Fast and Efficient Nucleic Acid Testing by ST's In-Check™ Lab-on-Chip Platform

Sabrina Conoci, Patrizia Di Pietro, Salvatore Petralia, Maria Grazia Amore, Floriana San Biagio, Georgia Alaimo, Giuseppe Iacono, Enrico Alessi, Daniele Ricceri, Giovanni Di Trapani, Flavio Di Francesco and Michele Palmieri

Lab-on-Chip R&D, Microfluidics Division, CPG Group STMicroelectronics, Stradale Primosole 50, I-95121 Catania (Italy), sabrina.conoci@st.com

ABSTRACT

In this contribution we report Nucleic Acid (NA) analyses by using the ST's In-Check™ lab-on-chip Platform. Such a platform is based on a miniaturized silicon Lab-on-Cchip (LoC) that integrates a Polymerase Chain Reaction (PCR) reactor - formed by buried microchannels 1 ul in volume – together with a customable microarray. Temperature Control System (TCS) and Optical Reader (OR) appliances, driven by a tailored bioinformatic system, complete the platform. These features allow achieving both DNA amplification and NA detection in an integrated cost effective and convenient format silicon chip.

Integrated detection (PCR and microarray) along with the reproducibility and sensitivity study of integrated PCR by using betaglobin gene from human genomic DNA in ST's In-Check™ platform are presented and discussed.

Keywords: lab-on-chip, miniaturization, PCR, microarray, silicon.

1 INTRODUCTION

Nucleic Acid Testing (NAT) represents one the most sensitive and effective method for several applications in the molecular diagnostic area, including clinical, industrial, veterinary and research applications [1-6]. In this area, the development of miniaturized devices can offer relevant advantages in terms of diagnostic performances (sensitivity and reliability), cost and speed.

In this context, ST's In-Check™ platform (http://www.st.com/stonline/prodpres/dedicate/labchip/labchip.htm) provides significant benefits allowing fast analysis of NA in an integrated cost effective and convenient format silicon chip. Such a platform is based on a miniaturized silicon Lab-on-Chip (LoC) that integrates a Polymerase Chain Reaction (PCR) reactor together with a customable microarray. The PCR reactor consists in four integrated micro-channels (1 ul in volume per channel) that have been buried in a silicon substrate by using MEMS technology. Such reactor is fluidically connected to a microarray area of 5x1.5 mm where, after PCR, DNA hybridization takes place. The chip contains integrated sensors and heaters and it is thermally driven by an external Temperature Control System (TCS) appliance that allows very accurate and fast thermal managing of the chip by a tailored bioinformatic system. The DNA detection in the microarray is based on fluorescent labels and it is performed by means of ST's Optical Reader (OR). In fig 1 the In-Check platform components are pictured.

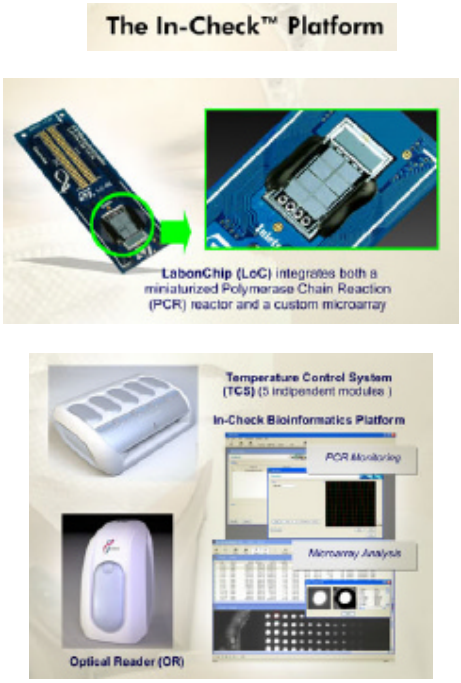


Figure 1 – The ST's In-Check™ platform

The due to a fast time-to-answer, portability the above described platform is then particularly suited for diagnostic applications based on NA in which it is useful to simultaneously screen a number of sequences.

Here we report results obtained in integrated detection of betaglobin gene from human genomic DNA by using ST's In-Check™ platform. Firstly, we show the reproducibility and sensitivity study of PCR in LoC. Finally, we present and discuss the fully integrated detection.

2 MATERIAL AND METHODS

LoC Production and Chemical Treatment

LoC device has been manufactured on a silicon wafer by using semiconductor processing techniques of MEMS technology.

The silicon chip is an electrically active system that integrates a PCR module and an hybridization area (chamber). The PCR module (12x8.5mm^2) consists in four independent PCR reactors (3 buried channels merged into one ~1µL reactor). Accurate temperature control is achieved through 3 resistive heaters and temperature sensors located above the PCR channels. The chip is covered by a glass substrate that delimit both the inlets into the channels and the microarray area. The inlets drilled in the glass cover allow channel loading with standard pipette tips and the outlets opened into the hybridization area delimited by a rectangular opening in the glass. The hybridization area (3.5x9mm^2) is a low-density microarray where oligonucleotide probes are spotted and covalently bound to the surface. A serpentine heater and a temperature sensor are connected to the microarray area in order to reach instantaneously the set point temperature for hybridization. The silicon chip is wire-bonded to a 25x75mm^2 printed circuit board (PCB) slide used as mechanical support and electrical interface with the temperature control system (TCS).

Before DNA-oligos spotting, LoC devices areproperly chemically treated for allowing both DNA probes anchoring and surface biocompatibility for PCR amplification.

Temperature Control System (TCS)

The Temperature Control System (TCS) is an electronic device for actuating controlled PCR Thermal cycling and hybridization temperatures. The system is driven by a microcontrloller which reads the embedded temperarute sensors and triggers embedded heaters through a closed loop control algorithm. Cooling is achieved with a ventilator fan. A simple graphical user interface, similar to a standard thermocycler interface, offers flexible parameter settings and real-time control. In particular, the TCS provides five Temperature Control Modules (TCMs) that allow to run five indipendent PCR/Hyb reactions (Fig. 1). Each module is controlled by an electronic board (TCM-EB) based on ST7 microcontrollers. The Temperature Control system is based on a control algorithm implemented as firmware (FIRM) for the ST7 microprocessor. The firmware in according to this thermal control algorithm will drive embedded heaters and an external cooling fan to allow high gradient and performance thermal cycles. The temperature cycle is controlled with high accuracy. Temperature Accuracy is 0.1°C. Fast ramping - either heating and cooling speed - improves PCR performance and reduces amplification time. Max positive gradient is 40°C/s, medium negative gradient is 10°C/s (average from 105 to 55°C - Ambient Temperature <30°C), temperature stability is 0.2°C. Heating phase is controlled by means of a PWM based algorithm.

Optical Reader

The Optical Reader is an optical device for acquiring images of LoC. It has been tuned in according to silicon oxide thickness to provide a limit of detection of 100 fluor/um2. This limit is enough for application needs. The camera offers up to 1392 x 1040 pixels and operates in 8-bits mode. It has two programmable outputs that are used for turning on excitation system based on two identical illuminator put symmetrically to sides of the camera and tilted of 45 degrees respect to that. Each illuminator is composed by a 5 mm white LED, a red LED, two aspherical lens and a excitation filter for Cy5. A dedicated software is used to drive the Optical Reader. Multi-shot acquisition is used to increade the dynamic linear range and the camera blooming effect. Below table summarizes main features of the Optical Reader.

PCR

β-globin gene from human genomic DNA, used as target in this study, was purchased from Novagen.

Oligonucleotide primers were designed with the Oligo primer analysis software, version 6.65, using the β-globin sequence (Accession no. AY260740). Table n.1 lists the oligonucleotide sequence and their position in the β-globin sequence. They were synthesized by MWG Biotech, Germany.

Protocol 1: symmetric PCR. The PCR mixture was prepared in 50 ul of volume containing 1 µM of each primer, 200 µM of each dNTP, 5 ul of 10X Qiagen reaction buffer (Tris.Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$ pH 8.7), 2U of HotStartTaq DNA Polymerase (Qiagen) and 0.25 ng/ul of human genomic DNA (Novagen). For the sensitivity study we use the same PCR mix except for the DNA concentration, it was diluted to the concentrations of 0.05 ng/ul, 0.025 ng/ul and 0.005 ng/ul.

Protocol 2: asymmetric labeled PCR. The PCR mixture was prepared in 25 ul of volume containing 1 µM of the reverseprimer, 0,0125 µM of the forward primer, 50 µM of each dGTP, dCTP and dTTP, 25 µM of dCTP and 25 µM of Cy5-AP3-dCTP (Amersham), 5 ul of 10X Qiagen reaction buffer (Tris.Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$ pH 8.7), 0.2 % of BSA, 2U of HotStartTaq DNA Polymerase (Qiagen) and 0.25 ng/ul of template.

Thermal Programs. All the PCR amplifications were conducted in TCS (Temperature Control System) module of In-Check platform under the following conditions: initial denaturation for 15 minutes at 95 °C followed by 35 cycles of denaturation for 30 sec at 95 °C, annealing for 45 sec at 61 °C and extension for 45 sec at 72 °C.

Microarray

Three specific oligonucleotides, catching the 240 bp fragment of the betaglobin gene, were designed with the ST's software Xprobedesig. Table n.1 lists the

oligonucleotide sequence, their position in the β-globin sequence and their modification. The oligos probes contain an appropriate modification that permit the covalent attachment of the oligonucleotide to the LoC surface, previously properly chemically treated.

Table 1.

Oligo name	Position	Modification	Sequence
BgloF	72	none	gaagagccaaggacaggta
BgloR	294	none	cagggcagtaacggcaga
Bglo1	97	amine	gcagagccatctattgcttac
Bglo2	131	amine	ctagggttggccaatctactc
Bglo3	198	amine	catcacttagacctcaccctg

Four kind of hybridization control oligonucleotides (3 positive and 1 negative) were designed from *Arabidopsis thaliana* sequence (data not shown). All the oligonucleotides were synthesized by MWG Biotech, Germany

The oligonucleotide probes were diluted in sodium phosphate buffer pH 9 and spotted in 4 replicas according to the layout shown in figure 4b. For the printing a non-contact piezorray microarraying system from PerkinElmer was used.

Array hybridization. The hybridization was carried out in 25 u l of total volume containing 2.5 µM of the high hybridization ctrl, 1 µM of the medium hybridization ctrl and 0.75 µM of the low hybridization in 20 mM of sodium phosphate buffer, 1M Nacl, 5.2 mM fo KCl, 0,1% of Tween20, 2x of Denardt's solution and 20µg/ul of ssDNA.

Washing solution. The washing solution after hybridization consist in a 5' at 40 ºC in 2X SSC, followed by a second wash for 5' at 40 ºC in 0.2X SSC.

LoC Handling

In all PCR experiments on LoC a total volume of 4 uL (1uL *per* each channel) of PCR mix was pipetted directly into the u-channels. These were then sealed by specific PDMF-clamps. Finally, the chip was inserted into the TCS to be thermally cycled.

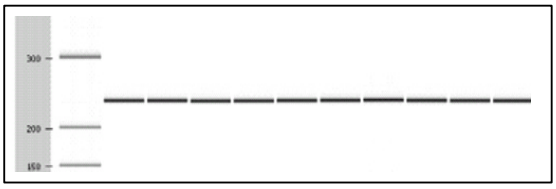
PCR Experiments. At the end of TCS run the chip was taken out from the sealing clamps, centrifuge in a 50 mL falcon tube and 1 uL the recovered volume analyzed by Agilent 2100-bioanalyzer. In parallel, for each set of experiments on LoC, a positive control consisting in 4uL of the same 50 uL PCR mix was run on a Applied Biosystem 2700 apparatus by using the same thermal cycling protocol of LoC. Also in this case, 1µL of this PCR solution was analyzed and quantified by Agilent 2100-bioanalyzer using the DNA500-kit.

PCR-Microarray Integrated Experiments. For the integrated experiment, at the end of TCS run the chip was taken out from the sealing clamps, loaded with 5,5 uL of the hybridization buffer in order to push the labeled PCR product in the detection area and sealed by specific PDMF-clamps. The chip was then inserted into the TCS to be hybridized. Immediately after the hybridization, the specific PDMF-clamps were taken out and the chip was washed by the washing solutions (*vide supra*), dried under air stream and the image of the microarray acquired by the ST's Optical Reader.

Data analysis. Sixteen-bit images were imported into the MAT (microarray analysis tool) module of Gene Platform and the signal/noise ratios for each spot were calculated. The average of the S/N for the four replicas were calculated and graphed in the chart 1 and 2.

3 RESULTS AND DISCUSSION

The first phase of the present investigation has been focused on the achievement of successful PCR amplifications in LoC by using a 240 bps amplicon of betaglobin gene from human genomic DNA as target. In this context, a preliminary phase of PCR set up in LoC has been carried out (data not showed), leading to definition of both protocol (protocol 1) and thermal program. After that, a reproducibility study of the above described PCR has been performed into the LoC. Fig. 2 reports the results obtained in such a study. The image represents the electrophoresis gel-like picture (Agilent Bioanalyser) of PCR products related to 10 repetitions of PCR reaction in 10 different LoC devices. The mean value of the PCR yields obtained in these 10 experiments corresponds to 19 ± 1 ng/ul. This is ca 75% of the mean of yield obtained in a conventional TC (25.6 ±0.8 ng/µl, 4 replicas). In terms of timing, this reaction takes ca 30% less then a conventional TC.



(a)

LoC Yields

Lane 1	18.15
Lane 2	22.40
Lane 3	19.36
Lane 4	20.56
Lane 5	18.24
Lane 6	18.68
Lane 7	20.05
Lane 8	19.38
Lane 9	19.52
Lane 10	20.15

Mean: 19 ± 1 ng/µl

TC Yields

Mean on 4 tests:
25.6 ±0.8 ng/µl

(b)

Figure 2 – Reproducibility of PCR amplification of 240bps betaglobin amplicon in LoC: (a) electrophoresis gel-like picture (Agilent Bioanalyser); (b) LoC-TC yield comparison

In order to test the PCR sensitivity of the ST's platform for the chosen betaglobine target, we have carried out some PCR tests in LoC with protocol 1 by reducing the concentration of DNA template, starting from 0.25 ng/ul (ca 333 copies/ reaction). Figure 3 depicts the electrophoresis gel-like (Agilent Bioanalyser) related to 0.25, 0.05, 0.025 and 0.005 ng/ul (ca 333, 66, 33.3 and 6.6 copies/reaction), respectively. In the light of these results we can conclude that, for the chosen PCR model, the LoC is able to amplify up to 0.005 ng/ul of DNA target detectable from Agilent bioanalyser (threshold limit 0.5-1.0 ng/ul).

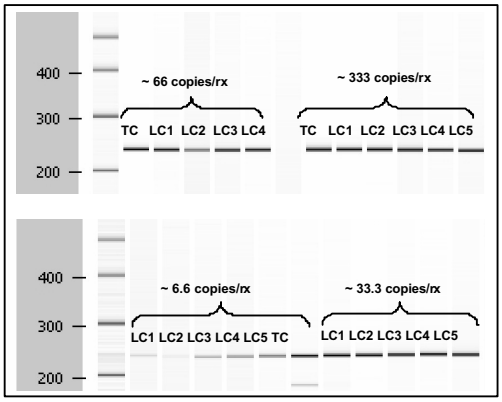
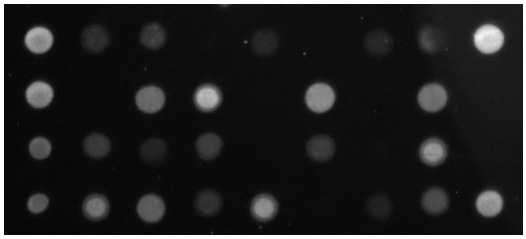
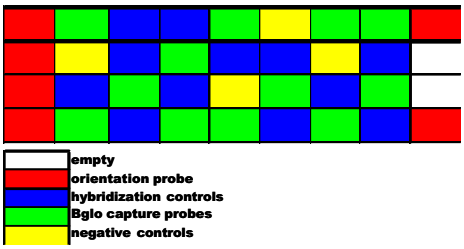


Figure 3 – Sensitivity of PCR amplification of 240bps betaglobin amplicon in LoC detected by Agilent Bioanalyser.

Finally, we have test the complete functionality of ST's In-Check™ LoC Platform in NA analysis by performing fully integrated experiments starting from the chosen betaglobine model. The integration strategy used in this experiment consisted in an asymmetric PCR with Cy5-dCTP multiple incorporation (protocol2) followed by hybridization of the specific microarray panel. (total time (PCR and hybridization) 2h). Fig. 4 depicts the optical image of the specific microarray of the above described experiment acquired by ST's Reader (a) along with the microarray scheme (b). Fig 5 reports the data anaysis of this experiment obtained by MAT. As expected for a positive test, all specific probes are well turned on (fig. 5 a). Moreover, the three specific hybridization positive controls (hyb ctrl) give the expected linear answer (fig.5 b).



(a)



(b)

Figure 4 – Results of fully integrated experiments (a); microarray scheme (b) .

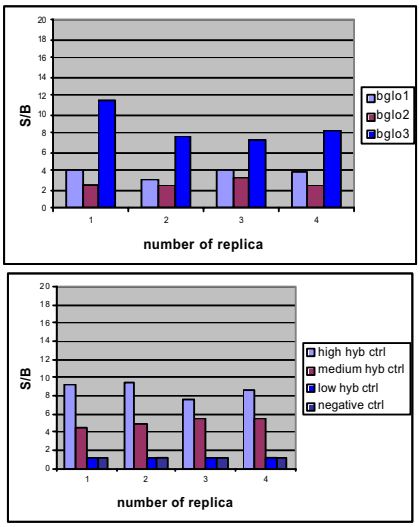


Fig5- Data Analysis of integrated tests from MAT: specific probes 8a); hybridization controls (b).

4 CONCLUSIONS

In this study we have demonstrated the suitability of ST's In-Check™ platform to perform accurate, sensitive and fast NA analyses. These features, therefore, along with its portability make this platform particularly suited for diagnostic applications involving a simultaneously screening of a number of DNA sequences.

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