A self-contained diagnostic platform for DNA concentration, elution, and qPCR inside a LabCard with stored reagents

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Abstract

This paper presents a self-contained system consisting of a portable platform and a disposable LabCard capable of performing a nucleic acid concentration, amplification, and detection. The LabCard comprises several inlets and outlets, integrated microvalves, two chambers and stored reagents. In the first chamber it is performed a magnetic beads-based DNA concentration, washing and elution. Then, the eluted DNA is transferred to the second chamber where it rehydrates with the pre-stored reagents. Finally, a real-time Polymerase Chain Reaction (qPCR) takes place and the fluorescence signal is recorded and displayed in real time. The magnetic bead protocol allows the LabCard to process a wide range of sample volumes (from 10 μl to 10 ml). The PCR reagents are stored inside the LabCard using a gelification technique allowing: (i) long-term storage of the LabCards, (ii) reduction of the reagent volumes, and (iii) significant simplification of the LabCard design and user operation. The cross contamination is avoided by making the LabCard disposable. On the other hand, the platform has been conceived as a comprehensive and generic solution able to perform a wide range of biological assays. These assays consist in an automated and programmable sequence of sample preparation phases followed by a DNA amplification and detection phase.

Keywords: LabonaChip; Sample preparation; System Integration; PCR.

1. Introduction

Miniaturization of biological assays in Lab on a Chips (LOCs) has very well known advantages. However, it has a simple disadvantage: it needs to select the target molecules out of a complex sample and placed them right on the biosensor surface or area. On top of that, the sample volume can be 100 times bigger than the final detection volume as it is in the case of this work. In the particular case of sample preparation and PCR, the most reported miniaturized devices for nucleic acid amplification are chips replacing the role of the PCR thermocycling as mentioned in the literature. Nowadays, the challenge resides on adding functionalities to these microPCR chips. Liu et al. integrated within a cartridge...
several sample preparation steps and PCR by a hybrid integration of paraffin valves, heaters, mixers and electrochemical sensors. However, this remarkable work demanded 3.5 hours to obtain a result and its hybrid fabrication was very complex ending in an expensive disposable cartridge. Kim et al. fabricated a Polymethylmethacrylate (PMMA) microchip where PCR reagents were stored under a paraffin layer in the same chamber where the amplification took place. LOCs in order to process a cell sample to obtain nucleic acid nanolitres ready for a PCR in tube were developed using Poly Dimethyl Siloxane (PDMS) or glass. Gulliksen et al. presented a Cyclo Olefin Copolymer (COC) LOC where RNA purification and a nucleic acid sequence-based amplification (NASBA) was carried out by injecting a crude E. coli cultured lysate. El-Ali et al. performed Cell dielectrophoresis and PCR in a SU8 LOC with a PDMS cover. Agirregabiria et al. fabricated a DNA sample preparation and PCR on a SU-8 chip for gram positive bacteria such as Salmonella spp. in human faeces. This chip design was further used to detect Campylobacter spp. in chicken faeces by PCR and identify influenza viruses in nasopharyngeal human sample by Retro Transcriptase qPCR. Lee et al. performed Cell dielectrophoresis and PCR in a SU8 LOC with a PDMS cover. More recently, Xu et al. presented a device that cleverly combined a cartridge and eppendorf tubes where RNA purification and reverse transcription PCR detection took place using crude patients’ nasopharyngeal swab samples.

This paper shows a portable device and a nucleic acid LOC in the form of a LabCard (See Fig. 1). The complex sample preparation and amplification is carried out in an automatic manner. Unlike other works mentioned above, this LabCard: (i) contains mastermix with fluorophores stored by gelification, allowing long-term storage and simplified LabCard design; (ii) has integrated microvalves; (iii) handles magnetic beads making the assay independent from sample volume and nature; (iv) has no clogging problems; (v) performs qPCR in a clean solution and (vi) is fabricated by three layer stack of COC polymer.

2. Materials and methods

2.1. LabCard Materials

The LabCard consists of a main substrate made from COC (Topas 5013, TOPAS Advanced Polymers) by injection moulding. A pressure sensitive adhesive (PSA) purchased from ProGene (Canada) was used to seal the COC substrate and prevent the deterioration of the PCR reagents gelified previously. The 50μm thick discs placed in the valve cavities were made from cyclic olefin polymer (COP) (Zeon chemical) using a 2.0 mm (diameter) puncher and a cutting mat.

2.2. Sample preparation and PCR reagents kits

The DNA template was a 66bp amplicon. The reason of using such a short DNA template was due to the fact this DNA probe was going to be used as an Immuno qPCR probe. Magnetic beads and elution buffer were purchased from Dynabeads SILANE genomic DNA kit (Invitrogen Dynal AS, Norway). The isopropyl alcohol was purchased from Scharlab (Spain). The enzymatic reaction was based on Lionprobes quantimix (Biotools B&M Labs, Spain). The fluorophore was FamCinmF and the primers were ClnmR.

2.3. Gelification process

The reagent immobilization and preservation sequence is achieved through mixing of the PCR mix together with a gelifying agent (Biotools, Spain) completed by a desiccation process. The content of gelifying agent inside the final mixture ranges between 10-20% according to the chemical composition of.
the reagent mix to be preserved. The mixture is dispensed in the center of the second chamber thanks to a dispenser module (PipeJet™ P9 Nanodispenser, Biofluidix, Germany) prior to desiccation.

3. System Description

The developed system is composed of two major subsystems (Fig.1): the microfluidic LabCard and the diagnostics platform (hereinafter referred to as the platform). It has been conceived as a comprehensive and generic solution able to perform a wide range of biological assays. These assays consist of an automated sequence of sample preparation phases followed by DNA amplification and detection phase. Each assay uses the identical diagnostics platform and LabCard design, whereas the reagents stored inside the LabCard chamber as well as the assay procedure are specific to each analysis.

Fig. 1. Pictures of the Diagnostic system components: Left) The packaged LabCard with a syringe with lysis buffer and magnetic beads; Right) The diagnostic platform with the Table PC and when the LabCard is inserted.

3.1. LabCard Description

LabCard dimensions are 85mm in length x 54mm wide x 1 mm thick (Fig. 2). The channels exhibit a depth of 300μm and width of 500μm whereas the volume of the chambers is 10 μl. The LabCard substrates are cleaned by sonication in an isopropyl alcohol (IPA) and nuclease free water (HyClone HyPure Molecular Biology Grade Water, Thermo Scientific, U.S) bath successively for 5min each. The diaphragm valves embed a 50 um thick disc of COP positioned on top of the valve seats. The primary function of this layer is to accommodate the valve seat to close the diaphragm valve and stop the liquid from flowing while preventing the PSA film from sealing permanently the valve upon actuation. The reagents are dispensed and gelified prior to adhesive film lamination. The adhesive film is laminated using a laminator or a rigid roller. Finally, a strip of adhesive of 8mm x 85mm is laminated on the groove located on the top side of the LabCard to seal the valve connecting channels.

3.2. Platform Description

The three building blocks listed below can be identified:

- **User interface block**: It is the control panel of the system, the major point of interaction between the users and the system. It allows them to visualize the system status and to define the system behaviour. It also stores results, assay procedures assay protocols, and device configuration data. It is
implemented as a software application for Windows operating system running on a Tablet-PC which is connected to the Control unit via USB.

**Control unit block**: It is the part of the platform in charge of receiving commands from the User interface and executing them in order to accomplish the assay protocols. It performs all of the hardware control tasks. It is implemented by means of a custom made printed circuit board. It also comprises a battery together with its management system to allow the system use in remote locations without mains availability. The Control unit executes the algorithms and provides the output control signals needed for the mechanical actuation of the valves and the magnets, and for liquid drawing and pumping. In addition, it controls a couple of heating units used for sample preparation and amplification, an optical detection system for real-time monitoring as well as a bar code reader for sample identification. These sensors and actuators, which are controlled by the Control unit, are the components of the LabCard holder.

- **LabCard holder block**: This part of the system interacts directly with the LabCard and the sample, reagents, and buffers that are stored or circulates through its channels and chambers. This insertion system guarantees a fluidic, electrical, magnetic, thermal and optical connection with the LabCard. It consists of an automatic LabCard insertion mechanism with regulated temperature, a fluorescence detector (FluoSens integrated, Qiagen, Germany), two micropumps (P625, Instech, USA), two heaters and temperature sensors, two magnets, five micromotors (RE6, Maxon motor, Switzerland) to actuate the valves, and three bottles. None of the LabCard holder’s components need to be replaced or washed from assay to assay.

![Fig. 2. Schematic representation of the LabCard layers and a picture of a LabCard with reagents gelified.](image_url)

**4. Experiment Description**

Before the first biological assay took place, it was needed to set the platform configuration parameters such as the DC current limit to feed to the valves’ micromotors in order to block the fluid flow without bending the LabCard, the calibration of the heaters in order to have the desired temperature in the chamber, adequate peristaltic fluid flow rate to carry out the concentration and the DNA transport, and finally the right configuration parameters of the FluoSens integrated fluorescence detector. Once these parameters were adjusted, it was possible to start performing the sample preparation and the PCR protocol (Fig. 3). The LabCard was verified using a volume of 1 mL of sample in order to simulate a real volume. The concentration was done by magnetic separation. Therefore, 5 μL of 109 copies/μL of template DNA (66bp amplicon) was added to 995 μL of nuclease-free water. Then, 10 μL of magnetic beads was added to the sample. To finish, 50 μL of isopropyl alcohol was added in order to fix the DNA to the beads. Then, the eppendorf tube was placed at 4°C for 10 minutes for incubation of the DNA on top of the beads surface. Subsequently, the LabCard was inserted in the Platform and the syringe filled
with the sample connected to the LabCard. The pumps, the magnets and the appropriate valves are activated and the liquid starts flowing inside the LabCard (Fig. 3).

Fig. 3: The steps are: 1) inject the sample (5 μL of DNA, 10 μL of magnetic beads, 50 μL of isopropyl alcohol are added to 995 μL of nuclease-free water) at (200 μL/min); 2) The DNA concentration is done by magnetic separation using two magnets placed on both sides of the first reaction chamber; 3) As the sample containing the magnetic beads and DNA passes through the chamber, the beads are retained in the magnetic field generated by the two magnets. The impurities leave the LabCards and only the beads and bound DNA remain inside the chamber. 4) The DNA is eluted using 40 μL of elution buffer, incubated and carefully transferred to the PCR chamber at 20 μL/min. 5) The DNA and PCR reagents are mixed rehydrating the stored reagents. 6) Once the amplification chamber is completely filled with eluted DNA, the appropriate valves are closed and the thermocycling begins.

5. Results and discussion

A verification plan was carried out in order to test and optimised the following steps: DNA concentration, elution, reagents rehydration and DNA amplification.

Bead-based DNA concentration was the first step to be optimised. Different flow rates were tested in order to achieve the maximum speed without losing any bead. An optimum flow rate of 200 μL/min was obtained, collecting the waste free of beads.

The elution step was tested by collecting the eluate in an eppendorf tube to perform a PCR instead of transferring it to the second chamber. Different elution steps were characterised injecting 10 μL of elution buffer each time and changing the incubation time. All the elution experiments were performed oscillating the magnets in order to improve the mixing and the releasing of the DNA from the beads conglomerate. These experiments demonstrated that the first 10 μL of elution buffer were not enough to release all the DNA retained on the beads. However, this was the maximum volume of the chamber, which was bigger than the sample volume that could be used in the next PCR. Due to this fact, 10 μL of elution buffer was considered as the optimised volume.

Regarding the incubation time, although long times could improve the elution efficiency; there was a compromise between the total analysis time and efficiency. A 5 minutes incubation time showed to be the best trade-off for the follow-up.

Gelification of the PCR reagents was tested in the second chamber of the LabCard. Hence, a LabCard with gelified reagents was placed in the platform. Then, the DNA was injected directly into the amplification chamber and mixed with the reagents (Fig. 5). Then, the proper valves were closed, the thermocycling started and the fluorescence detector activated. As expected by the gel provider (Biotools B&M Labs), the reactions with gelified reagents have a larger cycle threshold (Ct) than when using a fresh mastermix.

Fig. 5: Sequences of pictures where the gel is being rehydrated upon contact with the eluted DNA.
Finally, the whole experiment was carried out in the system: Automated DNA injection, concentration, elution, transport, amplification with stored LION reagents and optical detection. The result can be seen in Fig. 6. These experiments were confirmed by electrophoresis. The band had the expected size.

Fig. 6: Screenshot with a full protocol result. The DNA was injected, concentrated and purified in the first chamber and then eluted and transported to the second one for qPCR, using gelified reagents stored inside.

6. Conclusions

This platform with a LabCard with two chamber strategy, integrated valves and stored PCR reagents ensures (i) compatibility with complex sample by removal of PCR inhibitors from the crude sample, (ii) DNA concentration, (iii) automatic injection of reagents by the actuation of integrated valves, (iv) long shelf-life or uncontrolled temperature transportation of the LabCards by storing the PCR reagents and (v) disposability of the LOC due to its simple and inexpensive mass fabrication compatibility (injection molding and lamination).

Furthermore, the range of future applications of this LabCard is wide as long as the application is compatible with a sample preparation based on magnetic beads and lysis buffer (if needed). In fact, this work is framed within a European project where this LabCards are being validated for CEA protein detection by Immuno-qPCR, food pathogen typing by multiplex PCR and sea algae detection by NASBA. This validation will demonstrate that the platform is generic solution able to perform a wide range of biological assays.

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References

[1] Manz, A., Graber, N., Widmer, H.M., Sensors and Actuators, 62, pp. 978-994, 1990.
[2] Van den Berg, A., μTAS’96 Conference, 17-22 Nov, pp. 9-15, 1996.
[3] Effenhauser, C. S., Bruin, G. J. M., Paulus, A., Ehrat, M. Anal. Chem., Vol. 69, pp. 3451-57, 1997.
[4] Dempsey, E., Diamond, D., Smyth, M. R., Urban, G., Jobst, G., Moser, I., Verpoorte, E. M. J., Manz, A., Widmer, H. M., Rabenstein, K., Freaney, R., Anal. Chim. Acta, 346, pp. 341-49, 1997.
Roper, M. G.; Easley, C. J.; Landers, J. P. "Advances in Polymerase Chain Reaction on Microfluidic Chips" Analytical Chemistry 2005, 77, 3887-3894.

Huang, Y., Mather, E. L., Bell, J. L., Madou, M. MEMS-based sample preparation for molecular diagnostics (2002) Fresenius' Journal of Analytical Chemistry, 372 (1), pp. 49-65.

Auroux, P.-A., Koc, Y., DeMello, A., Manz, A., Day, P.J.R. Miniaturised nucleic acid analysis (2004) Lab on a Chip - Miniaturisation for Chemistry and Biology, 4 (6), pp. 534-546.

Zhang, C., Xu, J., Ma, W., Zheng, W. PCR microfluidic devices for DNA amplification (2006) Biotechnology Advances, 24 (3), pp. 243-284.

Chen, L., Manz, A., Day, P.J.R. Total nucleic acid analysis integrated on microfluidic devices (2007) Lab on a Chip - Miniaturisation for Chemistry and Biology, 7 (11), pp. 1413-1423.

[5] Roper, M. G.; Easley, C. J.; Landers, J. P. "Advances in Polymerase Chain Reaction on Microfluidic Chips" Analytical Chemistry 2005, 77, 3887-3894.

[6] Huang, Y., Mather, E. L., Bell, J. L., Madou, M. MEMS-based sample preparation for molecular diagnostics (2002) Fresenius' Journal of Analytical Chemistry, 372 (1), pp. 49-65.

[7] Auroux, P.-A., Koc, Y., DeMello, A., Manz, A., Day, P.J.R. Miniaturised nucleic acid analysis (2004) Lab on a Chip - Miniaturisation for Chemistry and Biology, 4 (6), pp. 534-546.

[8] Zhang, C., Xu, J., Ma, W., Zheng, W. PCR microfluidic devices for DNA amplification (2006) Biotechnology Advances, 24 (3), pp. 243-284.

[9] Chen, L., Manz, A., Day, P.J.R. Total nucleic acid analysis integrated on microfluidic devices (2007) Lab on a Chip - Miniaturisation for Chemistry and Biology, 7 (11), pp. 1413-1423.

[10] Liu, R.H., Yang, J., Lenigk, R., Bonanno, J., Grodzinski, P. Self-Contained, Fully Integrated Biochip for Sample Preparation, Polymerase Chain Reaction Amplification, and DNA Microarray Detection (2004) Analytical Chemistry, 76 (7), pp. 1824-1831.

[11] Kim, J., Byun, D., Mauk, M.G., Bau, H.H. A disposable, self-contained PCR chip (2009) Lab on a Chip - Miniaturisation for Chemistry and Biology, 9 (4), pp. 606-612.

[12] Hong, J.W., Studer, V., Hang, G., Anderson, W.F., Quake, S.R. A nanoliter-scale nucleic acid processor with parallel architecture (2004) Nature Biotechnology, 22 (4), pp. 435-439.

[13] Legendre, L.A., Bienvenue, J.M., Roper, M.G., Ferrance, J.P., Landers, J.P. A simple, valveless microfluidic sample preparation device for extraction and amplification of DNA from nanoliter-volume samples (2006) Analytical Chemistry, 78 (5), pp. 1444-1451.

[14] Guilliksen, A., Solli, L.A., Drese, K.S., Sörensen, O., Karlsen, F., Rogne, H., Hovig, E., Sirevåg, R. Parallel nanoliter detection of cancer markers using polymer microchips (2005) Lab on a Chip - Miniaturisation for Chemistry and Biology, 5 (4), pp. 416-420.

[15] J. El-Ali, I. R. Perch-Nielsen, C. R. Poulsen, M. Jensen, P. Telleman and A. Wolff. Microfabricated DNA amplification device monolithically integrated with advanced sample pretreatment (2003) 214, Transducers ’03 Boston MA, USA.

[16] Agirregabiria M., Verdoy D., Olabarri G., Berganzo J., Berganza J.I., Pascual de Zulueta M., Mayora K., Aldamiz-Echevarria P., and Ruano-Lopez J.M. Concentration, Lysis and Real-Time PCR on a SU-8 Lab on a Chip for Rapid Detection of Salmonella spp. in Faeces. (2007) MicroTAS Conference, Paris.

[17] Bang D., Høgberg J., Agirregabiria M., Berganzo J., Mayora K., Walczak R., Dziuban J.A., Bu M., Wolff A., Ruano-Lopez J.M. A total integrated LabOnaChip system for rapid detection of campylobacter spp. in chicken faeces. (2009) MicroTAS Conference, Jeju, Korea.

[18] Verdoy D., Barrenetxea Z., Fernández L., Agirregabiria M., Berganzo J., Ruano-López J.M., Marimón J.M, Montes M., Hammoumi S., Albina E., Olabarri G. LabOnaChip for fast RNA extraction and identification by one step RT-qPCR of influenza viruses in human samples. (2009) MicroTAS Conference, Jeju, Korea.

[19] Microchip-based one-step DNA extraction and real-time PCR in one chamber for rapid pathogen identification, Jeong-Gun Lee, Kwang Ho Cheong, Nam Huh, a Suhyeon Kim, Jeong-Woo Choi, and Christopher Koay, DOI: 10.1039/b515876a.

[20] A self-contained all-in-one cartridge for sample preparation and real-time PCR in rapid influenza diagnosis Guolin Xu, Tseng-Ming Hsieh, Daniel Y. S. Lee, Emril Mohamed Ali, Hong Xie, a Xing Lun Looi, Evelyn S.-C. Koay, Mo-Huang Li, and Jackie Y. Ying, DOI: 10.1039/c005265e.