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Integrated DNA and RNA extraction and purification on an automated microfluidic cassette from bacterial and viral pathogens causing community-acquired lower respiratory tract infections

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In this paper, we describe the development of an automated sample preparation procedure for etiological agents of community-acquired lower respiratory tract infections (CA-LRTI). The consecutive assay steps, including sample re-suspension, pre-treatment, lysis, nucleic acid purification, and concentration, were integrated on a microfluidic lab-on-a-chip (LOC) cassette that is operated hands-free by a demonstrator setup, providing fluidic and valve actuation. The performance of the assay was evaluated on viral, Gram-positive and Gram-negative bacterial broth cultures previously sampled with a nasopharyngeal swab. Sample preparation on the microfluidic cassette resulted in higher or similar concentrations of pure bacterial DNA or viral RNA compared to manual benchtop experiments. The miniaturization and integration of the complete sample preparation procedure, to extract purified nucleic acids from real samples of CA-LRTI pathogens to, and above, lab quality and efficiency, represents an important step towards its application on a point-of-care test (POCT) for rapid diagnosis of CA-LRTI.

1. Introduction

Community-acquired lower respiratory tract infections (CA-LRTI) cover a broad spectrum of diseases, ranging from acute bronchitis to community-acquired pneumonia, and are associated with high morbidity and mortality. Lower respiratory tract infections are the leading cause of burden of disease, accounting worldwide for 3.46 million deaths each year, thereby occupying third place globally and first place in low-income countries[1]. The etiologic agents of CA-LRTI can be bacterial or viral, and cannot be differentiated on the basis of clinical symptoms. Among the leading causative agents of CA-LRTI are Gram-positive bacteria, such as *Streptococcus pneumonia* (*S. pneumonia*) and *Staphylococcus aureus* (*S. aureus*), Gram-negative bacteria, such as *Haemophilus influenza* (*H. influenza*), and respiratory viruses such as *influenza A*[2]. Current gold standard diagnostic tests for bacterial pathogens causing CA-LRTI have a long turnaround time of at least 48 hours, viral pathogen tests even longer, because they are mostly based on culture, biochemical identification and susceptibility determination by diffusion or dilution methods[3]. Moreover, a laboratory diagnosis is often not conducted for patients presenting with symptoms of CA-LRTI at the point of primary care, which results in diagnostic uncertainty for clinicians. Thus currently, antibiotics are often prescribed even though a bacterial infection was not confirmed and over-prescription turns to a substantial source for the growing number of multi-resistant bacteria. Therefore, the development of better diagnostic tools for respiratory tract infections would have a great impact on treatment of such pathologies.

Since respiratory tract infections are the most common reason for prescribing antibiotics, and antibiotic resistance is strongly correlated to antibiotic consumption[4,5], improving diagnostics in this field could further help to curb antibiotic resistance[3]. Studies on the usage of point-of-care testing (POCT) in interaction with well-directed briefing of physicians and patient counselling show that significantly reduced prescription rates for antibiotics[6] can be achieved.

One of the main challenges in POCT is the usability of systems. The devices have to be comparatively self-explanatory and robust, to ensure the reliability of results, even if used by a non-medical laboratory expert. On the other hand, out-of-the-device sample pre-processing should be avoided and the POCT must be able to use the samples as they are attained from the patient

reducing sample handling sensitivity loss. Finally, these devices must provide at least lab-performance quality in a relatively short period of time (30 to 60 minutes) and at affordable cost. A great effort has been done to automate and integrate one or several laboratory operations into small devices during the last 20 years[7,8,9]. This was soon regarded as a means to obtain higher efficiency, faster analysis time, and lower reagent consumption, on existing analytical techniques. By definition, such microfluidic devices, able to perform complete laboratory assays, are also referred as, due to their application analytical nature, Micro Total Analysis Systems (μTAS), as introduced early in the 90's by Manz and colleagues[10]. However, little has been reported on systems performing the full preparation of the original biological sample prior to the measurement. More often, LOC performing few, or single, steps are presented isolated without proving the feasibility and the advantage of fully automated miniaturized systems[11,12,13].

In the work that we present here our efforts have focussed on the key challenge of performing the complete preparation of a clinical sample containing respiratory bacterial and viral pathogens to isolate and recuperate their genetic material. When evaluating CA-LRTI, swab samples are reliable sources of patient microbial content. Our LOC cassette, and its portable associated control system, performs all the needed operations with its self-contained reagents until the nucleic acids (NA) are obtained departing from an untreated sample-containing swab. Reagents, in particular the lysis buffer, were identified in order to be efficient for viruses and different bacteria types. Swab containing and cutting chamber was also optimized to maximize analytes release. The resulting system is able to outperform the standard lab techniques and reaches a higher integration of the full CA-LRTI analysis and protocol in contrast to previous, some less specific, μTAS approaches[14,15,16,17,18,19,20,21]. It is also the first LOC system, to the best knowledge of the authors, to successfully integrate a thin (80 μm) porous membrane to perform the NA extraction operation. The device was tested using cultured microbes as well as swab samples obtained from real CA-LRTI patients. Either fluorimetric assay or quantitative real-time PCR (RT-qPCR) were used to measure the NA concentrations that were recovered after the lab-standard and the cassette-based protocols for comparison.

2. Materials and methods

2.1. Biological Samples

Two types of samples were used to validate the system: bacterial or viral broth cultures and viral spiked sample. Pathogens were added to the sample collecting chamber, of the LOC cassette or the benchtop procedure, in the presence of a cut flocked swab (Copan Diagnostics, Brescia, Italy), during the experiments.

The broth cultures were made of Gram-positive bacteria (*S. aureus* ATCC 25923 and *S. pneumoniae* ATCC 49619), Gram-negative bacteria (*H. influenzae* ATCC 10211) or *influenza A* virus (ATCC VR-1520, LGC standards, Molsheim, France). Bacterial densities were determined by spectrophotometric measurement and spiral plating (Eddy Jet, IUL instruments, Leerdam, The Netherlands). Bacteria and virus test samples were made of 50 μl of 10^8-10^9 CFU/mL and 50 μl of $5.6x10^6$ TCID50/0.2 mL respectively.

For spiked samples, nasopharyngeal swabs (100 μl) obtained from patients suffering from CA-LRTI and stored in skimmed milk medium at -80°C were used. Samples were screened to confirm absence of the target pathogen, *influenza A* virus, by testing 80 μl on a 650-700 μl lysate aliquot. Reverse transcription RT-qPCR was applied for virus detection and quantification, following NA purification with Quickgene system (Quickgene-mini-80 and Quickgene DNA tissue kit S or RNA tissue kit SII, Fujifilm, Germany). Swabs that showed absence of *influenza A* were spiked with the viral reference (100 μl of $5.6x10^6$ TCID50/0.2 mL).

2.2. Benchtop sample preparation protocol

A benchtop manual sample preparation protocol was set for comparison purposes with our automated system. Samples (50 μl pure broth culture or 100 μl spiked sample) were subjected to pre-treatment with lysozyme buffer (250 μl PBS + 6 μL rlysozym, 30,000 U/μL, EMD Millipore, USA) in the presence of a swab and a subsequent guanidinium thiocyanate (GuSCN)-based chemical lysis (300 μl lysis buffer as described in Boom et al.[22]). Then, 96% purity ethanol (650 μl) (Merck Chemicals, Belgium) was added to the lysate solution container and forced over the nucleic acid binding-membrane by administering pressurized air using the Quickgene-mini-80 system (Quickgene DNA tissue kit S or RNA tissue kit SII, Fujifilm, Germany). After a wash step (Fujifilm wash buffer WDT, Quickgene DNA tissue kit S or RNA tissue kit SII, Fujifilm, Germany), nucleic acids were eluted in 50 μl Fujifilm Elution buffer CDT (Quickgene DNA tissue kit S or RNA tissue kit SII, Fujifilm, Germany).

2.3. LOC cassette sample preparation protocol

Flocked swab was cut by, and stored into, the swab-extraction chamber of the LOC cassette. Samples (50 μl pure broth culture or 100 μl spiked sample) were added and the chamber locked. In order to accomplish both bacteria and virus NA extraction on the LOC an automated sample preparation protocol was established (Table 1).

Table 1. Overview of the cassette integrated assay steps.

Assay step	Volume (μl)	Mixing	Flow rate (ml/min)	Incubation (min)
Cells lysis step 1 *Re-suspension & enzymatic pre-treatment*	300	Active	1.5	3
Cells lysis step 2 *Introduction of lysis buffer*	300	Active	1.5	3
NA capture step 1 *Addition of ethanol*	650	Active	4	-
NA capture step 2 *Filtering through SPE membrane & NA binding*	-	-	9	-
Washing step 1 *Loading wash buffer*	650	-	5	-
Washing step 2 *SPE membrane cleansing*	-	-	6	-
Elution step 1 *Transfer elution buffer to the SPE membrane*	50	-	2	1
Elution step 2 *Transfer elution buffer to the recovery chamber*	-	-	5	-

2.4. LOC cassette design and fabrication

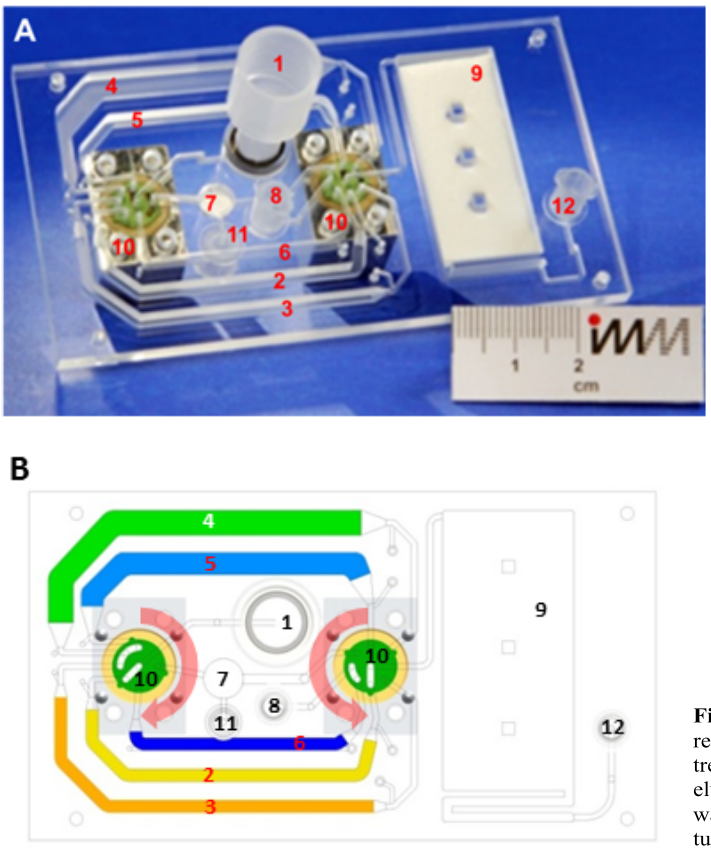
The fluidic circuit of the cassette was designed to address and integrate the previously stated sample preparation steps. It incorporates: reagent storage and waste reservoirs, sample handling microfluidics with embedded porous membranes and turning valves, and a lockable swab-extraction chamber.

2.4.1. Reagents storage and waste reservoirs

Reagent storage was done into five fluidic channels (2,3,4,5 and 6, Fig. 1A and 1B) milled (Fehlmann CNC 3 D Picomax 60-HSC) directly into Cyclo Olefin Polymer (COP, Zeonor 1420R) plates (100x56x4 mm^3) that conform the cassette main body. Channels were integrated into the more general fluidic circuitry and access, during their operation, was controlled via turning valves. The channels were sealed with pressure sensitive adhesive (Absolute TM QPCR Seal, Thermo Fisher Scientific Inc) or, alternatively, by solvent assisted bonding (proprietary technology of thinXXS Microtechnology AG) of thin COP film (ZEONOR 1420R film, 188 µm). Access to the reagents reservoirs for filling purposes was accomplished through inlets and outlets at the extremes of each reservoir. These ports were closed after reagent loading, and before cassette operation, with the same pressure sensitive adhesive. Reagents volume requirements to perform the sample preparation protocol were intrinsically set on the cassette design. Dimensions of the fluidic channels were set to correspond to the storage spaces (height, width and length, respectively): 2x3x56 mm^3 for the sample re-suspension and pre-treatment buffer (300 µl of 250 µl PBS + 6 µL rlysozym, 30,000 U/µL, EMD Millipore, USA), 2x3x65 mm^3 for the lysis buffer (300 µl of guanidinium thiocyanate (GuSCN)-based lysis buffer as described in Boom et al[22]), 3.5x3.6x58 mm^3 for the NA binding buffer (650 µl of 96% purity ethanol, Merck Chemicals, Belgium), 3.5x4x64 mm^3 for the wash buffer (650 µl of Fujifilm wash buffer WDT, Quickgene DNA tissue kit S or RNA tissue kit SII, Fujifilm, Germany), and 1x2x36 mm^3 elution buffer (50 µl of Fujifilm Elution buffer CDT, Quickgene DNA tissue kit S or RNA tissue kit SII, Fujifilm, Germany).
The waste cavity (9 in Fig. 1A and 1B) was designed with the capacity to contain all the resulting sub-products of the analysis (2000 µl) and was filled with absorbent material (VWR® Grade 707 Blotting Pads, VWR International).

4.2. Sample handling microfluidics and valves

The cassette main body also included all the microfluidic channels facilitating the transport of the sample and the reagents during the sample preparation procedure. Operations were driven through applying controlled, either negative or positive, pressure in a pneumatic port placed near the waste reservoir (12, Fig. 1A and 1B) and operating the custom-made turning valves externally. The originally milled port was sealed by thermally welding, on top of it, a hydrophobic membrane (Fluoropore Membrane Filter, PTFE, 0.45 µm, Merck Millipore) and then by ultrasonically welding an injection moulded commercially available COP (Zeonor 1420R) Luer adapter (Microfluidic ChipShop). The membrane acted as a security fluid stopper and the adapter as an interface to the controlling unit.
Two turning valves embedded into the cassette main body (10, Fig. 1A and 1B) were used for fluid routing. Each of these consisted of a metallic housing body (aluminium) and a round polymeric (polyether ether ketone, PEEK, REIFF Technische Produkte GmbH) turning body. The PEEK part embeds a green elastomeric sealing



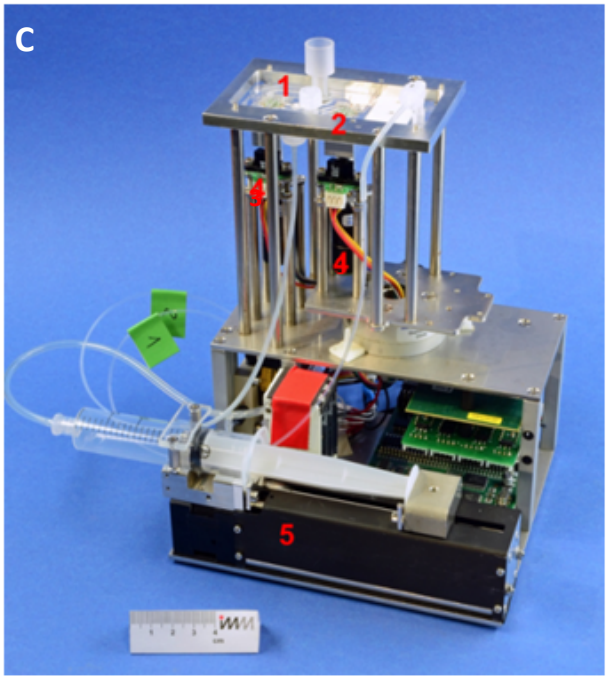


Fig. 1 A and **B**, are the final analytical cassette and its layout respectively, where: (1) swab/sample reservoir, (2) suspension/pre-treatment buffer, (3) lysis buffer, (4) ethanol, (5) washing buffer, (6) elution buffer, (7) SPE membrane, (8, 11, 12) Luer connections, (9) waste chamber, and turning valves (10). Red arrows in B show turning-valves rotating sense. **C** is the complete analytical tool, where: (1) analytical cassette, (2) cassette-holder, (3, 4) stepper motors, (5) syringe pump control.

(Viton 90° Shore A, Rala GmbH & Co. KG) which has engraved microchannels in one of its sides. Both, the main cassette body and the valves' housing and moving parts were constructed using mechanical structuring (mainly milling and drilling) with a **(**Fehlmann CNC 3 D Picomax 60-HSC**)** machine after accurate CAD design (Creo Elements). The turning valves sealing rings were manufactured by Laser ablation (Nd:YAG Laser, Q-Series, Lightwave Electronics). Each valve was fixated to the main cassette body through 4 screws.
For correct motion and complete control of the fluids on the cassette, ventilation outlets (8 and 11, Fig. 1A and 1B) were also designed and implemented on the channels. These ports on the COP plate were covered with PTFE membrane and COP Luer adapters, as done in the case of the pneumatic port.
Finally, in order to capture and purify the NA of the pre-conditioned sample, a 6 mm diameter cavity was designed and milled into the COP plate. A solid phase extraction (SPE) membrane (DNA Tissue Kit S DT-S, FUJIFILM Europe GmbH) was thermally welded into it (7, Fig. 1A and 1B) after being stamped to the required size (6 mm diameter).

4.3. Swab/sample extraction chamber

The swab/sample lockable chamber (1, Fig. 2A) is an independent sample holder where the original swab is cut and placed to perform the automated analysis. It serves both to load and to treat the swab sample. The chamber was manufactured by mechanical structuring (CNC EMCO E45) polycarbonate (PC, Makrolon 2458, REIFF Technische Produkte GmbH) and it can hold up to 1.8 ml of volume. A 3 mm diameter hydrophobic membrane (Fluoropore Membrane Filter, PTFE, 0.45 µm, Merck Millipore), thermally welded within the lid (2, Fig. 2B), allows for ventilation of the swab extraction chamber in the course of sample preparation once the chamber is plugged into the cassette.

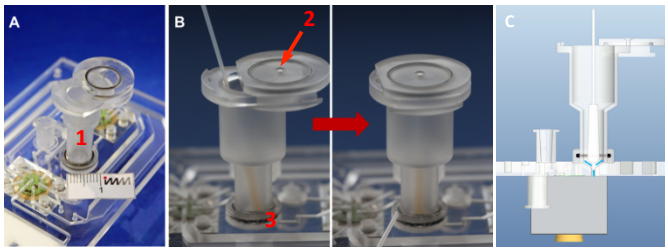


Fig. 2 A is the final analytical cassette with the plugged in extraction chamber. **B** shows the sequence of swab sample cutting and chamber locking. **C** shows the reagents and air introduction connection in the swab chamber once plugged in the LOC cassette.

The design consisted on a cylindrical reservoir with two different cavity diameters, an interface to the cassette, and a sliding cap. The smaller diameter (6 mm) at the base part of the chamber was designed to allow for sufficient buffer agitation relative to the swab surface for sample re-suspension and pre-treatment. The increased diameter (12 mm) on top of the chamber enabled increased volume capacity. Both chambers transition to a smaller diameter were conical (Fig. 2C). The plug-in interface of the chamber to the LOC cassette incorporated an O-ring (3, Fig. 2B) to prevent leakage during cartridge operation. The sliding cap was designed to be able to shaft the swab by shearing mechanism (Fig. 2B) and to seal the chamber. Finally, the hydrophobic membrane implemented within the lid allowed for ventilation in the course of cassette operation. This permitted sample re-suspension, mixing and homogenization during reagent treatment. These operations were based on agitation obtained by introducing air and forming bubbles through the bottom cassette interface (Fig. 2C). Bubble formation during mixing is not critical and further influences on the fluidic workflow are excluded since direct addition of ethanol to the swab extraction chamber results in elimination of remaining air bubbles in the most troublesome cases.

2.5. Controlling unit design, fabrication and implemented protocol

The disposable, sample and reagents pre-loaded, cassettes and the custom-made controlling unit (Fig. 1C) were designed to interface through the turning valves and the pneumatic port, connected to syringe pump tubing (PTFE Tubing biochem scientific). The portable controlling unit was fabricated in-house, as a proof of concept demonstrator, by combining and integrating an electronic control unit (hardware National Instruments, software Labview), an electronically controllable syringe pump (IMM syringe pump module), two stepper motors (Faulhaber stepper motors), and a pressure gauge (Fujikura XFPM700KPGR). The device was operated and programed using Labview (Version 2012, National Instruments Corp.). The overall system interacted and automatically performed the full assay by controlling the required parameters and the fluid position. The demonstrator allowed feasibility testing and improved repeatability.
Prior to the overall device operation, LOC filling of buffers was done manually and the extraction chamber was locked in. Single-use cassettes were then placed and connected to the controlling unit (1 and 2**,** Fig. 1C) and the sample loaded. The sample preparation workflow was then started by pressing the start button and the whole process ran automatically. A two-step lysis procedure was then performed. First, 300 µl of pre-treatment buffer was introduced at 1.5 ml/min to the swab-extraction chamber (1**,** Fig. 1A and 1B) from its original storing reservoir (2**,** Fig. 1A and 1B). The process continued by continuously flowing air to the chamber (producing a mixing effect) and then incubating the mix at room temperature for 3 minutes. In a second step, GuSCN-based lysis buffer placed in one reservoir (3, Fig. 1A and 1B) was added to the pre-treated sample using the same conditions. Once the cells were lysed, 650 µl of ethanol were added from its storing channel (4, Fig. 1A and 1B), to the swab-containing reservoir at 4 ml/min. This step was needed to stimulate NA binding conditions on the SPE membrane (7, Fig. 1A and 1B) embedded in the LOC. The overall mix was then flown at 9 ml/min through the membrane that was later rinsed with 650 µl of ethanol-based buffer (5, Fig. 1A and 1B), previously loaded at 5 ml/min into the swab-containing reservoir, at 6 ml/min. Finally, 50 µl distilled water (elution buffer), from storage channel 6 in Fig. 1A and 1B, were placed into contact with the SPE membrane (Fig. 3A), at 2 ml/min, and pushed through, at 5 ml/min, into the recovery chamber (8, Fig. 1A and 1B, Fig. 3B) after a 1 minute incubation. Then the sample could be easily recovered by pipetting for analysis.
In the course of development of the LOC cassette it was found out that by integration of an incubation step of the SPE membrane with the elution buffer, a significantly improved efficiency of nucleic acid purification can be achieved. This was not suggested in the standard Fuji purification kit benchtop protocol. To enable this incubation step, a hydrophobic membrane (Fluoropore Membrane Filter, PTFE, 0.45 µm, Merck Millipore) was thermally welded close to the SPE

membrane (11, Fig. 1A and 1B). While closing the outlet channels downstream the SPE membrane, the hydrophobic membrane serves as ventilation port and enables buffer positioning on top of the membrane. Correct buffer positioning is indicated via a pressure increase upon contact with the membrane. Position of the hydrophobic membrane relative to the SPE membrane was designed such, that the buffer contacts the hydrophobic membrane only, when the SPE membrane is fully wetted.

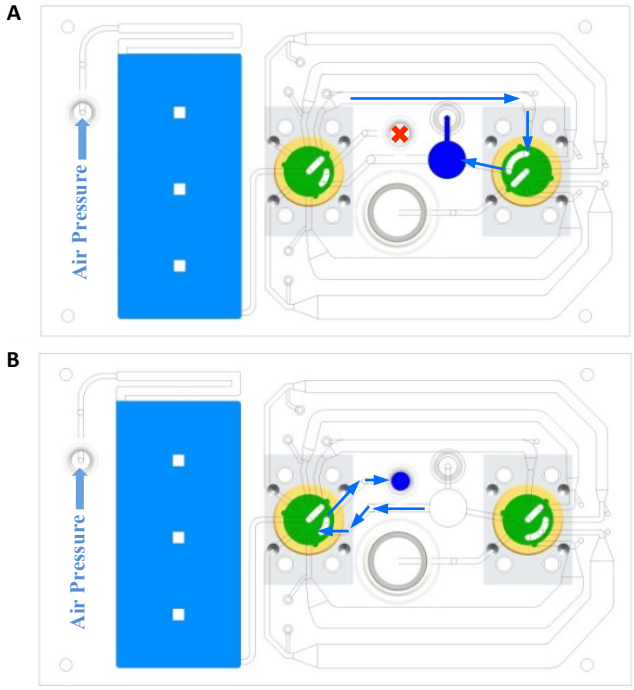


Fig. 3 Sequence of NA recovery from the SPE integrated membrane.

2.6. Nucleic acid quantification

Purified nucleic acids obtained from pure broth cultures were quantified by fluorometry (Qubit dsDNA HS or RNA assay kit, Invitrogen) on a ND-3300 fluorospectrometer (PEQLAB Biotechnologie GmbH). Target specific DNA obtained from spiked samples or pure *S. pneumoniae* culture were quantified using Sybr Green RT-qPCR. Eluted viral RNA was reverse transcribed into cDNA (Reverse Transcription System, Promega) and subsequently quantified using RT-qPCR. The specific intergenic fragment spn9802 of *S. pneumoniae* and M protein of *influenza A* virus were amplified using previously described primers[23,24]. PCR was carried out in a total volume of 25 µL containing 12.5 µL Sybr Green PCR Master Mix (2x concentration, Applied Biosystems), 400 and 800 nM concentration of *S. pneumoniae* and *influenza A* primers, respectively, and 5 µL of genomic DNA or cDNA template. A StepOnePlus instrument (Applied BioSystems) was utilized with the following cycling parameters: 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 61°C (*S. pneumoniae*) or 60°C (influenza A). Standard curves were set up using 10-fold serially diluted spn9802 and reverse transcribed M PCR product of *S. pneumoniae* (ATCC 49619) and *influenza A* (ATCC VR-1520), respectively, as described[25]. Samples were run in triplicate on RT-qPCR. Differences on quantified NA between benchtop and cassette results were always evaluated using the Mann-Whitney U statistical test (SPSS 15.0).

3. Results and discussion

3.1. Lysis protocol verification

In first instance, the lysis protocol was tested without the on-chip NA extraction steps for lysing both Gram-positive and Gram-negative bacteria broth cultures. Benchtop and cassette lysates were compared after purifying the resulting NA with the Quickgene benchtop system described previously. The LOC cassettes used for this comparison had their main plastic COP body sealed with pressure sensitive adhesive. NA extraction results were obtained by lab-standard fluorometry.
On-chip lysis resulted in similar NA yields compared to the benchtop procedure (Fig. 4), thus illustrating the identification of a suitable lysis procedure for Gram-positive and Gram-negative bacteria compatible with the developed LOC concept for sample pre-treatment and lysis. Non-significant differences were found between the two procedures for the three tested bacterial populations.

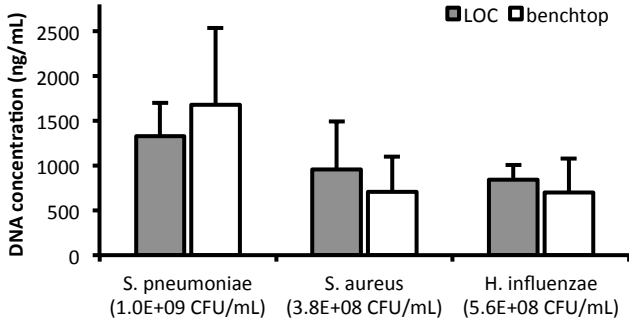


Fig. 4 Average concentrations obtained from broth bacterial cultures of *S. pneumoniae*, *S. aureus* and *H. influenzae* following lysis on the LOC cassette compared to a benchtop method. On-chip and benchtop experiments were performed in triplicate. Error bars depict 95% CI.

3.2. LOC cassette-based NA extraction from bacterial samples

Complete on-chip NA extraction from broth bacterial cultures was studied by fluorometric quantification of nucleic acids eluted from the adhesive film bonded LOC cassette. Results were compared to the concentrations obtained from the same broth culture with the benchtop protocol. For *S. aureus*, average NA yields were significantly higher with the on-chip protocol (992 ng/mL) than the benchtop protocol (407 ng/mL) ($p=0.03$). NA yields from *S. pneumoniae* and *H. influenzae* cultures were also higher on-chip although the difference with the benchtop protocol was not significant ($p \geq 0.05$) (Fig. 5).

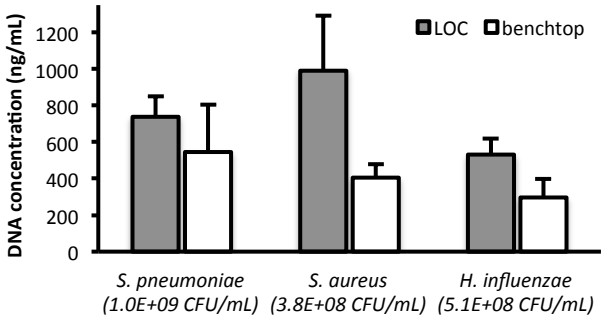


Fig. 5 Average concentrations of eluted NA obtained from pure bacterial cultures of *S. pneumoniae*, *S. aureus* and *H. influenzae* after sample preparation on the LOC cassette compared to a benchtop method. On-chip

and benchtop experiments were performed in quintuplicate and triplicate, respectively. Error bars depict 95% CI.

Since some components of the adhesive film bonding seemed to have an inhibitory effect on PCR, we also tested the microfluidic assay on solvent-assisted bonded LOCs to be able to use PCR both as a downstream application and as a method to quantify the obtained nucleic acids to assess assay performance. For this, a pure broth culture of *S. pneumoniae* ($1.2x10^8$ CFU/mL) was subjected to microfluidic sample preparation on these cassettes having their main COP part sealed with solvent bonded COP sheets. This experiment was done in duplicate and the obtained NA was quantified in triplicate using RT-qPCR targeting the spn9802 fragment of *S. pneumoniae*. On average, $4.5x10^7$ NA copies/mL (range: $2.9x10^7$–$6.2x10^7$ NA copies/mL) were recovered after microfluidic sample preparation on the LOC cassettes in comparison to $2.5x10^7$ NA copies/mL (range: $3.4x10^7$–$1.6x10^7$ NA copies/mL) using the benchtop procedure. Thus, demonstrating that the sample preparation procedure is compatible with the solvent bonded LOC cassette and comparable amounts of NA were obtained using microfluidic and benchtop protocols. Moreover, downstream applications such as RT-qPCR are compatible with the microfluidic sample preparation on solvent-assisted bonded LOC.

3.3. LOC cassette-based NA extraction from viral samples

The performance of the microfluidic sample preparation assay was also examined by on-chip lysis of pure viral culture ($5.6x10^6$ TCID50/0.2 mL). The eluted RNA was reverse transcribed to cDNA and quantified in triplicate by qRT-PCR targeting the matrix protein (M protein) after each experiment. A median value of $2.9x10^5$ cDNA copies/mL was retrieved after performing three solvent-bonded cassette-based lysis and NA extractions, while the benchtop protocol yielded a value of $4.4x10^5$ cDNA copies/mL (Fig. 6A). Differences between the two methods were not significant ($p \geq 0.05$).

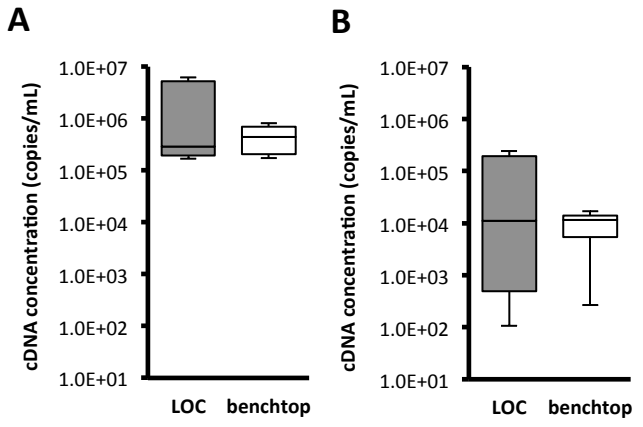


Fig. 6 NA concentrations obtained after sample preparation of pure viral culture (A) and nasopharyngeal samples spiked with *influenza A* virus (B). Experiments were done in triplicate using the microfluidic cassette assay (L.) and benchtop method (b.). Quantitative real-time PCR for quantification of target pathogen was performed in triplicate; data range, median and quartiles depicted.

In a different experiment, NA extraction from the target pathogen in the complex background of a nasopharyngeal swab was also tested on the cassette. Spiked samples were prepared by adding *influenza A* virus to a nasopharyngeal sample that tested negative for the target pathogen. Median values of $1.1x10^4$ cDNA copies/mL and $1.1x10^4$ cDNA copies/ mL were obtained for the microfluidic and benchtop experiments, respectively (Fig. 6B). Again, differences were not significant in statistical terms ($p \geq 0.05$).

4. Conclusions

Using an automated microfluidic assay designed for direct insertion of swab samples, we were able to rapidly lyse both Gram-positive and Gram-negative bacteria. NA recovery results obtained after the LOC cassette-based lysing process showed no significant differences with regular lab procedures thus proving their equivalence.

Improvement of the overall recovered genetic material happened in the cassette after integrating the NA extraction. Average NA yields were significantly higher for the on-chip protocol when confronted to the benchtop protocol ($p=0.03$) for *S. aureus*. Similarly, NA yields obtained with *S. pneumoniae* and *H. influenzae* cultures were also higher for the cassette protocol but could not be considered significant after statistical testing.

Similarly, we performed *influenza A* virus NA extraction on the LOC cassette. Results with samples of both virus pure cultures and nasopharyngeal spiked swabs showed no significant differences between LOC and benchtop procedures after reverse transcription of the obtained RNA into cDNA and subsequent qRT-PCR quantification.

Resuming, experimentally mimicking real sample conditions by performing the sample preparation assay in the presence of a swab, we were able to confirm the feasibility of universal sample re-suspension, lysis, and NA purification and concentration with the presented LOC cassette design and performance parameters. Hence, to the best of our knowledge, being the first time to show a complete miniaturized assay from direct swab insertion, without the need for transfer medium, to the purified NA extraction of any type of bacteria and virus. Similar miniaturized assays could be found in literature for simulated samples of urinary tract infections (urine sample spiked with *E. coli*) or sepsis (whole blood sample spiked with *E. coli*, *Bacillus subtilis* or *Enterococcus faecalis*[26,27]). These performed also comparably or better than a benchtop method, but the NA yield obtained for Gram-positive *E. faecalis* was lower than for Gram-negative *E. coli* so the performance of the assay to extract NA was dependent on the composition of the cell wall and less efficient for Gram-positive than Gram-negative bacteria tested[28].

We also proved that, even with background nasopharyngeal cells and debris, as in the case of viral RNA spiked swabs, the automated and miniaturized device performed equally or better than benchtop equivalents. Eluted NA could also be amplified and detected using RT-qPCR, which is promising for its application on an integrated nucleic acid-based in-vitro diagnostics point-of-care test (IVD-POCT) for rapid diagnosis of CA-LRTI. The incorporation and miniaturization of multiple steps on chip-based assays provides significant advantages in terms of less hands-on time, reduced risk for contamination, less reagent and sample consumption, and short time to result[29].

Abbreviations

CA-LRTI community-acquired lower respiratory tract infections; LOC lab-on-a-chip; POCT point-of-care test; RT-qPCR quantitative real-time PCR; SPE solid phase extraction;

S. pneumonia Streptococcus pneumoniae; S. aureus Staphylococcus aureus; H. influenza Haemophilus influenzae; IVD-POCT in vitro diagnostics point-of-care test.

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Notes and references

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