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Construction of a microfluidic chip, using dried-down reagents, for LATE-PCR amplification and detection of single-stranded DNA

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LATE-PCR is an advanced form of non-symmetric PCR that efficiently generates single-stranded DNA which can readily be characterized at the end of amplification by hybridization to low-temperature fluorescent probes. We demonstrate here for the first time that monoplex and duplex LATE-PCR amplification and probe target hybridization can be carried out in double layered PDMS microfluidics chips containing dried reagents. Addition of a set of reagents during dry down overcomes the common problem of single-stranded oligonucleotide binding to PDMS. These proof-of-principle results open the way to construction of inexpensive point-of-care devices that take full advantage of the analytical power of assays built using LATE-PCR and low-temperature probes.

1. Introduction

Point-of-care (POC) diagnostic testing is a driver of innovation in the field of medical technology. POC devices aim at rapid and cost effective generation of high levels of information in any setting. In the realm of infectious diseases immediate information identifying the type, strain, and drug resistance status of a pathogen can make it possible for physicians and healthcare workers to maximize the chances of patient survival and minimize the risk that the infection will spread to other members of the community. Achieving these goals in a POC device requires integration of novel technologies at every level, including the chemistry used for molecular detection, the engineering of the devices themselves, the software used for analysis and management of the results, and the training of individuals who perform the test.

PCR (polymerase-chain-reaction) is a fundamental method for amplification and analyses of specific DNA and RNA targets. Not surprisingly many innovative microfluidic chips have been designed to achieve both static and dynamic PCR amplification in small chambers and moving droplets for POC.[1–7] In some of these systems the chips are preloaded with reagents needed for PCR amplification and related steps. For instance, Hoffmann *et al.* pre-stored liquid reagents on-chip for DNA extraction.[8] Brivio *et al.* demonstrated on-chip PCR amplification with freeze-dried reagents stored in polymer PCR chips.[9] Kim *et al.* preloaded paraffin-sealed dried reagents on-chip for PCR running at a later time.[10] But the process of preloading in all of these cases was carried out during the chip manufacturing, which increases the difficulty of chip assembly and reproducibility. Most importantly all previous microfluidics based PCR systems, whether preloaded or not, have been designed to amplify products *via* symmetric (conventional) PCR. In reactions of this type, high concentrations of two primers having equivalent melting temperatures are used to generate double-stranded DNA products. In microfluidic devices the initial concentrations of both primers are kept particularly high in order to compensate for the loss of these single-stranded oligonucleotides on the surfaces of the chip.

This report describes our first steps in the design of micro-fluidic devices that carry out Linear-After-The-Exponential (LATE) PCR.[11,12] LATE-PCR is an advanced form of non-symmetric PCR that uses a limiting primer (at 100 nM or less) and an excess primer (at 1000 nM) to efficiently amplify double-stranded DNA followed by single-stranded DNA. This is made possible by the fact that the melting temperature of the limiting primer at its initial concentration is equal to or higher than the melting temperature of the excess primer at its initial concentration. LATE-PCR reactions automatically switch from exponential amplification of double-stranded products to linear amplification of single-stranded DNA when the limiting primer is used up. Thereafter the single-stranded DNA accumulates and outnumbers the double-stranded DNA by 10 to 20 fold. Each strand can readily be sequenced by a convenient Dilute-'N'-Go protocol.[13,14]

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The accumulated single-stranded DNA can also be probed by using either low temperature sequence-specific molecular beacon probes, or low temperature mismatch tolerant probes which hybridize to sequence variants at different temperatures. Lights-On/Lights-Off probes are a novel class of low temperature mismatch tolerant probes that are used in sets to coat long stretches of DNA and thereby scan a target sequence for both known and unknown mutations. All of the Lights-On/Lights-Off probes in a set are labelled in the same fluorescent colour.[15]

This paper also reports for the first time that it is possible to preload already manufactured microfluidic chips with all of the reagents needed for LATE-PCR amplification and product detection. The problem of reagent loss during the steps of preloading, drying and suspension was intensively evaluated and overcome. Both monoplex and duplex LATE-PCR assays were successfully run on chip with and without dried reagents.

2. Materials and methods

2.1 Chip design and fabrication

The devices, shown in Fig. 1a were designed in AutoCAD software and prepared as double-layer PDMS (RTV) on glass using multiplayer soft lithographic techniques, following the protocols described previously.[16] Flow channels (green) and PCR chambers (red) are in the upper thick (5 mm) layer. Reservoir and control valves (blue) are in the lower thin (40 μm) layer separated from the thick layer by a 15 μm thick PDMS membrane. Fig. 1b depicts the cross section of the device and its basic operation principle. Because PDMS is water and air permeable, water in the PCR chamber would evaporate mainly through the thin membrane to the empty reservoir channels. There is also a small flux through the 5 mm thick PDMS layer to the exterior. If the PCR chamber is filled with PCR reagents, it would dry up in the chamber when the water totally evaporates out. For the same reason, water would evaporate away during the PCR thermal cycling, which is undesirable. Under this situation, the reservoir is filled up with DI water to compensate the loss of water in the PCR chamber to keep all the reagents concentrations concrete during PCR cycling.

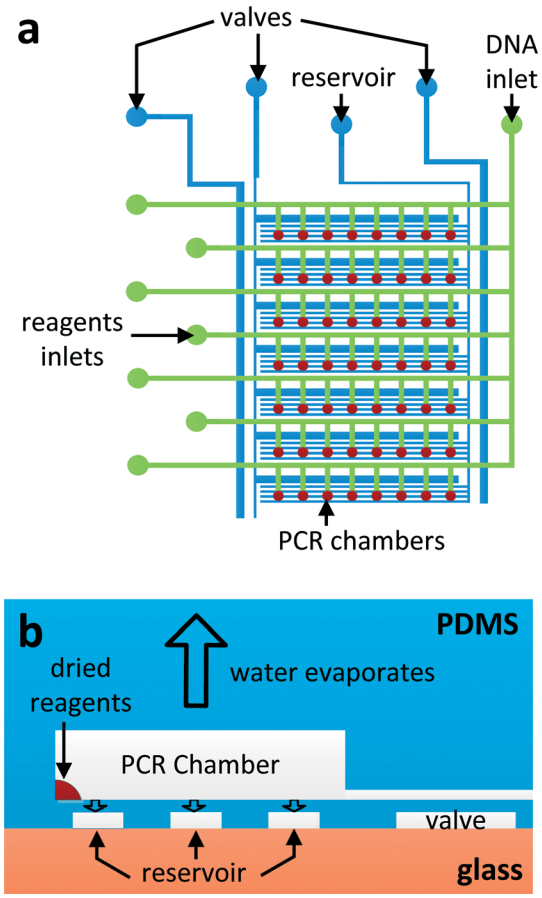

Fig. 1 Chip design: a) plane view of the chip; b) cross section of the channel and the schematic, showing how the chip works for drying down reagents and for PCR amplification. The two layers are separated by a 15 μm thick membrane. The upper layer contains the flow channels and the PCR reaction chambers while the bottom layer has the control valves and reservoirs. The fluidic flow channel is 100 μm wide and 15 μm high, while the control channels are slightly larger, *i.e.* 150 μm wide and 30 μm high. The PCR chamber is pancake shaped 300 μm in diameter and 100 μm high. The reservoirs have a width of 75 μm. At the positions where the control channels meet the flow channels while no valves are involved, the control channels are 75 μm in width so that it does not close the flow channel with an air pressure of less than 25 psi. Holes are punched at the inlets and outlets of the flow, control and reservoir channels are included for sample loading and controlling sample flowing.

2.2 Loading, drying and re-suspending PCR reagents on chip

The samples were loaded through the reagents inlet holes using 0.02'' Tygon tubing (Cole-Parmer, IL) fitted with blunt-end, glass tubing (0.20 mm ID, 0.63 mm OD) (Drummond Scientific Company, PA). 10 psi nitrogen air pressure was used to load the samples and 25 psi was used to close the valves as needed.

During the drying experiments, different composites of the PCR reagents were loaded by 10 psi air pressure into the PCR chambers through the reagents inlet holes of the chip kept on ice, while the DNA inlet hole was closed by on-chip valves. Then the DNA inlet valve was opened to flush out the excess solutions in the tubing and to let air pass through the channels to dry the drop in the chamber. Flushing of the flow channel lasted about 5 min, which was sufficient to shrink the drops till all the contents were totally in the PCR chamber. Then the chip was put on a hot plate at 95 °C for 5 min to thoroughly dry the reagents in the chamber.

After drying, the preloaded chips were stored in a sealed jar filled with $CaCl_2$ packs for later use. When re-suspending the reagents, solutions were filled with 10 psi pressure to the chamber until all the air bubbles had totally disappeared. It took roughly 5 minutes or less with a larger air pressure. The chip was kept on ice during the entire re-suspending process. Once the chambers were filled up, they were closed by a 25 psi pressure in the control channels before running PCR. A 10 psi pressure was given to the reservoir channels filled with DI water to keep compensating for the water lost during PCR cycling.

2.3 PCR testing system

Proof-of-principle monoplex experiments were carried out using a previously described sequence within the human G269 gene that was 312 bp long in order to demonstrate on-chip LATE-PCR amplification, as well as on-chip reagent dry down and re-suspension.[13] Proof-of-principle duplex experiments used two pairs of primers within the same segment of the G269 gene target, as illustrated in Fig. 2. One amplicon generated with LP1 and XP1 (Table 1) was 94 bp long and was detected with a Taqman® probe labelled with a 5' Cal Red 590 fluorophore and a 3' Black Hole Quencher 2 (BHQ2). The second amplicon generated with LP2 and XP2 (Table 1) was 85 bp long and was detected with Taqman® probe labelled with a 5' Quasar 670 fluorophore and a 3' BHQ3.

Primer design followed the criteria for LATE-PCR[11,12] such that the T_m of the limiting primer was 3–5 °C higher than that of the excess primer at their respective initial concentrations. Probes were designed in accord with the design criteria for Taqman® probes and the T_m of each probe was 8–10 °C higher than that of the excess primer and 10–12 °C higher than the annealing and extension temperature of the thermal cycle. These probes hybridized to the limiting primer-strand and because they remained hybridized at the temperature of excess primer extension, they are cleaved during both the exponential phase and the linear phase of the amplification. A low T_m molecular beacon probe with a T_m 10 °C lower than the annealing temperature was used in one set of experiment so that it did not interfere with the amplification. All primers and probes were purchased from Biosearch Technology (CA). All the primers were desalted and purified and all the probes were double HPLC purified.

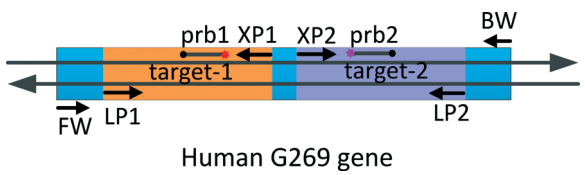

Fig. 2 On-chip LATE-PCR testing system.

The template in the proof-of-principle monoplex experiments was the 312 bp G269 target sequence in its double-stranded form. This target was prepared by 25 cycles of symmetric PCR amplification using 25 nM of each of a forward (FW) and backward (BW) pair of primers (Table 1) with a thermal profile of 95 °C for 5 min followed by 60 cycles of 2-step PCR with 95 °C, 10 s and 60 °C, 20 s.

The complete PCR mixture was comprised of 1× PCR buffer (Invitrogene, Carlsbad, CA), 3 mM $MgCl_2$, 200 nM dNTPs, 100 nM each limiting primer and 1 μM each excess primer, 500 nM each probe, 1× additive reagent (AR), see below, and 1 unit of Platinum Taq DNA polymerase (Invitrogene, Carlsbad, CA) with different amounts of DNA target. 1× AR consists of 0.2 mg ml^{-1} BSA, 150 mM Trehalose and 0.2% Tween-20. The initial concentration of the DNA target was established by serial 10 fold dilutions in standard PCR tubes. The dilution at which only about one third of the replicates gave a signal was judged to be the single target molecule level.

The off-chip thermal profile of LATE-PCR was optimized on a Stratagene Mx 3500P PCR machine. The on-chip PCR was run on a flat-surface PCR machine, AmpliSpeed 200D (Advalytix AG, Munich, Germany). In light of the temperature difference between the plate of the PCR machine and in the PCR chamber, we optimized the thermal profile on-chip by running a series of experiments with different annealing temperature and annealing time to obtain the brightest fluorescent signal. The optimized thermal profiles for on-chip analysis are two-step PCR including 5 min at 95 °C followed by 50 cycles of 95 °C, 10 s and 58 °C, 40 s. After PCR amplification, the microfluidic chip was then put under a fluorescence microscope (Olympus) with advanced imaging software Slidebook (Olympus) for the fluorescence reading.

3 Results

3.1 Re-suspending dried reagents

The drying process has been described in Method and materials section. For running PCR, the dried reagents were

Table 1 PCR target, primers and probes (5'–3')

Sequence name	Sequence
G269 gene piece	CGAGGTCATTGAATACGCACGGCTCCGGGGTATCCGTGTGCTTGCAGAGTTTGACACTCCTGGCCACACT TTGTCCTGGGGACCAGGTAAGAATGATGTCTGGGACCAGAGGGACTCTGCTTGTTATGCTCAGAGTGAAG CTTCAGGGCACTGGCTCATGGAAGTGGCATATCCCAGCTTGGTCCTTAGAAGAATGTTTTCCATCGACTTC TTCCACCTGGGAATTTAGATAGGAAGAACTCACTTTGGACAATGGAGGCTGCTTCTTACTATTAAAATATGT ACTGTTAGACTATGTAAGGGCACAGCGC
FW	GAATGATGTCTGGGACCAGA
BW	GACCAAGCTGGGATATGCC
LP1	GAATGATGTCTGGGACCAGAG
XP1	GACCAAGCTGGGATATGCC
Prb1	Cal red 590-CCATGAGCCAGTGCCCTGAAGC-BHQ2
LP2	TGCCCTTACATAGTCTAACAGT
XP2	CCACCTGGGAATTTAGATAGG
Prb2	Quasar 670-GGACAATGGAGGCTGCTTCTTAC-BHQ3
LPMB	CTGTGCCCTTACATAGTCTAACAGT
XPMB	ATCGACTTCTTCCACCT
PrbMB	Quasar 670-CGTGCTCCATTGTCCAAACACG-BHQ2

re-suspended at the same time that DNA was added. Fast mixing of the reagents during re-suspension would be desirable. To test the time it takes for mixing the reagents in a microfluidic chamber after re-suspension, we used a DNA oligonucleotide with a fluorophore attached at one end as a model. This DNA was dried in the chambers. Then water was refilled through the DNA inlet hole. The chambers were monitored under microscope with the time lapse fluorescence reading. Fig. 3a shows a typical chamber when the labelled oligonucleotide was re-suspended in water. Before the water reached the dried DNA pellet, the pellet was much bright with a saturated fluorescence. Once the water hits the pellet, the probe begins to quickly diffuse in the chambers, making the chambers more homogeneous. The fluorescence was averaged in each region labelled in Fig. 3a and shown in Fig. 3b. The strongly inhomogeneous fluorescence appears in the first couple of minutes when the water just contacts the pellet and the fluorescence begins to diffuse. After about 20 min, the distribution was stable with time. There was a slight difference between regions. This is attributed to the inconsistencies in the imaging system. All these images were obtained at room temperature. In the later experiments, we observed that mixing is much faster when the chip is heated during PCR. Thus, the mixing time is not a problem if PCR started immediately after reagent suspension.

3.2 The function of additive reagents during drying and re-suspension

To assess how much of the reagent oligonucleotides are lost by adhesion to the PDMS during the processes of drying and suspension, the level of fluorescence in each chamber was monitored before drying and after suspension. Additive reagents (AR) including 0.2 mg ml^{-1} BSA, 150 mM Trehalose and 0.2% Tween-20 were also tested to minimize the loss of DNA during the preloading process. As shown in Fig. 4, without AR, the fluorescence after suspension is about 80% of its level before drying. This indicates about 20% of these single-stranded DNA oligonucleotides are lost during the drying process. Furthermore, 50 nM probe has similar fluorescence intensity as the device background intensity, both before and after drying. This indicates that when the probe is less than 50 nM the majority of the probe was lost during the loading process. In contrast, in the presence of AR, there is no fluorescence difference before drying and after suspension. Moreover, probe fluorescence in the presence of AR increases linearly with probe concentration and the fluorescence of even 50 nM probe is now well above background. We conclude that the cocktail of AR significantly decreases adhesion of single-stranded oligonucleotides to the PDMS during loading, drying and suspension.

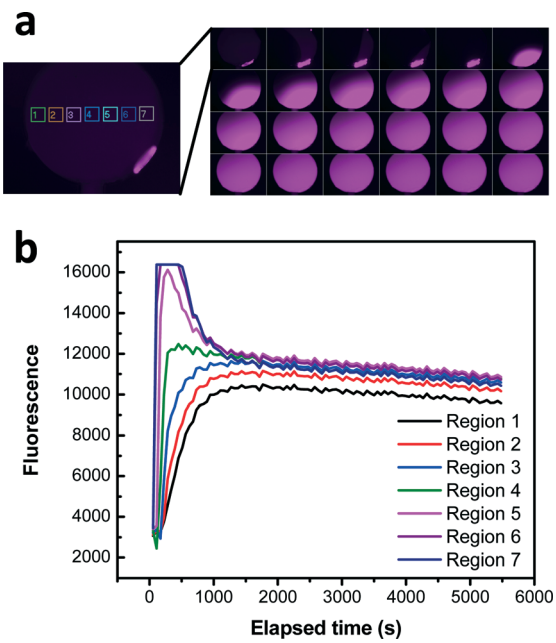

Fig. 3 Re-suspension of dried DNA in water: a) time elapsed images of one chamber as the dried DNA is re-suspended in water, 55 s between each frame; b) fluorescence at different locations labelled in a) with time elapsed.

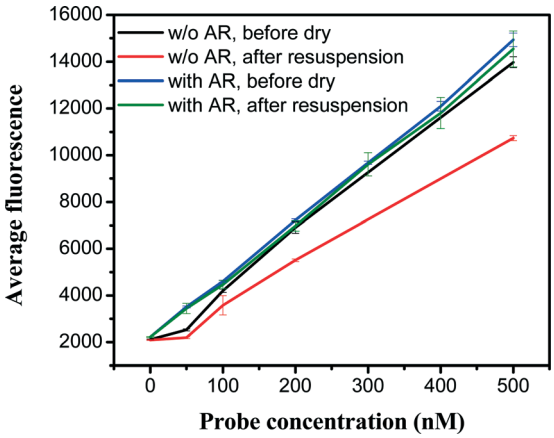

Fig. 4 DNA lost during the dry down and re-suspension process. 6–8 samples on average at each condition.

3.3 On-chip LATE-PCR

On-chip LATE-PCR was tested next using a mixture of freshly prepared reagents comprised of 100 nM limiting primer (LP1/LPMB), 1000 nM excess primer (XP1/XPMB), 500 nM probe (Prb1/PrbMB), 1× AR, and different concentration of G269 double-stranded DNA. All the chambers in each row on chip (Fig. 1a) have the same PCR cocktail, which give us eight replicates for each condition.

As shown in Fig. 5a, the fluorescence of samples containing template DNA is much brighter than no-template control (NTC) samples. Even those samples receiving a single target molecule generated clearly detectable signals. In contrast to symmetric PCR, LATE-PCR can be used for end point detection of single-stranded DNA. The intensity of these end-point signals correlates with the number of target molecules added to the reaction.[12] As can be seen, the average fluorescence is quite linear in the range of 1–1000 initial DNA molecules in each chamber although the error bars are relatively large.

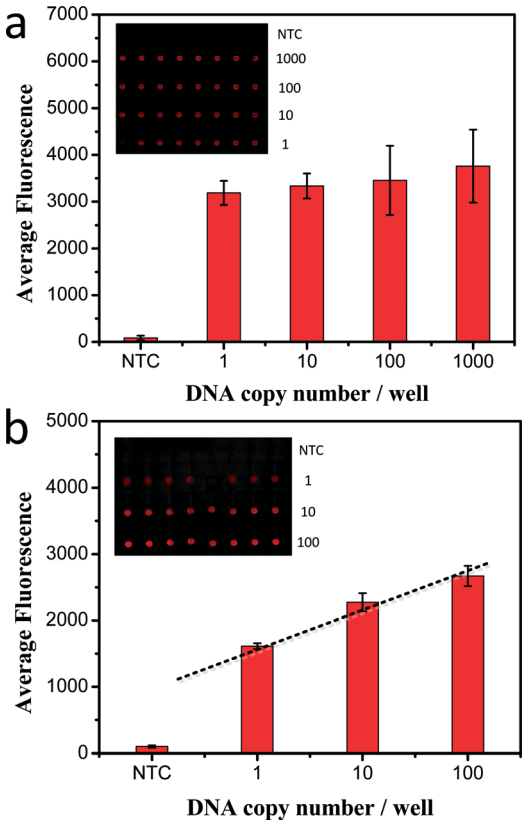

Fig. 5 Average fluorescence intensity after LATE-PCR in chambers having different initial concentrations of DNA. Only the chambers with fluorescence higher than the background were considered in calculating the average intensity.

This is due to the fact that Taqman® probes were used in these proof-of-principle experiments. Taqman® probes measure product accumulation during both exponential and the linear phase of LATE-PCR amplification. The linearly accumulating single-stranded products therefore generate only a small increase in signal intensity over the background of already accumulated double-strands.

In order to be able to utilize end-point analysis in LATE-PCR, we designed a set of primers (LPMB and XPMB) plus a low-T_m molecular beacon probe (PrbMB), T_m 48 °C, which only detects the single-stranded products. These reagents were tested using replicate tests in a serial dilution. The whole chip was incubated at 45 °C for 10 minutes after amplification and before taking images allowing the probe to bind to completion. As can be seen from Fig. 5b, the fluorescence signal increased linearly with target concentration from single copy to 100 copies per well. Thus, in accord with LATE-PCR reactions in standard tubes, the initial concentration of the target on-chip can be judged from the intensity of the signal at end-point.

3.4 What can be dried on chip?

In order to determine what can be dried on chip and what has to be loaded just before PCR amplification, different combinations of reagents were dried on chip. These preloaded chips were prepared in advance and were kept in a sealed drying container for a month before addition of the appropriate missing components at the time that the template DNA was added and the reaction was run. For a comparison, two rows were left not preloaded, so that a freshly prepared mixture of LATE-PCR reagents, with or without DNA, or just DNA was loaded immediately before running PCR. In the other empty rows, chambers were preloaded with the following components: primers only, primers and probe, primers and probe and dNTPs in PCR buffer, or everything except DNA. The other needed reagents for each row were loaded along with 1000 copies of DNA just before PCR. The reagent inlets were used to load DNA in this case, because each row needed a different mixture.

As shown in Fig. 6, dried primers and primers with probe generate relatively low intensity signals after PCR, as compared to fresh reagents. But the fluorescence intensity of samples prepared from dried primers and probes along with dNTPs in buffer is close to that of freshly prepared reagents. The most interesting version of dried reagents was that containing all reagents, including Taq polymerase. In this case, the AR cocktail contained the non-ionic surfactant Tween-20 and 1× PCR buffer contained 3 mM $MgCl_2$. This means that immediately after re-suspension of these reagents the local salt concentration would be very high. Fortunately, Taq polymerase appears to tolerate these conditions. This is consistent with the fact that Kim *et al.* have successfully dried all reagents on chip during the chip manufacturing.[17] Previous work has shown that KlenTaq from *Thermus aquaticus* is very stable in even 4 M guanidine hydrochloride and the chemical denaturation is reversible for KlenTaq.[18]

3.5 Converting monoplexes to multiplexes on-chip

Now that all reaction components except the DNA template could be combined and dried on chip, it was possible to test

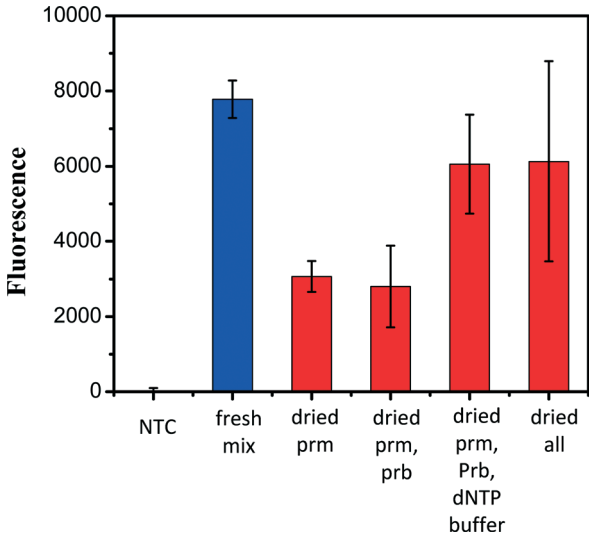

Fig. 6 Average fluorescence intensity after LATE-PCR in chambers with different initial DNA concentration. 8 samples on average.

LATE-PCR multiplexing on preloaded chips. Two targets were run in parallel and in a mixture. Fig. 7a and b show the fluorescent signal of amplification of 1000 and single copy target in each well respectively. The first two rows were for the amplification of the first target (target-1) from LP1, XP1 and monitored with Prb1. The second two rows were for the second target (target-2) from LP2, XP2 and monitored with Prb2. The last two rows were for duplex co-amplification of both targets. In all cases the whole gene piece were filled in from the DNA inlet holes and the reagents inlet holes were closed when filling up the chip. The top rows in each condition were controls in which no primers were included in the preloaded reagents.

As can be seen from Fig. 7a, all the amplifications ran well and gave clear fluorescence. In the case of the duplex reactions, both of the targets were amplified efficiently with the preloaded reagents. Further diluting the targets to the single copy level does not affect the amplification efficiency of either target, as shown in Fig. 7b. At the single copy level, some of the chambers, see arrows, did not give a signal because they lacked a target. Occasionally, at the single copy level one assay in the duplex was more competitive than the other, as shown in circles. Competition between targets was not observed at the 1000 copy level. Fig. 7c shows the average fluorescence of both targets amplified in monoplex and duplex assays of Fig. 7a. The amplification of both targets in the duplex assay is slightly less efficient than that in the monoplex assay, causing a decrease in fluorescence of about 10%.

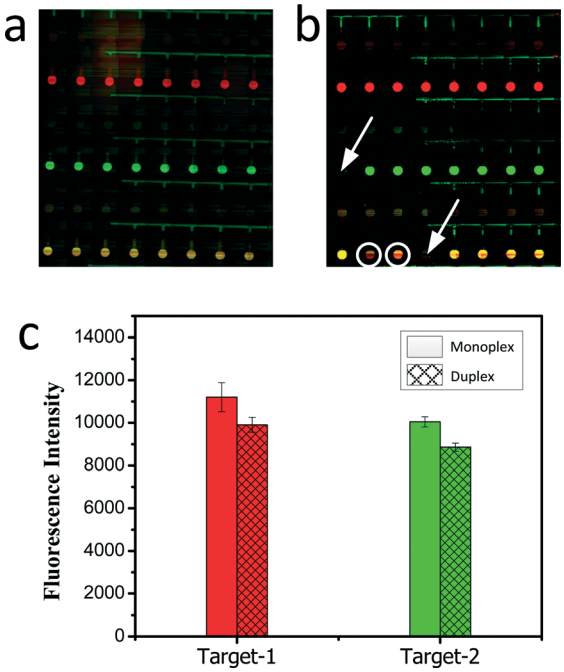

Fig. 7 Monoplex and multiplex LATE-PCR with all reagents preloaded on-chip. (a) 1000 copies DNA/chamber; (b) single copy DNA/chamber; (c) average fluorescence of each target in monoplex and duplex. The PCR conditions from the top row to the bottom in (a) and (b) are: NPC for target-1; target-1; NPC for target-2; target-2; NPC for both targets; duplex. NPC: No primer control.

4. Discussions and conclusions

In this report, we demonstrate that the complete cocktail of reagents needed for LATE-PCR amplification can be dried in preassembled chips and stored at room temperature for at least a couple of weeks. The reagents can then conveniently be re-suspended *in situ* immediately before use. These are important technical steps toward our goal of constructing cost-effective microfluidics POC devices using this new amplification chemistry.

Currently highly informative LATE-PCR multiplex amplification and analysis is carried out in 25 μL closed-tube reactions which are expensive on the reagents and time consuming to construct. On the basis of the findings presented here we envision that it will be possible in the future to reduce the volume of each reaction at least 2500 times from 25 μL to 10 nL. This, in turn, will make it practical to run a great many 10 nL reactions in parallel in order to analyze a great many target sequences in a single sample. These improvements alone reduce the reagent cost per reaction by at least 1000 fold.

In addition, the most common experimental approach to optimize a multiplex is to first optimize the single-target amplifications and then to iteratively combine primer sets to determine which primer sets are incompatible. Thermocycling and buffer conditions also need to be adjusted to accommodate all the primer and probe sets.[19] With such an approach, the optimization of a 10-plex PCR typically takes a PhD level scientist 3–6 months (or more), with a significant chance of failure.[20] The time required for assay development could be dramatically shortened and the success rate greatly increased by running monoplex assays in parallel on a chip.

The advances described here point to an exciting future in the design of POC devices. Such devices can now have single colour optical system that can analyse many small chambers over a range of temperatures. Devices along these lines will make it possible to simulate end-point melting curves using fixed temperature gradients. Indeed, preliminary experiments carried out in our laboratory point the way to adding sets of Lights-On/Lights-Off probes to microfluidics chips. Lights-On/Lights-Off analysis of sequence variants generate fluorescent signatures that serve as characteristic "virtual sequences" of these targets.

Acknowledgements

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