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Parallel Picoliter RT-PCR Assays Using Microfluidics

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The development of microfluidic tools for high-throughput nucleic acid analysis has become a burgeoning area of research in the post-genome era. Here, we have developed a microfluidic chip to perform 72 parallel 450-pL RT-PCRs. We took advantage of Taqman hydrolysis probe chemistry to detect RNA templates as low as 34 copies. The device and method presented here may enable highly parallel single cell gene expression analysis.

Although there are numerous approaches available for detecting the presence of nucleic acids, the methods most commonly utilized are PCR and reverse transcriptase-PCR (RT-PCR) for DNA and mRNA, respectively.[1] Microfluidic technology offers added advantages for PCR assays, including, but not limited to, decreased cycling times due to high surface-to-volume ratios and, therefore, rapid thermal energy transfer, smaller reaction volumes, portability, and lack of contamination issues.[2] Our group has previously demonstrated microfluidic devices with integrated micromechanical valves as a viable solution to perform parallel PCR in volumes as small as 3 nL.[3,4] Although microfluidic PCR has been demonstrated by numerous groups,[2] there has been little previous work on integrating the reverse transcription enzymatic step into microfluidics.[5−7] In previous studies, material was removed from the chip for detection, starting templates were on the order of 10 million gene-specific RNA molecules, and RT reaction volumes were 5 μL.

Here, we have fabricated microfluidic devices by multilayer soft lithography (MSL)[8] to demonstrate sensitive, highly parallel RT-PCR. We have capitalized on the advantages of small reaction volumes available with microfluidic assays and performed 72 parallel RT-PCR reactions in 450-pL reactors using a design suggested by Unger et al.[9] Using endpoint detection, we were able to detect less than 50 β-actin transcripts from a total RNA template and, thus, achieved the detection sensitivity[10] required for single cell analysis.

EXPERIMENTAL SECTION

Device Fabrication. All devices were fabricated using the process of MSL.[8] Devices were composed of three layers of the silicone elastomer poly(dimethylsiloxane) (PDMS) bonded to a glass coverslip and were fabricated as previously described,[11] but with push-up valve geometry.[12] Negative master molds were fabricated out of photoresist by standard optical lithography and patterned with 20 000 dpi transparency masks (CAD/Art Services). SU8-2025 (Microchem) (24 microns high) was utilized for the control molds. The flow master molds were fabricated out of 10-μm features that were defined in 5740 photoresist (Shipley).

General Device Operation. The on−off valves within each device are controlled by individual pressure sources (Fluidigm). The pressure sources are actuated by a NI-DAQ card (National Instruments) and Labview graphical interface.

RT-PCR Device Operation. Each chip contains eight flow channels which are filled in a west to east fashion with a master RT-PCR reaction. There are two control channels, which form valves when crossing their respective flow channels. When initially filling a specific flow channel, the vertical control channel (Figure 1, green) is opened to allow for purging of the respective flow channel with RT-PCR cocktail. The vertical channel is then closed, and the horizontal reaction partition control channel (Figure 1, blue) is opened, allowing for dead end filling of the nine 450-pL reactions. The reaction partition control channel is closed once the reactors are filled. The 50-μm-wide guard channels[9] (Figure 1, channels filled with yellow food dye) are then pressurized to prevent flow channel evaporation. All control and guard channels are dead-end filled with water.

RNA Amplification and Detection. We utilized human male total RNA as template and probed for a 240-bp portion of the

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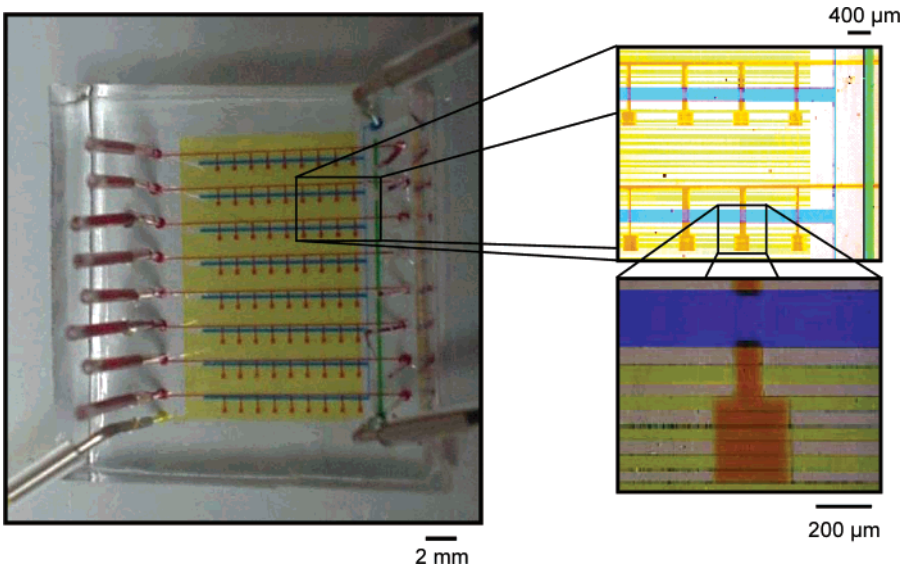

Figure 1. RT-PCR device. Left: Photograph of the device loaded with food dye. Guard channels, which supply water vapor to the porous PDMS, are filled with yellow dye, flow channels are filled with orange dye, and the two control channels are filled with blue (vertical control channel) and green dye (horizontal reaction partition control channel). Right: Optical micrographs of eight reaction chambers (upper) and one reaction chamber (lower).

β-actin gene using primers and Taqman probe supplied in an Applied Biosystems kit (Part no. 401846). Each probe contains a reporter dye (Alexa 488) covalently attached to the 5′ end and a quencher dye covalently attached to the 3′ end of the probe. Fluorescence resonance energy transfer (FRET) between the two dyes enables the shorter wavelength reporter to be quenched by the longer wavelength dye when the probe is intact. The 5′-to-3′ exonuclease activity of *Taq* polymerase cleaves the probe and thereby increases the distance between the two dyes, and in so doing augments the fluorescence emission of the reporter. Once the chip was loaded, thermal cycling was performed on a flat plate thermal cycler according to the following protocol: 50 °C for 30 min, 95 °C for 10 min, and 30 cycles of 94 and 58 °C for 20 and 50 s, respectively. The reaction chambers on the chip were then probed for fluorescence with a modified flat-bed ArrayWorx scanner (Applied Precision).

RESULTS AND DISCUSSION

We fabricated microfluidic devices with the purpose of performing RT-PCRs in parallel picoliter reactions. A prototype device, with its control and flow lines filled with food dye, is shown in Figure 1. Each chip has eight flow lines, so multiple templates and master mixes can be varied up to 8-fold on a single device. This multiplexing ability allows for standard curve generation, utiliziation of multiple primer sets, and microfluidic reaction optimization on a single device. The RT-PCR cocktail is mixed off-chip, loaded onto the device in a west to east manner, and divided into nine 450-pL chambers, for a total of 72 chambers per chip. Because the chip's chambers have a geometrically defined volume, error due to pipetting variability is not an issue.

Initial experiments with these devices demonstrated proof of principle. We verified the PCR chemistry and cycling parameters by performing PCR on a β-actin cDNA template and detected DNA templates ranging from 6 to 18 000 molecules (data not shown).

We then utilized three devices to demonstrate RT proof of principle and to determine RNA detection sensitivity. Results from these experiments are given in Figure 2. Figure 2a shows a fluorescence scan of a chip utilized to verify RT feasibility. We kept the RNA template constant at 50 ng/μL (Figure 2a, middle four lines) and employed the first two and last two lines as no-template controls. We then used a hydrolysis probe to detect a segment of the β-actin gene, which is present in high abundance[11] inside most cells, with copy numbers ranging from 2000 to 3000 per individual cell.[13] When considering the amount of mRNA in one cell is ~1 pg and 1% of total RNA,[14] we estimate the positive template reactors to contain 680 β-actin copies each. The scan shows a 2.4-fold difference in fluorescence between the positive and negative template reactors.

Next, we determined the sensitivity of the device by varying the total RNA template concentration. RNA was serially diluted to establish a range of 0−4 pg of total RNA per reaction chamber (Figure 2b). Using the same reasoning as above, we estimate the β-actin copy numbers per reactor to be 0, 34, 68, and 136 copies, respectively. Figure 2b shows a graph of mean fluorescence values for the respective chambers of a chip after RT-PCR. This detection sensitivity is comparable to conventional real time RT-PCR in our hands (50 copies/reaction, Figure S-1 of the Supporting Information). There was an increase in fluorescence up to ~50 copies/chamber, followed by a plateau region. We interpret the fluorescence plateau at higher template concentrations to mean our reaction conditions were not optimal or we were detecting in the plateau phase of the reaction. If conditions were not optimized, it is reasonable to speculate reagents depleted before the cycling ended. Accumulation of product may have inhibited further synthesis of the β-actin gene segment. Nevertheless, the mean fluorescence values for the different positive templates were significantly different from the no-template control, as measured

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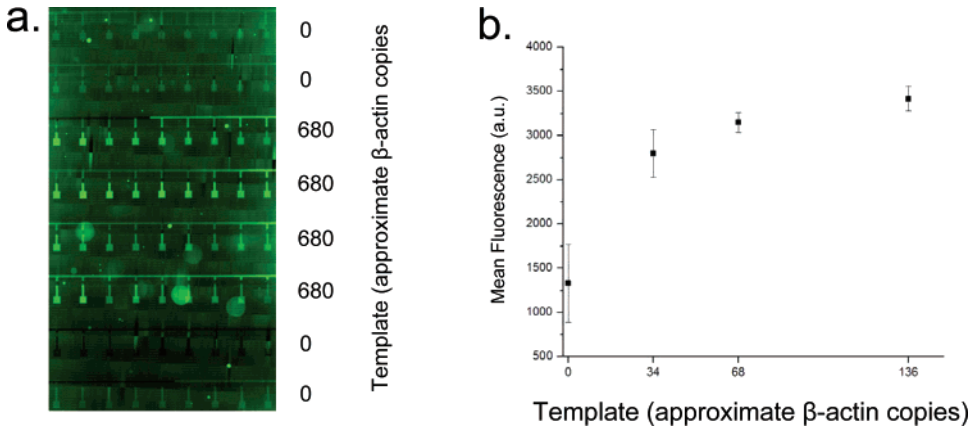

Figure 2. RT-PCR results. A. Scan of a chip after RT-PCR. We are detecting fluorescence at 530 nm, the emission wavelength of the reporter dye (FAM) attached to the Taqman probe. B. Mean fluorescence data from the low-end serial dilution experiment. Points are mean fluorescence of chambers ± standard error after RT-PCR.

by paired *t*-tests. To rule out the possibility of false positive results due to genomic DNA (gDNA) contamination, we ran off-chip real time RT-PCRs with no RT enzyme along with serially diluted human male total RNA templates. Amplification was limited to reactions with 500 pg or 50-pg templates (Figure S-1). We calculated the percent of gDNA contamination by plotting the Ct values of the amplified no-RT reactions onto a standard curve generated with the RT enzyme (Figure S-1). We then divided the calculated template by the initial template present in the respective reaction and arrived at a value of 0.3% for gDNA contamination. Therefore, 333 β-actin molecules would need to be present in a reaction chamber to achieve amplification of 1 gDNA molecule.

It has become popular to use real time PCR, in which detection of products is performed after each thermal cycle, to obtain quantitative information about the amount of starting material. In the work described above, detection was performed at the end of thermal cycling, but it would be straightforward to modify the scanner to include a thermocycling capability such that real time data could be acquired from our device.

CONCLUSIONS

These results demonstrate that RT-PCR assays can be implemented with success on a microfluidic platform, in a regime useful for single cell analysis. We were able to detect a signal for low-abundance mRNA templates in a parallel fashion. The ability to detect low and medium copy transcripts, as presented here, may enable future studies on gene expression in individual cells.

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SUPPORTING INFORMATION AVAILABLE

Additional information as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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