DETECTION OF OIL-UTILIZING MICROORGANISMS BY NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION IN A TOTAL ANALYSIS LAB-ON-A-CHIP DEVICE

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ABSTRACT

A real-time nucleic acid sequence-based amplification (NASBA) application integrated in SelfPOCNAD, a sample-in, answer-out lab-on-a-chip (LOC) solution is presented. Extraction of nucleic acids in the same LOC system is also described. With this application we expect to detect gene activity from oil-utilizing microorganisms in various oil samples. We have successfully extracted nucleic acids from 1 ml sample in the extraction chamber of the cassette and to run amplification and detection in a 2 µl NASBA chamber identical to one of the eight NASBA chambers in the microfluidic chip of the SelfPOCNAD system.

KEYWORDS: Lab-on-a-chip, DNA-/RNA extraction, real-time amplification, NASBA, detection, oil, oil-eating, microorganisms, genes, gene-activity, point-of-care (POC), point-of-need (PON).

INTRODUCTION

Development of point-of-care (POC) lab-on-a-chip (LOC) platforms are giving us various simple point-of-need (PON) solutions for molecular biological analyses and detection of microorganisms. A micro-/nano fluidic device based on pre-treatment and tagging of cells and electro-kinetic trapping to detect bacteria was developed by Wang and co-workers [1]. There are also reports of microfluidic platforms were sample preparations, amplification and detection are integrated in one chip. Dimov and his team have developed microfluidic platforms for integrated RNA purification and NASBA amplification to rapidly detect pathogens [2, 3]. However, these platforms require manual application of reagents; there is no automatic analysis. By developing fully automatic LOC platforms, so-called sample in, answer out solutions, manual handling and contamination can be decreased. This also enables operation by people with minimal training, and on-site analysis in the field. Such an automated LOC system for point of care detection of pathogens based on real time PCR was developed by Chen and co-workers [4].

We here describe the integrated nucleic acid extraction handling more than 1 ml sample and real-time duplex NASBA reaction as part of SelfPOCNAD, a fully automatic sample-in, answer out platform. Our previous solution consisted of two chips, for extraction and duplex NASBA reaction, respectively [5], which now has been assembled into one chip and cassette system. The full system includes sample collection device, sample concentration from up to 20 ml sample, sample mixing, refining technology [6], RNA extraction, real-time duplex 8X NASBA and quantification using molecular beacons. An industrial prototype control instrument and associated software has been developed. Our optical detection system is capable of emitting and detecting at wave lengths of 470 nm and 590 nm in eight parallel chambers, enabling it to detect up to 16 different mRNA targets in one run. Purified RNA including selected genes related to housekeeping and activity from microorganisms of interest are subjected to amplification and quantification inside the chip.

THEORY

A microfluidic chip is enclosed by a plastic cassette, constituting the cartridge. Reagents are stored as 300 nanoliter freeze dried spheres inside the chip or in liquid form in the storage chambers of the cassette (Figure 1). Transport of reagents for isolation of nucleic acids, and amplification and detection is carried out in the same microfluidic chip (Figure 2). The cartridge is placed in an accompanying instrument for fully automatic run.

The extraction method of nucleic acids is based on NucliSens® (Biomérieux, Lyon, France). The sample is inserted and the cells are collected in a filter membrane at the sample inlet on top of the cassette. Lysis buffer is sucked through the chip to the filter (Figure 2: (2) to (1)) using the screw pump in the controlling instrument in order to lyse the cells. One millilitre sample of lysed cells is pumped through the chip to the extraction chamber (3) containing silica beads onto which nucleic acid binds, as residues are transferred to a 20 ml large waste chamber (4) in the bottom part of the cassette. The silica beads are entrapped by an elevating magnet located in the instrument as the extraction chamber is washed with wash buffer 1 (6), wash buffer 2 (7) and elution buffer (8). Waste is sucked to the waste chamber in the bottom cassette through a waste outlet (4).

Extracted nucleic acids and reagents (9) are transferred to the mixing chamber (10) and blended. The mixture is transferred to a broad channel (11) prior to filling up eight pre-NASBA chambers (12). Primers and probes stored as lyophilized spheres inside the chambers are mixed with the sample, and thermal element heats the sample to 63 °C to separate double-stranded nucleic acids. The contents are transferred to the succeeding NASBA chambers, and mixed with enzymes stored as lyophilized spheres. A heating element holds 41 °C (+/- 0.5 °C) to run the NASBA reaction. Fluorescence from molecular beacons in real-time NASBA is read with an fibre-optic optical detection system [7] in the controlling instrument.

Figure 1: POCNAD cartridge. Reagents are stored in the top part: lysis buffer (blue); NASBA reagents (yellow); elution buffer (green); extraction chamber with silica beads (orange); and wash buffers (purple and pink).

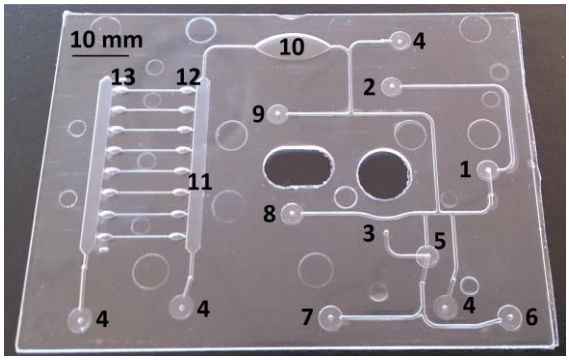

Figure 2: Microfluidic chip. (1) Sample inlet valve; (2) lysis buffer inlet valve; (3) extraction chamber transfer point; (4) waste outlet valves; (5) valve; (6) wash buffer 1 inlet valve; (7) wash buffer 2 inlet valve; (8) elution buffer inlet valve; (9) reagents inlet valve; (10) mixing chamber; (11) broad channel; (12) pre-NASBA chambers; (13) NASBA chambers.

EXPERIMENTAL

Microfluidic chips were fabricated in polycarbonate (PC) by injection moulding. Sealing valve membranes of 40 µm cyclic olefin polymer (COP) disks and PCR film covering the top and bottom side completed the microfluidic chip. The enclosing cassette was fabricated in a mixture of transparent acrylonitrile butadiene styrene (ABS) and acrylonitrile styrene (AS) by injection moulding.

Extraction of bacterial (*E. coli*) nucleic acids was carried out in the extraction chamber of the POCNAD cassette with the NucliSens® extraction kit (Biomérieux, Lyon, France). The magnet in the associated instrument entrapped the silica beads, reagents were added and removed with a syringe, and the amounts of reagents were adjusted to the 1 ml volume of the extraction chamber in the cassette. The extraction was otherwise carried out according to the NucliSens ® protocol. A control sample of the same material as the sample was collected with the NucliSens® protocol and the NucliSens miniMAG (Biomérieux, Lyon, France). To verify that the nucleic acids were extracted from the samples NASBA was run according to NASBA Basic kit protocol, with reagents supplied by the PreTect HPV-Proofer assay (NorChip, Norway) and Basic kits (Biomérieux, Lyon, France). General primers Grm neg 16S P1 and -P2 and molecular probe Grm neg MB (Biosearch Technologies, Inc., Novato, CA, USA) were used in 0.2 µM concentrations. NASBA was run on a Bio-Tek FLx600 reader with KC4^{TM} Data Analysis software (Bio-Tek Instruments, Inc., Winooski, VT, USA).

To verify the correct functioning of the NASBA chamber, a 2 µl COP chamber with 300 nanoliter freeze-dried enzymes, as seen in Figure 3, was used. This was performed in a way similar to the POCNAD device, ensuring ready transference of the results. First, 2 µl of the nucleic acid-containing solution was pipetted, filling the channels and the chamber partially. Next, a small amount of air (~1 µl) was injected with a pipette to centre the solution in the chamber and dissolve the freeze-dried sphere. Upon filling, the chamber was loaded in to the reader, shown in Figure 4, which contains a heater and a fluorescence reading unit.

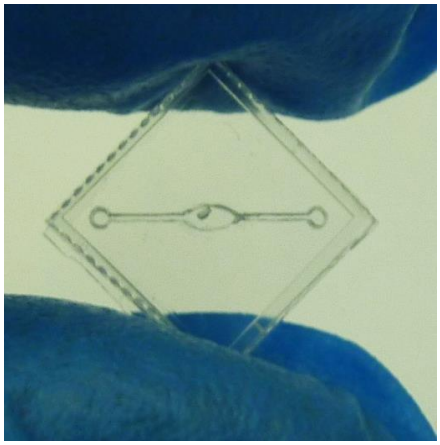

Figure 3: Freeze-dried sphere inside a 2 µl COP chamber bonded with PCR film, developed at IK4-Ikerlan S.Coop, supplied by MicroLIQUID., Spain.

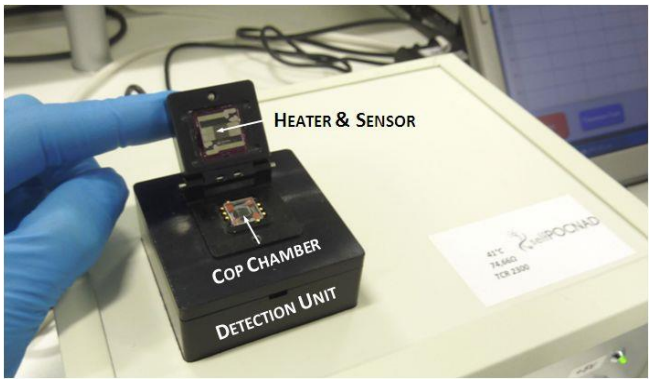

Figure 4: The NASBA reader for the testers. The device performs a customized thermal cycling and double channel fluorescence reading, developed at IK4-Ikerlan S. Coop.

Microfluidic paths in the chip were tested and verified. Flow rates vary from 20-120 µl/min, depending on the step of the protocol.

RESULTS AND DISCUSSION

Extraction of nucleic acids from 1 ml sample was performed in the extraction chamber of the POCNAD cassette. By running conventional NASBA on the extracted samples (Figure 5) we verified that this extraction method was successful. The results proved most comparable to our conventional extraction method.

NASBA amplification was successfully performed in a 2 µl chamber using freeze dried reagents, as shown in Figure 6. This result is easily transferable to the full microfluidic chip, and we expect to be able to detect up to 16 different oil-utilizing bacteria in on run by incorporating primers and probes targeting active genes and housekeeping genes of these microorganisms.

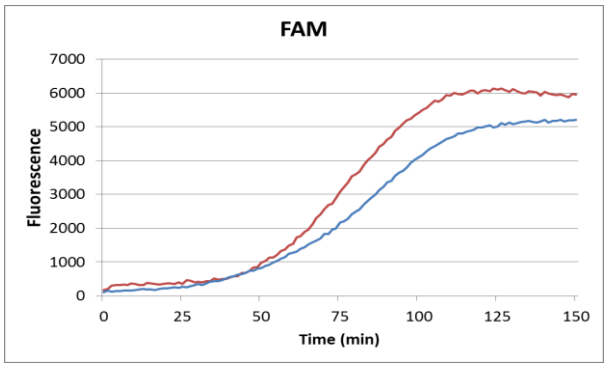

Figure 5: Conventional NASBA run on bacterial nucleic acids extracted in the POCNAD cassette (red line) and by conventional extraction method (control, blue line).

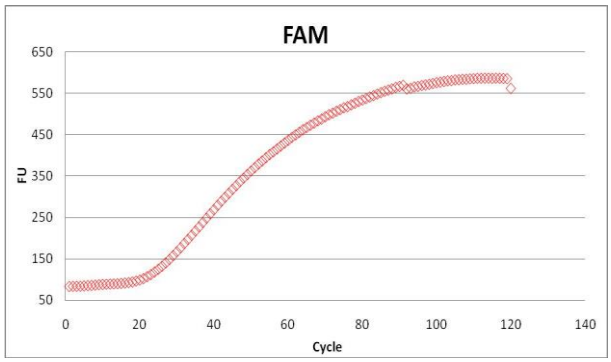

Figure 6: Plot of the amplification curve using freeze dried enzymes in the 2 µl COP chamber.

We have obtained a sealed cartridge, and verified that the liquid is drawn from the storage chambers in the cassette through the enclosed chip and into the waste by a pumping device.

CONCLUSION

Isolation of nucleic acids was successfully done in the extraction chamber of the POCNAD cassette of the LOC sample in, answer out SelfPOCNAD system. NASBA reaction was successfully run in a 2 µl reaction chamber with freeze dried reagents.

Our next step will then be to combine the nucleic acid extraction with subsequent NASBA amplification in the full system, in one run.

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