

Hagan et al., An Integrated, Valveless System for Microfluidic Purification and Reverse Transcription-PCR of RNA for Detection of Infectious Agents

Supplemental Materials

Materials and methods

Reagents

Hyperprep silica beads (15-30 μm) were purchased from Supelco (Bellefonte, PA). Potassium chloride was purchased from Malinckroft (Paris, KY). Low molecular weight chitosan (chitosan oligosaccharide lactate) ($M_n < 5000$, deacetylation $> 90\%$), SigmaCote® and 3-(glycidyloxy-propyl)trimethoxysilane (GPTMS) were purchased from Sigma-Aldrich (St. Louis, MO). Triton X-100, trizma base, hydrochloric acid (HCl), acetone, sodium hydroxide, 2-(4-morpholino)-ethane sulfonic acid (MES), and diethylpyrocarbonate (DEPC)-treated water were purchased from Fisher (Fairlawn, NJ). Quant-iT™ RiboGreen® RNA Assay Kit containing Quant-iT™ RiboGreen® RNA reagent fluorescent nucleic acid stain, 20X Tris-EDTA (TE), and ribosomal RNA standard (16S and 23S rRNA from *E. coli*) was purchased from Invitrogen (Carlsbad, CA). TaqMan® Control Total RNA (Human) was purchased from Applied Biosystems (Foster City, CA). Random decamers, SuperTaq™ Polymerase (with 10X PCR buffer and dNTPs), M-MLV RT, SUPERase-In™, and TURBO DNA-free™ Kit were purchased from Ambion (Austin, TX). Bovine Serum Albumin (BSA) was purchased from Promega Corporation (Madison, WI). Primers for a 353-bp segment of β -actin, a 153-bp segment of the protamine-1 gene, and a 240-bp segment of the influenza A nucleoprotein gene were purchased from MWG-Biotech, Inc. (High Point, NC). Buffers were prepared in Nanopure water (Barnstead/Thermolyne, Dubuque, IA), and all working solutions were prepared in DEPC-treated water or autoclaved deionized water treated

with UV for 20 min. Madin-Darby canine kidney cells were infected with influenza A/PR/8/34 at a multiplicity of infection of 0.1 as described previously.¹ Cells were harvested after 48 h, washed in phosphate buffered saline and pelleted. Purified RNA from semen and from the influenza A-infected cells was obtained via Qiagen extraction performed in-house using the Qiagen RNeasy® MinElute™ Cleanup Kit and the Qiagen QIAamp® Viral RNA Mini Kit, respectively (Valencia, CA).

Microchip preparation

Microdevices were fabricated with Borofloat glass (Telic Company, Valencia, CA) using standard photolithographic techniques to pattern microstructures on the substrate, followed by wet chemical etching of the structures.² For solid phase extraction chips, the resulting channel dimensions (measured using a profilometer) were 1.5 cm effective length, 225 μm deep, a top width of 650 μm , and a bottom width of 200 μm . A 1.1 mm-diameter diamond-tip drill bit (Crystallite Corp., Lewis Center, OH) was used to drill access holes at both ends of the channel. A Borofloat glass cover plate was cut to fit the device and thermally bonded to the etched bottom plate. The distance from the top of the weir to the cover plate was approximately 5-20 μm . A photo of the SPE device is shown in **Figure S-2A**. Following fabrication, chitosan-coated silica beads, 15-30 μm , were suspended in DEPC-treated water and packed against the weir in the channel using vacuum. The channel was filled with new chitosan-coated silica beads prior to each extraction.

The PCR device channel dimensions were 200 μm deep with a 500 nL chamber volume. An additional chamber (reference chamber) was etched with the same dimensions parallel to the PCR chamber to allow for temperature monitoring during

infrared (IR)-mediated heating. A window 100 μm deep was etched around the PCR and reference chamber after bonding each device to a glass cover plate to reduce thermal mass. Access holes 1.1 mm in diameter were drilled at both ends of the channels. A photo of the PCR device is shown in Figure S-2A. After fabrication, and prior to each amplification, the device was dried and passivated with SigmaCote®.

The integrated SPE-RT-PCR device channel dimensions were 225 μm deep with a bottom width of 200 μm for both the SPE and side-arm channels. The SPE channel and side-arm channel had an effective length of 1.5 cm and 1.8 cm, respectively. The channels surrounding the PCR chamber were 200 μm deep, with a bottom width of 75 μm and 1.5 cm in length on each side of the PCR chamber. A reference chamber was etched with a channel of 4 mm for thermocouple insertion on the sample outlet end of the device, and a 5 mm channel on the opposite side. A window 100 μm deep was etched around the PCR and reference chamber to reduce thermal mass after bonding each device to a glass cover plate. Access holes 1.1 mm in diameter were drilled at both ends of the channels. After fabrication, and prior to each amplification, the device was dried and the surface passivated with SigmaCote®. Chitosan-coated silica beads, 15-30 μm , were suspended in DEPC-treated water and packed against the weir in the SPE channel using vacuum. The SPE channel was filled with new chitosan-coated silica beads prior to each extraction.

Apparatus

The microchip-solid phase extraction (SPE) apparatus consisted of a SP100i model syringe pump (WPI, Sarasota, FL) with a 250 μL Hamilton gastight syringe

(Hamilton, Las Vegas, NV). The syringe was connected to the microchip using PEEK™ tubing and mini-tight fittings (Upchurch Scientific, Oak Harbor, WA).

The non-contact IR mediated-PCR system was built in-house as previously described in Easley, et al.^{3, 4} and aluminum foil placed over the chambers was used to enhance heating of solution.

Microchip solid phase extraction procedure

Microdevices were filled with chitosan-coated silica beads (coating procedure described below) and conditioned with 70 μL 10 mM MES, pH 5.0. A 75 μL load solution containing purified influenza A viral RNA in a 10 mM MES buffer was first loaded onto the chitosan bed. Next, a wash of 25 μL 10 mM MES was flowed through the bed to remove any unbound material. Elution was performed with 10 mM Tris/50 mM KCl, pH 9.0, and 2 μL fractions were collected. All extraction steps on the chitosan phase were performed at a flow rate of 5 $\mu\text{L}/\text{min}$. For the two-chip method, the third 2 μL fraction was collected into a tube already containing RT-PCR reagents making up a 5 μL total volume master mix.

A similar procedure was used for the extraction of viral RNA from nasal swab eluate. Autoclaved swabs were used to collect nasal swab specimens from inside the nose by swabbing a single nostril 3 times. The cells were eluted from the swab in 500 μL PBS, and a 40 μL aliquot of cells from the stock solution in PBS was mixed with influenza viral RNA (4 ng) and 10 μL Proteinase K (20 mg/mL, Qiagen, Valencia, CA). Next, 50 mM MES, 1% Triton X-100 was added to the cell solution to bring the volume up to 150 μL (0.027 ng/ μL influenza viral RNA) and the mixture was incubated at room temperature for 30 minutes. The wash of 10 mM MES to remove cellular debris was

increased to 50 μL when using nasal swabs. For the clinically-relevant sample, the same load solution preparation was performed, except only 0.4 ng of viral RNA was added.

Chitosan-coated silica bead fabrication

Silica beads, 15-30 μm , were coated with chitosan following the procedure in Hagan, et al.⁵

Fluorescence detection and sample amplification

Fluorescence detection was performed on a NanoDrop 3300 Fluorospectrometer (NanoDrop, Wilmington, DE) using a RiboGreen® fluorescence assay for RNA to determine the concentration of RNA in purified samples. Microchip RT-PCR of elution fractions from all viral RNA extractions were performed using a master mix consisting of the 2 μL elution fraction, 0.625 μM random decamers, 1 μM dNTPs, 1X PCR buffer containing 1.5 mM MgCl_2 , 0.1 U/ μL SUPERase-In™ RNase inhibitor, 0.24 μM each forward and reverse primer, 1 U/ μL M-MLV RT, 0.24 mg/mL BSA, 1 U/ μL SuperTaq™ Polymerase and 0.16 μL water. This mix of PCR reagents and purified DNA was loaded into the PCR sample chamber of a PCR device while 1X PCR buffer was loaded into the temperature reference chamber. A thermocouple (Model T-240C, PhysiTemp Instruments, Inc., Clifton, NJ) was inserted into the reference chamber, and mineral oil was placed over each open reservoir to prevent sample evaporation. The filled PCR microchip was then placed on a stage seated over the IR lamp of the IR-PCR system.

Thermal cycling was performed using the following conventional protocol, for 2.5 hr total RT-PCR time: 42 °C for 60 min (reverse transcription), 94 °C 10 min (RT inactivation), 35 cycles of denaturing at 94 °C for 20 s/annealing at 55 °C for 30 s/extension at 72 °C for 40 s, followed by 72 °C for 5 min (final extension). A more

rapid protocol for 60 min RT-PCR time was used for some of the amplifications as described below: 42 °C for 1 min (reverse transcription), 94 °C 1 min (RT inactivation), 35 cycles of denaturing at 94 °C for 20 s/annealing at 55 °C for 30 s/extension at 72 °C for 40 s, followed by 72 °C for 5 min (final extension). The most rapid protocol used for 39 min total RT-PCR time was as follows: 42 °C for 1 min (reverse transcription), 94 °C 1 min (RT inactivation), 35 cycles of denaturing at 94 °C for 5 s/annealing at 55 °C for 20 s/extension at 72 °C for 30 s, followed by 72 °C for 1 min (final extension). For the clinically-relevant nasal swab eluate sample, when only 0.2 ng viral RNA was loaded on the two-chip system or 2 ng loaded using the integrated device, the cycles of the above protocol were increased to 40. This increased the time required for RT-PCR to 45 min. Primer sequences for protamine-1 are as referenced⁶, β -actin primers used were: sense – 5'-GCTCGTCGTCGACAACGGCTC-3'; antisense – 5'-CAAACATGATCTGGGTCATCTTCTC-3') and influenza A nucleoprotein primers were: sense – 5'-TGCTTCAAACAGCCAAGTG-3'; antisense – 5'-GCCCAGTACCTGCTTCTCAG-3'. All RT-PCR products were analyzed via microchip gel electrophoresis on a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) using the commercially available DNA Series II kit according to the manufacturer's instruction.

Integrated SPE-RT-PCR procedure

For SPE performed during integration on the SPE-RT-PCR microdevice, a solution of 67 ng influenza A viral RNA in 10 mM MES was loaded onto the device (in 35 μ L) after the chitosan phase had been conditioned with 50 μ L of 10 mM MES, pH 5, with all flow through the SPE channel performed at a flow rate of 5 μ L/min. A wash step was performed to remove any unbound material with 25 μ L 10 mM MES. During both

the load and wash steps, DEPC-treated H₂O was flowed through the sidearm at a flow rate of 7.5 $\mu\text{L}/\text{min}$ to maintain a fluidic connection within the device. For elution, 10 mM Tris/50 mM KCl, pH 9, was flowed through the SPE bed to elute RNA from the chitosan at a flow rate of 2.5 $\mu\text{L}/\text{min}$. RT-PCR master mix was flowed from an 100 μL syringe treated with SigmaCote® at a ratio of 3:2 to the eluted RNA (3.25 $\mu\text{L}/\text{min}$) to ensure all reagents would be present in the PCR chamber at a concentration determined to be successful for RT-PCR on a microdevice from RT-PCR studies performed in this work. The fraction of RNA and RT-PCR master mix determined to contain the maximum mass of eluted RNA was trapped in the PCR chamber and the mixture subjected to RT-PCR using the IR-PCR system after all reservoirs were covered with mineral oil to prevent evaporation.

When nasal swab samples were used, the load solution preparation differed from above. Autoclaved swabs were used to collect nasal swab specimens from inside the nose by swabbing a single nostril 3 times. The cells were eluted from the swab in 500 μL PBS, and a 40 μL aliquot of cells from the stock solution in PBS was mixed with an aliquot of RNA purified from the influenza A-infected MDCK cell line containing 4 ng influenza viral RNA and 10 μL Proteinase K (20 mg/mL, Qiagen, Valencia, CA). Next, 50 mM MES, 1% Triton X-100 was added to the cell solution to bring the volume up to 150 μL and the mixture was incubated at room temperature for 30 minutes. The load was increased to 75 μL , and the wash of 10 mM MES to remove cellular debris was increased to 50 μL when using nasal swabs.

Results and discussion

Microchip RT-PCR

RNA purified in-house from semen was chosen for initial experiments, as conventional RT-PCR had been successfully performed from this sample in previous work.⁷ The RNA was heat-denatured and then mixed with the reagents required for both RT and PCR at the appropriate concentrations, loaded into the 500 nL PCR chamber on the microdevice, the device placed on the IR-PCR system, and RT-PCR carried out as a single process to amplify a portion of the semen-specific protamine-1 gene⁶. The PCR product was removed from the microdevice and separated using an Agilent 2100 Bioanalyzer. The resulting electropherogram in **Figure S-1** shows a 153-bp fragment product specific to the protamine-1 gene expected if mRNA from semen is present. Although the peak height for the amplified product is small, it is important to note that this amplification uses primers specific for the mRNA transcript of a portion of the semen-specific gene, protamine-1. The mass of total RNA that is present in the 0.5 μ L PCR chamber during the amplification is \sim 2.4 ng total RNA, but only 1-5% of this is mRNA. Therefore, a mass of only \sim 24-120 pg mRNA is present in the PCR chamber for RT-PCR. These results help demonstrate the sensitivity of the chip-based RT-PCR analysis. These results demonstrate that RT-PCR can be successfully combined into a single-chamber reaction on a microdevice, which is more conducive to future integration of SPE with RT-PCR on a single device and, importantly, provides the first example of IR-mediated heating to RT-PCR.

The above experiments were performed using either random decamers or gene-specific primers for the mRNA transcript of a portion of the protamine-1 gene to determine which approach functions best for priming the RT reaction. This was explored

because the manufacturer's instructions for use with the RT-PCR reagents suggest that a greater yield of RT product will be observed if the random decamers were used, but that this advantage may not be apparent after PCR is performed.⁸ It was determined that there was no (\pm one standard deviation) difference between the results obtained using either approach after comparing resultant PCR product concentration determined through the semi-quantitative method used by the Agilent 2100 Bioanalyzer for analysis (data not shown). Consequently, random decamers were used throughout the remainder of this work.

Due to the more rapid temperature transitions of the IR-PCR system, the reaction time was reduced from 3.5 hours on a conventional thermal cycler to 2 hours simply by using the IR-PCR system with conventional times for each step. There is a clear advantage to simply using a system that allows for faster temperature transitions in microchip PCR, and this will be further demonstrated throughout the rapid RT-PCR amplifications performed in this work. Previous work has demonstrated that the time necessary for each step involved in the PCR process can be reduced when performing the amplification on a microdevice to decrease analysis time,^{9, 10} but as yet it had not been determined if the RT time could be reduced as well. Studies were performed in-tube to ascertain whether decreasing RT time would negatively affect downstream PCR amplification. RNA isolated from semen was subjected to RT, initially for the manufacturer-recommended time of 60 min, and then reduced to 30, 15, and 2 min. The resulting cDNA was then amplified conventionally and the Agilent 2100 Bioanalyzer was used to determine the resulting concentration of the 153-bp PCR product from the electropherograms depicted in **Figure S-3**. There was no significant difference in the

resulting amplicon concentration when RT times were reduced from 60 min to 30 or 15 min [4.5 (\pm 0.7) ng/ μ L, 3.8 (\pm 2.5) ng/ μ L, and 3.4 (\pm 0.2) ng/ μ L, respectively]. There was a slight difference between the concentration of amplicon from the 2 min incubation [2.5 (\pm 0.7) ng/ μ L] relative to that resulting from the 60 min sample [4.5 (\pm 0.7) ng/ μ L], but this difference is acceptable when the corresponding benefit is to reduce the RT time by 58 min.. These results suggest that the same would hold true for microchip RT-PCR and that the total analysis time could be successfully reduced further.

Two-chip system for SPE and RT-PCR

For initial two-chip system experiments, a load solution containing 67 ng of human total control RNA was loaded onto the SPE device, followed by a wash step to remove any unbound material. The elution buffer (10 mM Tris/50 mM KCl, pH 9) was heated to 90 °C prior to being pulled up into the syringe immediately before the elution step to ensure heat denaturation of the RNA while being eluted from the chitosan phase. As the RNA was eluted from the chitosan, 2 μ L fractions were collected from the outlet of the device. From previous elution profile studies⁵, it was determined that the third 2 μ L fraction would contain the most concentrated fraction of the eluted RNA. Therefore, this fraction was collected in a tube containing reagents for RT-PCR and a portion of this mixture was loaded into the 500 nL chamber on a PCR device. RT-PCR was performed using the IR-PCR system, and after analysis of the PCR product, the electropherogram shown in **Figure S-2B** depicts the successful amplification of a 353-bp portion of the β -actin gene. The results in **Figure S-2B** demonstrate the first example of microfluidic solid phase extraction using chitosan followed by RT-PCR amplification on a second microdevice utilizing the IR-PCR system. To facilitate future integration of SPE and RT-

PCR on a single microdevice, tests were performed to determine whether the heat denaturation step (performed here by eluting with heated elution buffer) was necessary. Heat is commonly used to denature any secondary structure that might hinder RT from making cDNA copies of RNA template, but this step can often be eliminated with no negative impact on PCR product yield.⁸ The same two-chip experiment as above was performed using human total control RNA with room-temperature elution buffer. **Figure S-2C** shows that the results of the two-chip process without heating the elution buffer are comparable to, if not better than, results with heated elution buffer, indicating that no heat denaturation step is necessary to perform successful two-chip analyses with these samples. Based on these results, the heat denaturation step was eliminated from the procedure for all further experiments.

To simulate a two-chip method for identification of biowarfare agents, 67 ng of RNA from influenza A virus-infected cell culture was used as a sample. Additionally, as control experiments to ensure any PCR product was the result of amplification of the influenza virus RNA, 33.5 ng RNA from non-infected cells (mock) were tested as well as a blank (no RNA) sample. Primers were used for a fragment of segment 5 of the influenza A viral RNA genome, which encodes the nucleoprotein (NP) which acts as a viral RNA binding protein and participates in nuclear import regulation.¹¹ In **Figure S-4**, the 240-bp amplicon indicative of the nucleoprotein gene from influenza viral RNA is present demonstrating the success of using the two-chip method for viral RNA purification and amplification. The inset panels in **Figure S-4** show a clear-cut absence of an amplicon in both mock and blank control experiments, not surprisingly, as expected neither contained viral RNA. These results indicate that viral RNA present in samples

infected with virus from viral biowarfare agents can be detected and identified using a two-chip microfluidic method for SPE and RT-PCR.

Perhaps the most significant result of the two-chip experiment was the substantial decrease in total analysis time achieved using the microfluidic system and IR-PCR instrumentation. The RT-PCR amplification with the two-chip system was reduced from 2 hours to 60 min (largely due to the 1 min for the RT instead of the manufacturer-recommended 60 min). The 5 min hold time at 94 °C to inactivate the RT was also reduced to 1 min, contributing to the overall time reduction for RT-PCR.

Integrated SPE-RT-PCR on a single microdevice

Integration of SPE-RT-PCR was attempted using a load solution containing 67 ng of influenza A viral RNA. This large mass of viral RNA was tested first in this proof-of-concept experiment to guarantee a sufficient mass of viral RNA would be present in the RT-PCR chamber. During elution of RNA, master mix for RT-PCR was simultaneously flowed through the sidearm channel and allowed to mix with the eluting RNA in a ratio of 3:2 (master mix to eluted RNA) – again, the same ratio of master mix to RNA present when successful RT-PCR was performed on a microdevice without integrated SPE. While the mixture of master mix and RNA eluted from the device, twelve 2 µL fractions were collected to ensure that the 25th µL of eluting RNA and master mix would be trapped in the PCR chamber of the device. RT-PCR amplification of the trapped fraction was then performed utilizing the IR-PCR system under conditions allowing for 39 min total RT-PCR analysis as previously discussed. The resulting PCR product was removed from the device and the Agilent 2100 Bioanalyzer was used to detect whether the 240-bp amplicon indicative of the influenza A nucleoprotein was present. **Figure S-6** shows the

resulting electropherogram where, indeed, the 240-bp fragment expected is present. These results demonstrate the success of the integrated SPE-RT-PCR system for both RNA purification and amplification of cDNA on a single device for the identification of a simulant for biowarfare agents. This is also the first example of integration of SPE with RT-PCR performed in less than 40 minutes, establishing a prototype for more rapid analysis of RNA. However, since this sample was at the high end of the range explored previously on the two-chip system, the single chip system for integrated SPE-RT-PCR was evaluated using mock clinical samples containing a 35-fold reduction in the mass of the loaded RNA.

Supplemental Material- Figures

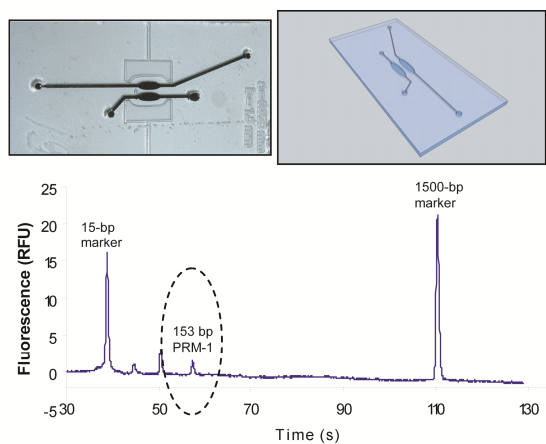


Figure S-1. The 153-bp amplicon indicative of the semen-specific protamine 1 gene is present in the resulting electropherogram after RT and PCR were performed as a single reaction on a microchip utilizing the IR-PCR system (representative of $n=3$). A 2-fold reduction in RT-PCR analysis time is demonstrated.

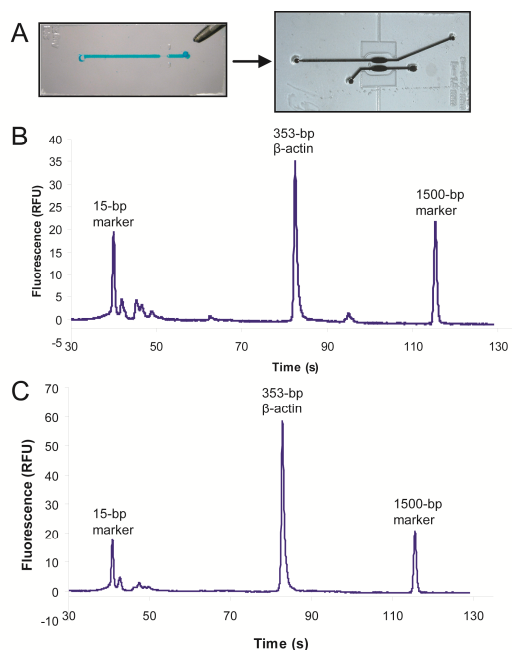


Figure S-2. A two-chip system was used to perform microchip SPE followed by microchip RT-PCR utilizing the IR-PCR system. A 353-bp fragment of the β -actin gene is present in the electropherograms after the two-chip system was used to process human total RNA (representative of $n=3$). A) SPE and PCR microdevices used for the two-chip system. B) Electropherogram resulting after a heated elution buffer was used during SPE (representative of $n=3$), and C) after the use of room-temperature elution buffer (representative of $n=3$).

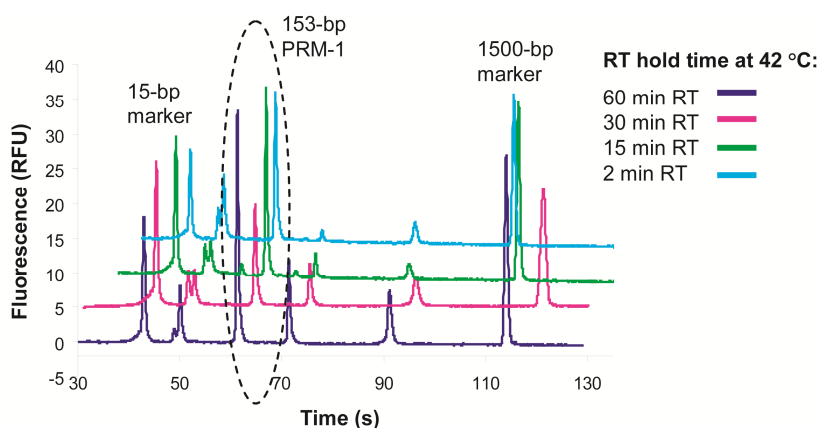


Figure S-3. Conventional RT-PCR was performed with decreasing RT time reduced from 60 min to 30, 15, and 2 min ($n=3$). From the concentration of PCR product in the 153-bp protamine 1 amplicon for each RT time, it was determined that there was no statistically-significant (\pm one standard deviation) difference when RT was performed for 60 min as compared to 30 or 15 min. PCR after RT performed for 2 min was also successful, indicating that RT times can be significantly reduced.

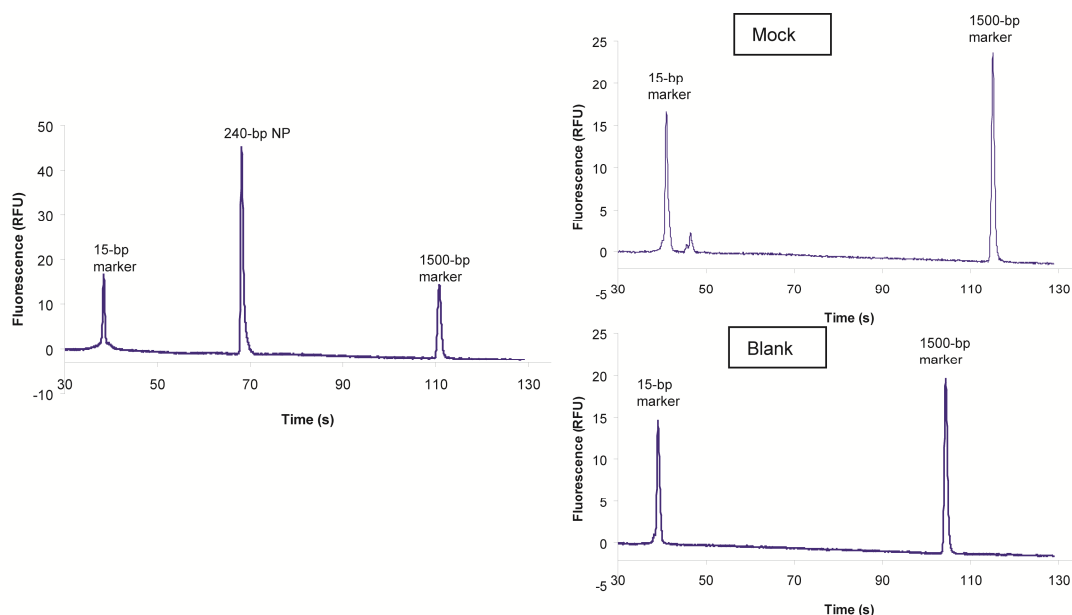


Figure S-4. A two-chip system was used to perform microchip SPE from 67 ng influenza A viral RNA, followed by microchip RT-PCR in 60 min using the IR-PCR system. A 240-bp amplicon of the nucleoprotein indicative of influenza A is present in the resulting electropherogram (representative of $n=3$). The two-chip system was also used to perform SPE and RT-PCR from “mock” (non-infected) cells and a “blank” (no RNA) (representative of $n=3$). The 240-bp amplicon is absent in both (insets).

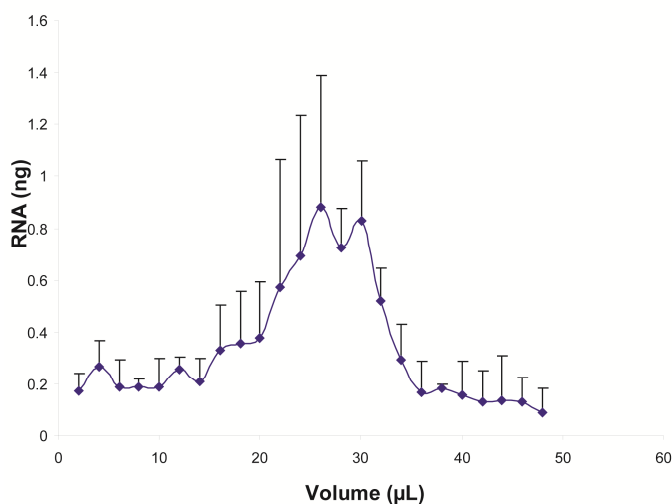


Figure S-5. Elution profile of influenza A viral RNA on the integrated SPE-RT-PCR device. The most concentrated portion of the eluted RNA spans the 24th-30th μL .

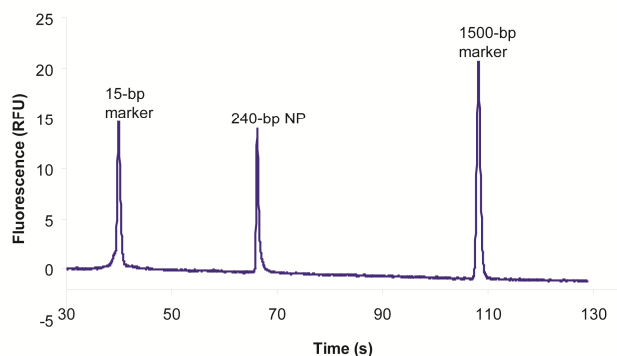


Figure S-6. Integrated SPE-RT-PCR was performed on a single device using 67 ng influenza A viral RNA, and in the resulting electropherogram, the 240-bp amplicon indicative of the influenza A nucleoprotein is present demonstrating successful sample purification and amplification on a single microdevice (representative of n=3).

References

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