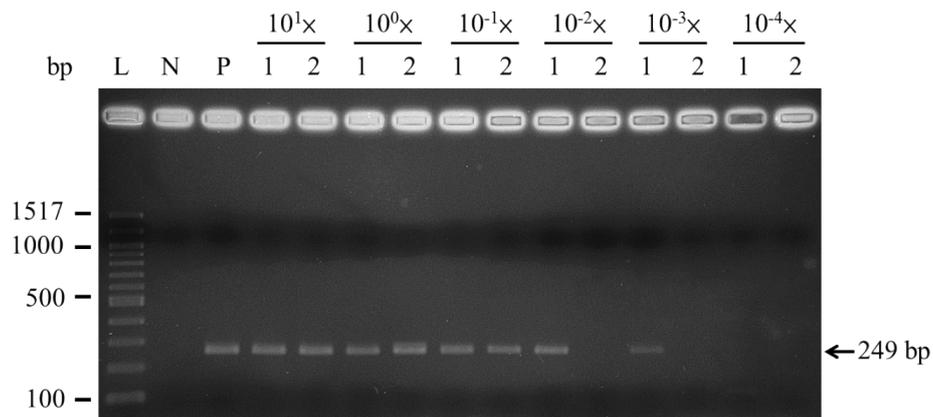
Sensitivity tests of the influenza A/H1N1-specific aptamer

To validate sensitivity results of the fluorescence assays, PCR with electrophoresis were performed (Supplementary Fig. 6(b)). In the gel image, the desired 249-bp amplicon band was only observed in lanes 1 to 5, which corresponded to 6.4×10^1 to 6.4×10^{-3} hemagglutinin unit (HAU). Lane N contained the negative control with ddH₂O as the template, and lane L contained the 100-bp DNA ladder to determine the amplified sizes. Note that the amplified size of the detected gene for InfA/H1N1 was 249 bp. According to the both of the analytic results, the LOD for this developed system using InfA/H1N1-specific aptamer conjugated magnetic beads was determined to be 6.4×10^{-3} HAU. Furthermore, the intensity of the band increased along with the increasing titer, which agreed with the optical signals.

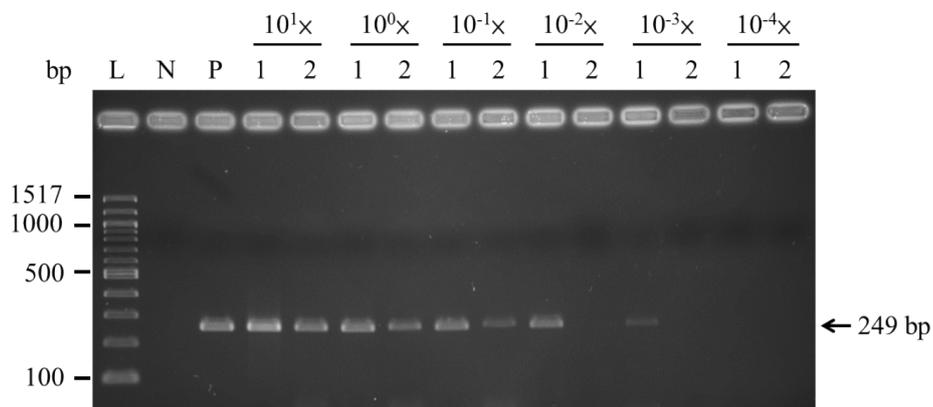
For comparison, manual sensitivity tests were performed using the anti-NP-A mAb conjugated beads with three strains of InfA/H1N1 (Supplementary Fig. 7). Three independent tests of different InfA/H1N1 strains (89N364H1, 88N35IAH1, and 94S320H1) were used to compare the sensitivity between the InfA/H1N1-specific aptamer conjugated magnetic beads and anti-NP-A mAb conjugated beads using a 2% agarose gel. In this study, a 10-fold series dilution (6.4×10^1 ($10^1\times$), 6.4×10^0 ($10^0\times$), 6.4×10^{-1} ($10^{-1}\times$), 6.4×10^{-2} ($10^{-2}\times$), 6.4×10^{-3} ($10^{-3}\times$), 6.4×10^{-4} ($10^{-4}\times$) HAU) of InfA/H1N1 virus was used. The results of the InfA/H1N1-specific aptamer conjugated magnetic beads incubation with different titers of InfA/H1N1, in which the dilutions varied from $10^1\times$ to $10^{-4}\times$, were shown in lane 1. The electrophoretic results of the sensitivity tests with anti-NP-A mAb conjugated beads incubated with different 10-fold serial dilutions of InfA/H1 were shown in lane 2. Lane N contained the negative control using ddH₂O and lane P contained the positive control with viral RNA from InfA/H1N1 virus, which was used to verify the accuracy of RT-PCR. Lane L contained a 100-bp DNA ladder. Note that the amplified size of the detected gene

for InfA/H1N1 was 249 bp. These results demonstrated that the sensitivity tests were reproducible for the detection of InfA/H1N1 using the InfA/H1N1-specific aptamer conjugated magnetic beads with three repeated measurements. All of the electrophoretic results demonstrated that the InfA/H1N1-specific aptamer was 100-fold more sensitive than the antibody-based assay for the detection of target in this study. Namely, this selected aptamer showed 100-fold improvements in sensitivity when pegged against the anti-NP-A mAb in a similar reverse-transcription PCR (RT-PCR)-based detection assay.

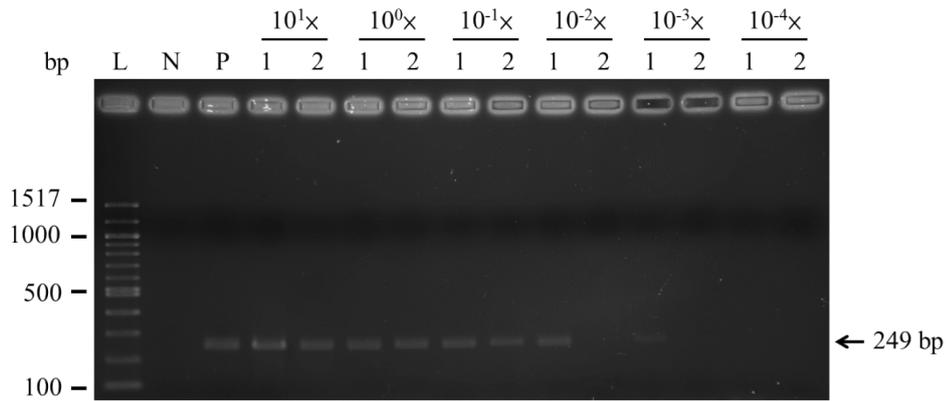
(a)



(b)



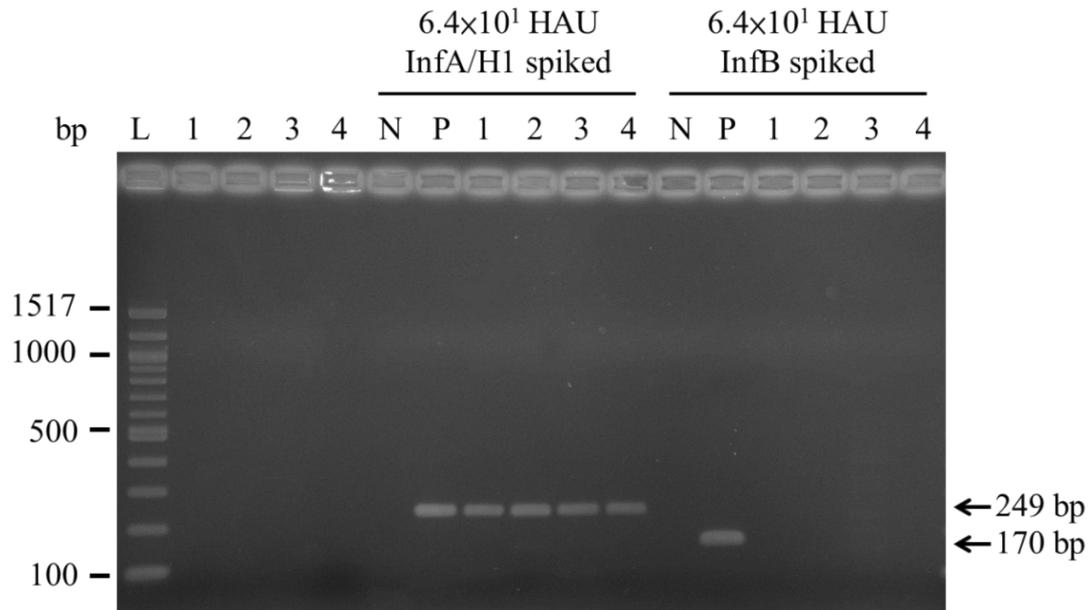
(c)



Supplementary Figure 6: Comparison of the detection sensitivity of InfA/H1N1-specific aptamer (lane 1) and anti-NP-A mAb (lane 2) conjugated magnetic beads by incubating with three strains of InfA/H1N1 at 10-fold serial dilutions, followed by RT-PCR and slab-gel electrophoresis analysis. (a) 89N364H1, (b) 88N35IAH1, and (c) 94S320H1 were the different strains of InfA/H1N1 diluted from 6.4×10^1 HAU ($10^1 \times$) to 6.4×10^{-4} HAU ($10^{-4} \times$), respectively. These electrophoretic results showed that the aptamer-based assay was able to detect down to 6.4×10^{-3} HAU for all three strains of InfA/H1N1 virus and presented 100-fold improvements in sensitivity when pegged against the anti-NP-A mAb, which was only able to detect 6.4×10^{-1} HAU. Note that Lane N contained the ddH₂O negative control, lane P contained the positive control using viral RNA of InfA/H1N1, and lane L was the 100-bp DNA ladder for all gels.

Spiking biological samples with known influenza virus particles to test the influenza A/H1N1-specific aptamer

The electrophoretic results from various the biological samples with InfA/H1N1 and InfB, including 1× PBS buffer, normal throat swab, normal sputum, and normal serum samples were labeled as 1, 2, 3, and 4, respectively (Supplementary Fig. 8). The experimental results confirmed the validity of the fluorescence signals for the different biological samples spiked with known influenza virus particles tests. However, no amplified PCR products were detected in the four bio-samples including 1× PBS buffer, normal throat swab, normal sputum, and normal serum samples that were not initially spiked with viruses. The RT-PCR products from these bio-samples that contained InfA/H1N1 particles, including 1× PBS buffer, normal throat swab, normal sputum, and normal serum samples were detected on a 2% agarose gel stained with EtBr. Furthermore, no RT-PCR products were observed in 1× PBS buffer, normal throat swab, normal sputum, and normal serum samples when they were spiked with InfB. Note that Lane N indicated the negative control using ddH₂O, and lane P contained the positive control using viral RNA from InfA/H1N1 and InfB. Lane L was the 100-bp DNA ladder. The amplified size of the detected genes in InfA/H1N1 and InfB were 249 and 170 bp, respectively. These electropherograms showed that InfA/H1N1 could be successfully captured by the InfA/H1N1-specific aptamer conjugated magnetic beads in different kinds of biological samples. According to both of the analytic results, the InfA/H1N1-specific aptamer-based assay effectively detected InfA/H1N1 virus from clinically-relevant sample matrices.



Supplementary Figure 7: Slab-gel electrophoresis showed that the InfA/H1N1-specific aptamer-based assay effectively detected InfA/H1N1 virus from clinically-relevant sample matrices. Lane N contained the negative control using ddH₂O, and lane P contained the positive control using viral RNA of InfA/H1N1 (249 bp) or InfB (170 bp). Lane L was the 100-bp DNA ladder. The experimental results demonstrated its potential utility for the diagnosis an influenza infection in clinical samples.

Comparison of the SELEX for traditional methods and the microfluidic system

Specific aptamers are usually screened by SELEX by performing iterative rounds of amplification and selection from a pool of single-strand DNA (ssDNA). However, the traditional SELEX processes are usually time-consuming and labor-intensive in their washing and incubation steps. Additional drawback is that manual operation may cause the loss of collected beads. Furthermore, it also needs a number of large-scale equipment such as a PCR machine, shakers, and pipettes, and generally requires large reagent consumption. The entire SELEX procedure takes almost 160 minutes and sample consumption is 30 μ l. By using the proposed microfluidic chip, the entire procedure for SELEX only needs 80 min. In addition, the sample consumption with this microfluidic system (10 μ l) is relatively low when compared with the traditional methods (30 μ l). The user can also expect a smaller sample volume and faster screening by using the integrated microfluidic chip. A comparison of the traditional methods and the microfluidic system is listed in Supplementary Table 2.

Supplementary Table 2: A comparison of the traditional methods and microfluidic systems for the screening of aptamers in this study (for an entire procedure of the SELEX process)

	Traditional methods	Microfluidic system
Incubation time		
Influneza virus and magnetic beads	30 min	10 min
Virus-beads complex and ssDNA library	30 min	10 min
Sample consumption		
ssDNA library	30 μ l	10 μ l
Influenza virus	30 μ l	10 μ l
Magnetic beads	30 μ l	10 μ l
Wash (3 times)	15 min / 3 times	5 min / 3 times
PCR time by 20 cycles amplification	90 min	60 min

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