

Citation for published version:

Des Brennan, Helena Coughlan, Eoin Clancy, Nikolay Dimov, Thomas Barry, David Kinahan, Jens Ducreé, Terry J. Smith, and Paul Galvin, 'Development of an on-disc isothermal in vitro amplification and detection of bacterial RNA', *Sensors and Actuators B: Chemical*, Vol. 239:235-242, February 2017.

DOI:

<https://doi.org/10.1016/j.snb.2016.08.018>

Document Version:

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Development of an on-disc Isothermal *In Vitro* Amplification and Detection of Bacterial RNA.

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Abstract

We present a centrifugal microfluidic “Lab-on-a-Disc” (LoaD) system capable of implementing nucleic acid *in vitro* amplification using non-contact heating and fluorescence detection. The system functionality is verified by implementing a Nucleic Acid Sequence Based Amplification (NASBA) reaction, targeting the tmRNA transcript of *Haemophilus influenzae*. The NASBA assay incorporates fluorescent molecular beacon probes reporting target tmRNA amplification for endpoint detection. The system implements non-contact IR heating to heat the NASBA reaction to the required target temperatures during denaturation and amplification steps. The LoaD control system facilitates spin speed and chamber positioning for heating and fluorescence detection. The LoaD alignment system uses magnetic fields to locate and lock the chamber in the required position (heating or detection). The NASBA assay was implemented on the system using *Haemophilus influenzae* tmRNA over the range 10^2 – 10^4 cell equivalent (CE) units. For comparison, identical qNASBA assays were implemented on a Roche LightCycler 2.0 over this concentration range.

Keywords: Isothermal amplification, Lab-on-a-Disc (LoaD), tmRNA, IR heating, fluorescence detection.

1. Introduction

First generation point of care (POC) immunoassay diagnostic tests were relatively complex to perform and typically demonstrate poor sensitivities (25-65%), when compared to established laboratory based nucleic acid (NA) tests [1,2,3]. Second generation POC tests have adapted NA amplification for rapid disease diagnosis. The sensitivity and specificity of NA tests are typically > 90%, with multiplex detection and analytical sensitivities in the range 1 – 100 cells [4]. Recently a number of commercial PCR platforms have emerged [5,6] for POC infectious disease screening from Cepheid and BiofireDX. Many of these use complex fluidic pump and valve systems to implement biochemical protocols, increasing system cost. The ability to integrate liquid manipulation on the

fluidic device has increased interest in centrifugal microfluidics, where centrifugal forces manipulate liquid reagents [7,8]. The LoaD system is an open platform where liquid moves outward towards the disc edge expelling air as it progresses. This facilitates on-disc liquid storage, negating the need for external pumps, valves or reservoirs found in hydrodynamic systems [9]. Furthermore, valving can be implemented and pressure gradients controlled through careful device design and optimized spin protocols [8,9,10]. At elevated temperatures in open systems, liquid loss by thermo-capillary pumping or evaporation can be an issue, thus on-disc PCR requires robust valving [11].

Isothermal amplification offers advantages over PCR, removing thermal cycling reduces power consumption, eliminates complex electronics [12], and reduces liquid leakage/evaporation. Isothermal amplification methods achieve sensitivities and specificities matching or exceeding that of PCR and are capable of delivering results in <10 minutes [13]. Nucleic Acid Sequence based Amplification (NASBA), employs the simultaneous action of three different enzymes to amplify sequences from an original single-strand RNA template. The addition of molecular beacon probes to a NASBA reaction enables real-time and multiplex analysis [14]. NASBA has been employed for detecting microorganism RNA from a range of biological samples [15,16]. Since NASBA is an RNA specific amplification technology it offers several advantages over conventional DNA amplification technologies; (i) enabling quantitative detection of microorganism RNA [15,16,17], (ii) no amplification interference from contaminating background DNA [18], (iii) direct detection of RNA transcripts [19], (iv) yields of up to 10^{12} copies [13] and (v) has the potential to only detect viable micro-organisms [20].

Previous reports of NASBA in microfluidic devices use direct contact heating to maintain reaction temperatures [21,22]. Non-contact heating facilitates LoaD NASBA amplification, allowing spin without the complexity of contact heating. Approaches to noncontact microfluidic heating include; microwave [23], induction [24] and infrared [25]. Microwave and induction heating require complex electronic components e.g. high frequency oscillators (26GHz) with high power inputs (20-70W). More recently, noncontact IR heating was applied to the rapid amplification of nucleic acids in microfluidic devices [26] and IR heating facilitates rapid heating/cooling of nanolitre sample volumes [27,28].

Previously a LoaD system was demonstrated for solid phase purification of total RNA from MCF7 cell lysates with integrated dissolvable film and hydrophobic membrane valves [29,30]. This highlighted the possibility of integrating complex biochemical protocols on a LoaD system for downstream applications such as realtime amplification. In this work non-contact IR heating and fluorescence detection was applied to endpoint NASBA on LoaD. A software/hardware system implemented:(i)heating,(ii)detection,(iii)spin-control and (iv) chamber alignment for heating/detection. A LabVIEW™ programme controlled system timing, signal acquisition and data analysis. For detection performance endpoint NASBA assays were implemented on LoaD over the range 10 to 10^4 *H. influenzae* CE to determine system detection limits. For comparison, identical qNASBA assays were also carried out on a Roche LightCycler 2.0. The system demonstrated suitability for implementation of the NASBA heating protocol and optical fluorescence detection. This paper outlines the materials and methods used in: (i) device chamber fabrication/assembly,(ii) biological test samples and (iii) system components to implement heating, detection and LoaD spin control. Results presented detail the system performance for detection of *H. influenzae* tmRNA over a clinically relevant range.

2. Materials and methods

2.1 Microfluidic chamber fabrication

The NASBA reaction chamber was injection moulded using Zeonor™ (1060R, Zeon Chemicals Europe Ltd) on a Babyplast 6/10P machine (Babyplast; Molteno, LC, Italy), pellets were dried for sixty minutes at 55°C. The system parameters were set for Zeonor™ [31]: Injection nozzle 195°C, injection chamber 210°C, plastic melt chamber 220°C, with three second injection at 60bar. The manufactured components were cleaned as follows: (i) forty minutes at 50°C in an ultrasonic bath (10% methanol) (ii) forty minutes at 50°C in an ultrasonic bath (0.1% TWEEN 20), (iii) forty minutes at 20°C in an ultrasonic bath (de-ionised water) and (iv) ten minute ozone clean (ProCleaner™ BioForce Nanoscience, UT). The microfluidic component was designed using computer-aided design (CAD) software (Solid Edge; Siemens, Plano, TX) and the injection mould was milled in brass using a Computer Numerical Control (CNC) milling machine (Bridgeport GX 480 VMC; Elmira, NY). The chamber dimensions were 500µm deep, 4mm wide and 15mm long (figure 5). The chamber design tapered from 4mm to 500µm wide at the inlet/outlet channels minimising air bubble trapping. The reservoir had inlet and outlet ports drilled for pipette sample loading and was mounted on a 4mm thick Load substrate using double sided pressure sensitive adhesive (MH-92712-3, Adhesives Research Ltd, Limerick, Ireland).

2.2 *Haemophilus influenzae* culture and RNA extraction.

Haemophilus influenzae (DSMZ23393) was cultured overnight in liquid haemophilus test medium (HTM) at 37 °C under microaerophilic conditions. Following this the organism was subcultured in HTM broth by transferring 100 µl of overnight culture to 10 ml fresh HTM broth and allowed to grow to exponential phase (~ four hours). Once in exponential phase, 1ml aliquots of culture were harvested and the *H. influenzae* cells were collected by centrifugation for two minutes at 12,000 x g. Total RNA was isolated from the cell pellet using the RiboPure yeast kit (Ambion, Austin, TX, USA) according to the manufacturers' instructions. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). RNA with an RNA Integrity Number (RIN) above 7.0 was used for NASBA assays. RNA concentrations were determined using the Qubit RNA BR Assay kit and the Qubit 1.0 fluorometer (Life Technologies, Carlsbad, CA) CEs were calculated on the basis of 1 cell containing 100 fg of RNA [32].

2.3 NASBA assay design

A pair of primers and a molecular beacon were designed to specifically amplify and detect the tmRNA transcript of *H. influenzae*. The primers (5'-3') P1; AATTCTAATACGACTCACTATAGGG-AGAAGG-CTTCGATCCTCAAACGGT, P2; GCAGCTTAACCT and a molecular beacon 5'FAM CCGAGT-GGGGATAACGCGGAGTCA-ACTCGG DAB 3' were designed according to recommended guidelines [33,34]. Primer and molecular beacon probes were supplied by Eurofins MWG Operon (Ebersberg, Germany).

2.4 qNASBA *H. influenzae* assay

NASBA assays were performed using the NucliSENS EasyQ Basic Kit V2 (Biomérieux, Marcy l'Etoile, France) to manufacturer's instructions. qNASBA reactions were performed in a total volume of 20 μ l. Target RNA (5 μ l) was added to 10 μ l reagent/KCL (70 mM final concentration)/primers and molecular beacon probes (0.2 μ M and 0.1 μ M concentration respectively) mixture. The reaction was incubated on an iCycler thermocycler (Bio-rad, Hercules, CA) for 65°C for five minutes to denature the RNA secondary structure followed by 41°C for five minutes to allow primer and probe annealing. Subsequently, the enzyme mixture (5 μ l) was added to the reaction. The reaction was then incubated on a LightCycler 2.0 (Roche Diagnostics) at 41°C for 60 minutes with a fluorescent measurement (530 nm) recorded every minute. A no template control (NTC) consisting of water instead of RNA was included in each experiment.

2.5 On-chip NASBA

NASBA reactions were performed using the NucliSENS EasyQ Basic Kit v2.0 (BioMérieux, Marcy l'Etoile, France). A 40 μ l sample volume consisting of 20 μ l reagent/KCL (70mM final concentration)/primers and molecular beacon probes (0.2 μ M and 0.1 μ M concentration respectively) mixture, 10 μ l RNA template, and 10 μ l enzyme mix was prepared and used to fill the NASBA chamber. Reagent/KCL/primer mix and RNA template were premixed prior to loading on the chip. The reaction was incubated on the IR heater at 65 °C for five minutes and then cooled to 41°C for five minutes. Following the 65°C denaturation step, the enzyme mix was added directly to the chip and placed back on the IR heater at 41°C for one hour. Endpoint fluorescence on the NASBA product was measured at room temperature using the detection system.

2.6 System functionality

The instrument implemented denaturation/amplification temperature profiles using a PID controller to modulate heater power. A LabVIEW™ clock function held the target chamber at 65°C for five minutes, it then cooled & held the chamber at 41°C for five minutes for primer annealing. The motor spin parameters were set to generate centrifugal forces facilitating sample manipulation and chamber repositioning between the heating & detection locations. Chamber repositioning was achieved by rotating the LoaD in discrete 18 degree steps, while activating the position magnet (section 2.9). With the LoaD locked in position the sample chamber is heated to 41°C for 55 minutes. After amplification, the system rotates chamber rotated to the fluorescence detection and activates the excitation source and acquires the fluorescence signal from the PMT detector (section 2.8) via the daq card (NI-DAQ6229) analogue input.

2.7 Non-contact IR heating system

The non-contact heating element is a broadband NIR filament emitter from Sci-Tech Instruments (IR-12K) positioned at the inner focus of a parabolic reflector. The PID controller receives a signal from the IR thermal sensor (Omega OSR137-1-MA) and modulates power to the heater and cooling fan (20watt) via two high voltage relays (MOSFET 100V). A calibrated thermocouple (K type, Chauvin

Arnauk TK2000 instrument) was placed in the microfluidic chamber (filled with deionised water) the controller was set to hold the IR heater output fixed for five minutes before the temperature was recorded. For calibration, forty measurements were made over the range 20°C to 70°C. The heater to LoaD distance was fixed at 10mm while the sensor to LoaD distance was fixed at 15mm, identical materials and chamber designs were used for calibration and test. The PID parameters were tuned to optimise system response minimising overshoot/oscillation around set-points.

2.8 Fluorescence detection system

The detection system (figure 1), is controlled via LabVIEW™ through the NI-daq card. The optical setup uses a dual band (FAM/CY5) fluorescence emission filter (Omega optics XF 535-700DBEM). Two laser diodes; (i) 635nm (Thorlabs CPS182) and (ii) 450nm (Thorlabs CPS450) excite sample fluorescence for detection. Laser output was defocused illuminating the entire chamber (15mm x 4mm), a microscope objective focused light from the chamber onto the detector (PMT H10721-01, Hamamatsu photonics, Japan), via the filter. A 1mm diameter circular aperture between the filter and detector reduced stray light. The PMT was biased to 450V with 10msec integration time, the PMT current was converted to voltage using a transimpedance (X10) amplifier (C7319, Hamamatsu photonics, Japan) and measured using the daq card analogue voltage input (0 – 10V).

Figure 1: The system (left & centre) rotates the LoaD counter-clockwise (right) repositioning the NASBA test chamber between the heating and detection locations as outlined in the centre and right images.

Figure 1 illustrates the system implementing NASBA heating and detection within a chamber mounted on a Zeonor™ LoaD substrate. The fluorescence signal is recorded after amplification is completed. The LoaD was mounted in the system and chamber aligned to the detection position for initial measurement and subsequently rotated to the heating position for amplification. Upon NASBA completion the chamber was rotated to the detection position for fluorescent measurement.

2.9 LoaD spin control

The LoaD was mounted on DC motor spindle (2657W024CR, Faulhaber, Switzerland) with a 1000 point line encoder (HEDM5500B, Faulhaber, Switzerland) the maximum motor speed was set to 6000rpm. The acceleration/de-acceleration was set below 50rpm/sec. The LoaD positioning was automated by activating the motor in timed pulses, relocating the chamber from the heating to the fluorescence detection position.

Figure 2: The LoaD motion control (NI-DAQ 6229) and positioning system (RS232) is driven by LabVIEW™ software to actuate the solenoids/positioning magnets and control the motor e.g. acceleration, deceleration, speed, duration etc.

Figure 2 outlines system hardware implementing spin control, the programme implements the spin protocol (speed, acceleration, duration etc.) for sample manipulation and chamber repositioning.

Permanent magnets (MOD4, Magnet Expert Ltd, UK) attached to the LoAD aligned the chamber as the motor rotated the LoAD in short pulses (figure 3). After each pulse, an external magnet fixed on a linear solenoid (Black Knight™ 122, BLP components Ltd, UK) was actuated when LoAD & external mounted magnets overlapped the LoAD/chamber was locked in place. Two magnets mounted on the LoAD in combination with identical solenoid actuated magnets, allowed the chamber to be repositioned between heating and detection points from a random starting location. The motor was controlled via a motion controller (MCDC3006S, Faulhaber, Switzerland) for spin speed, acceleration, de-acceleration etc. The solenoids were controlled via the DAQ card analogue output (6V). All commands were executed in a LabVIEW™ to interface via an RS232 port & DAQ card. The sequence of timed pulses (figure 3) to the external solenoids and timed motor rotation allowed the sample chamber to be repositioned between heating and detection locations. The LoAD rotated by 18° per step, achieved by motor rotation (10rpm) for one second. The solenoid was activated every 500msec when the chamber was aligned the magnets overlap and the chamber locks in place.

Figure 3: The rotation control of the LoAD and the position of the sample chamber were implemented by pulsed (500msec) solenoid actuation of the attached “seek” magnet and the timed rotation (10rpm for 1sec) of the LoAD motor under LabVIEW™ control.

3. Results

3.1 System heating – Temporal Profile

The heating control system implemented the NASBA temperature profile within the sample chamber, from room temperature the sample is heated to 65°C at a rate of 0.45°C/sec as outlined in figure 4. The sample was maintained at 65±0.5°C for five minutes denaturing target RNA and then cooled and held at 41°C for five minutes. For amplification the sample was maintained at 41±0.5°C for 55 minutes. For each power setting the temperature within the microfluidic chamber was measured using a calibrated K-type thermocouple (Chauvin Arnauk TK2000). The power input was slowly adjusted and left to equilibrate for ten minutes before the temperature was recorded.

Figure 4: The NASBA heating profile was applied for the protocol duration (60 minutes), the ramp time from room temperature to the denature temperature of 65°C was 100seconds and the cooling time to the incubation temperature of 41°C was 31 seconds. The sample was maintained at 65°C for five minutes then cooled and held at 41°C for 55 minutes implementing the complete NASBA heating protocol.

3.2 System heating – chamber heating uniformity

To confirm uniform heating, calibrated thermocouples were placed at three locations in the microfluidic chamber and temperature measured as illustrated in figure 5. The chamber heating profile using the IR heater was compared to that of a 1450nm laser (Roithner LaserTechnik L145T600M). Water has a strong infrared absorption peak at this wavelength making it suitable for microfluidic sample heating. For test, the beam from the laser and IR heater were centred on the

chamber (long & short axis). When inverted on the long axis, similar temperature profiles were observed within the chamber.

Figure 5: The thermal profile across the chamber heating with the 1450nm laser is illustrated. The left profile (A) is from the laser focused on the fluidic chamber (2mm spot diameter). A significant fall-off in sample temperature is observed off centre of illumination. In (B) defocusing the laser spot on the chamber generates a more uniform profile. However, the sample temperature also decreases off axis. The temperature profile across the chamber using IR emitter (C) achieved a uniform temperature profile across the chamber.

The focused laser spot size heats approximately 3mm². However, the measured temperature fell rapidly from 65°C at the centre to 54.5°C - 2mm off centre and 47°C - 4mm off centre (figure 5A). Defocusing the laser beam (Figure 5B) spread the beam over a larger area, resulting in a decrease in the intensity per unit area. Power was increased to 3.8W to maintaining the reservoir centre at 65°C. The measured temperature 2mm and 4mm above centre was 62°C and 54°C respectively. Further defocusing wasn't possible due to the laser power limit. The IR broadband emitter achieved a uniform temperature profile (65°C) across the chamber (figure 5C) when measured with the thermocouple.

3.3 Fluorescence detection

To verify detection sensitivity, qNASBA reactions were carried out on a Roche Lightcycler 2.0 at concentrations of 10⁴, 10³ and 10² CE. The reactions confirmed successful amplification of the *H. influenzae* tmRNA concentrations tested. The qNASBA products were then loaded onto the LoAD chamber, positioned in the optical fluorescence detector path and the signal recorded (figure 6). This established that the fluorescence system could detect 10² CE for end point detection.

Figure 6: The fluorescent signal from NASBA amplifications undertaken in a bench-top thermocycler and subsequently analysed on the fluorescence detection system (endpoint).

In figure 6 the fluorescence signal increases from the empty chamber (figure 6 – blank) to the NTC sample (figure 6 – control) due to molecular beacon characteristics: (i) the quencher may not completely absorb the reporter dye, (ii) there may be a native fluorescence from the quencher itself and (iii) the possibility some reporter dye molecule detach from the beacon structure. Thus the NTC sample exhibits decaying fluorescence signal during measurement similar to the samples.

3.4 On disc endpoint NASBA using non-contact IR heating

Following independent optimization and validation of the IR heater and fluorescence detection components, on LoAD endpoint NASBA was carried out in the system. Reactions were carried out at 1 x 10⁴, 1 x 10³, 5 x 10², and 1 x 10² CE. A NTC (molecular grade H₂O) was also included. For on LoAD tests RNA was extracted from cultured *H. influenzae* cells and amplification was carried out as outlined in section 2.5. After NASBA incubation the spin control system was used to relocate the sample chamber from the heating position to the detection location. The endpoint fluorescence

intensity for each of the on-chip NASBA reactions, including the NTC, was measured. To determine the absolute fluorescence signal from the NASBA product reactions, the NTC fluorescence signal measurement was subtracted.

Figure 7: The fluorescence intensity for *H. influenzae* RNA amplified and detected on the integrated heating / detection system. The RNA concentrations analysed were equivalent to 10^2 , 5×10^2 , 10^3 and 10^4 cells. The error bars are standard deviation of three repeated measurements per test.

This established increasing fluorescence signal over the range 1×10^2 to 10^4 CEs (figure 7). However, at 10^2 CE no detectable fluorescence signal above the NTC reaction was observed, thus the current detection limit of on-chip NASBA using this system lies between 100 and 500 CEs.

4. Discussion

This work demonstrates end point NASBA amplification of tmRNA transcript (*H. influenzae*) on a LoAD system. tmRNA, encoded by the *ssrA* gene has previously been shown as to be a useful molecular diagnostics target for NASBA [35,36]. It is highly expressed, significantly more stable than mRNA and contains conserved and variable regions making ideal for the sensitive and specific detection of bacterial species [22, 36]. The microfluidic LoAD system offers the possibility to integrate fluidic functionality for assay protocol implementation without the need for the external pumps, valves etc., traditionally used in hydrodynamic systems. In this approach the LoAD chamber is rotated between a heating and fluorescence detection locations using LoAD mounted magnets to align the NASBA chamber, eliminating the need for complex position control. A low temperature isothermal microfluidic amplification approach reduces: (i) bubble formation, (ii) liquid evaporation, (iii) high chamber thermodynamic pressure. Cost is a key consideration for disposable diagnostic devices and plastics are preferred over silicon and glass devices. Thus injection moulded Zeonor [37] devices were manufactured in this work, the devices were biocompatible, optically transparent with low auto-fluorescence.

Laser and broad wavelength IR sources were evaluated, while the 1450nm laser diode achieved the required chamber temperature non-uniform chamber heating was an issue. Thermal gradients within a chamber can create localised diffusion, causing spatial variation in reagent concentration which may impact reaction efficiency [38]. Isothermal amplification doesn't require rapid thermocycling thus slow heating rates are acceptable ($2.5^\circ\text{C}/\text{sec}$). The detection system was evaluated with monoplex FAM assays, but it could detect duplex assays e.g. FAM/CY5, CY3/CY5. The system demonstrated fluorescence sensitivity down to 100CE with tmRNA (*H. influenzae*) sample amplified on a LightCycler 2.0 (Roche Diagnostics). To establish the system performance with NASBA heating and detection, a sample set were tested over the range 10 to 10^4 CE. The full NASBA heating protocol was implemented on the LoAD chamber using the IR heater, the fluorescence signal was recorded before and after incubation. In this work the sample was pipetted onto the LoAD which was loaded onto the system for denaturation, upon completion the sample was removed from the LoAD by pipette mixed with the enzymes and reloaded onto the system for incubation/amplification.

The LoAD motion control system outlined in section 2.9 was used to relocate the chamber between heating and detection zones. This demonstrated a system sensitivity of between 100 & 500CE. The ideal molecular diagnostics assay should be highly accurate (reproducible, sensitive and specific). One characteristic of a highly accurate assay is its analytical sensitivity. In blood stream infections, bacterial load can very often be less than 100 CE/mL [39]. Low bacterial load may be compounded when sample volumes are limited which is often the case with neonates and children [40]. By further optimising the NASBA assay presented here by for example altering the primer/probe concentration or through the addition of betaine to the reaction cocktail should enable improvement of the analytical sensitivity of the assay [36]. The heating system generated temperature profiles compatible with the open chamber LoAD system without air bubble formation, liquid evaporation. Initially on-chamber NASBA tests demonstrated no amplification, a stringent wash protocol prior to test eliminated RNase chamber contamination delivering successful amplification. Biomolecule-surface interaction presents a challenge for microfluidic diagnostics [12]. Thus a published microchip treatment protocol was carried out [41] giving good amplification with comparable results obtained from on-chip and Lightcycler NASBA assays.

Conclusions

A non-contact IR heating approach compatible with fluorescence detection implemented the NASBA amplification protocol within a microfluidic LoAD system for endpoint detection of *H. influenzae* RNA samples between 10^2 and 10^4 CEs. A stringent chamber wash protocol was required to eliminate contamination for amplification. The IR heating approach lends itself to non-contact heating and uniformly heated the sample chamber to the target temperatures suitable for the NASBA assay implementation. Currently the system implements endpoint detection but future work will investigate the possibility of real time heating and detection by rotating the chamber between heating and detection locations. Thus the sample was loaded for denaturation, then extracted for enzyme addition and reloaded onto the chamber for amplification. Future work will implement all functionality on the LoAD. The ultimate goal of such a point-of-care LoAD system is to implement a complete test, incorporating biological sample preparation i.e. extraction, purification and detection. Future work will also focus on achieving a detection limit below 100 target cells.

Acknowledgements

This work was supported by Science Foundation Ireland as part of the Biomedical Diagnostics Institute Centre for Science Excellence and Technology (10/CE/B1821).

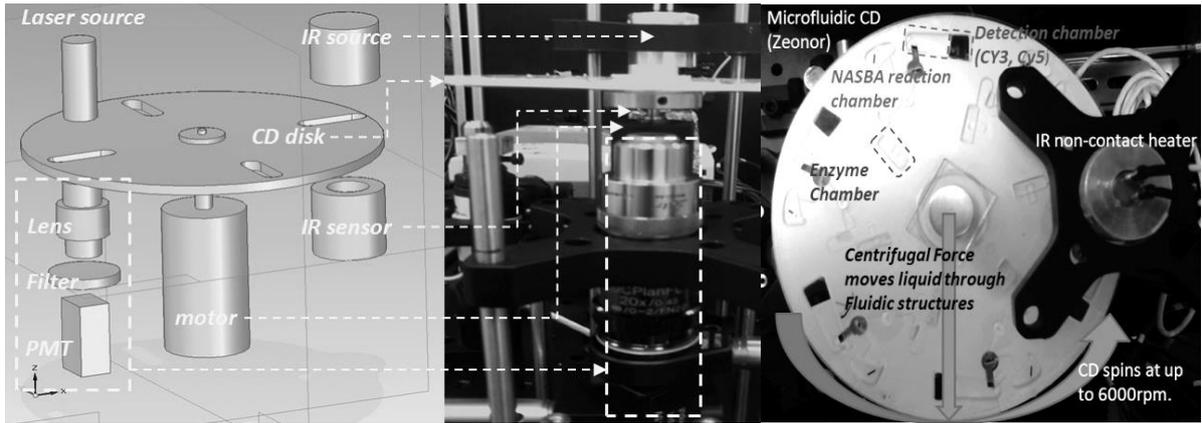
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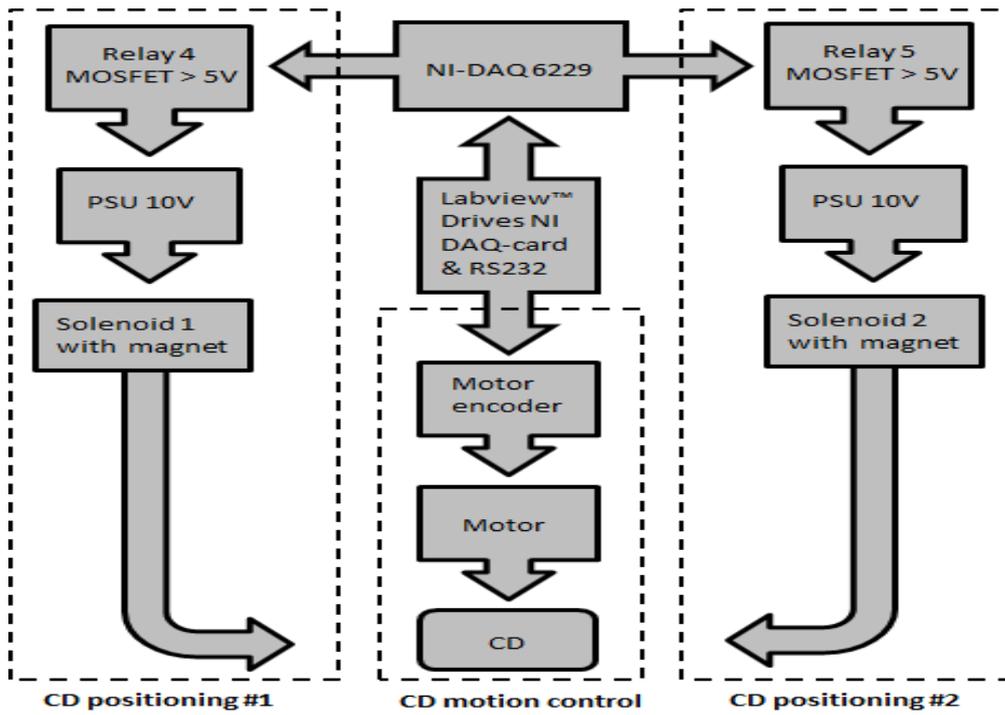
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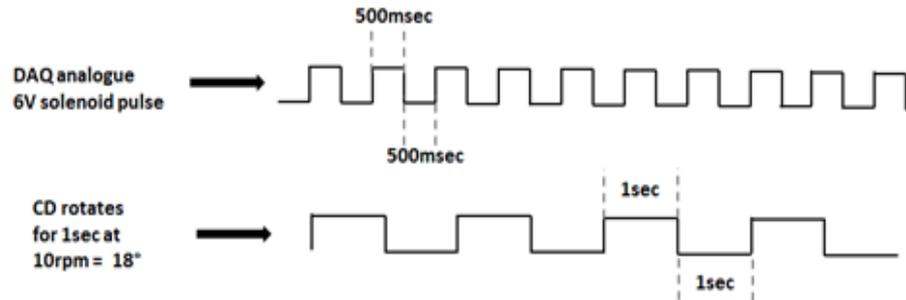
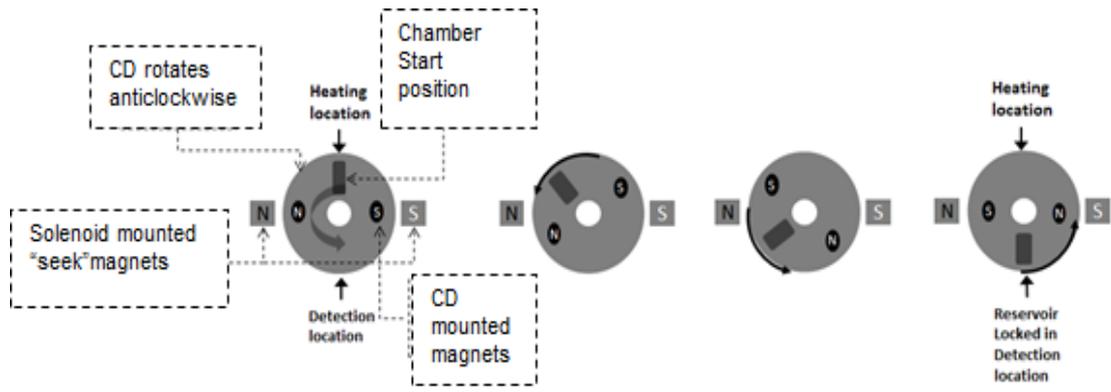
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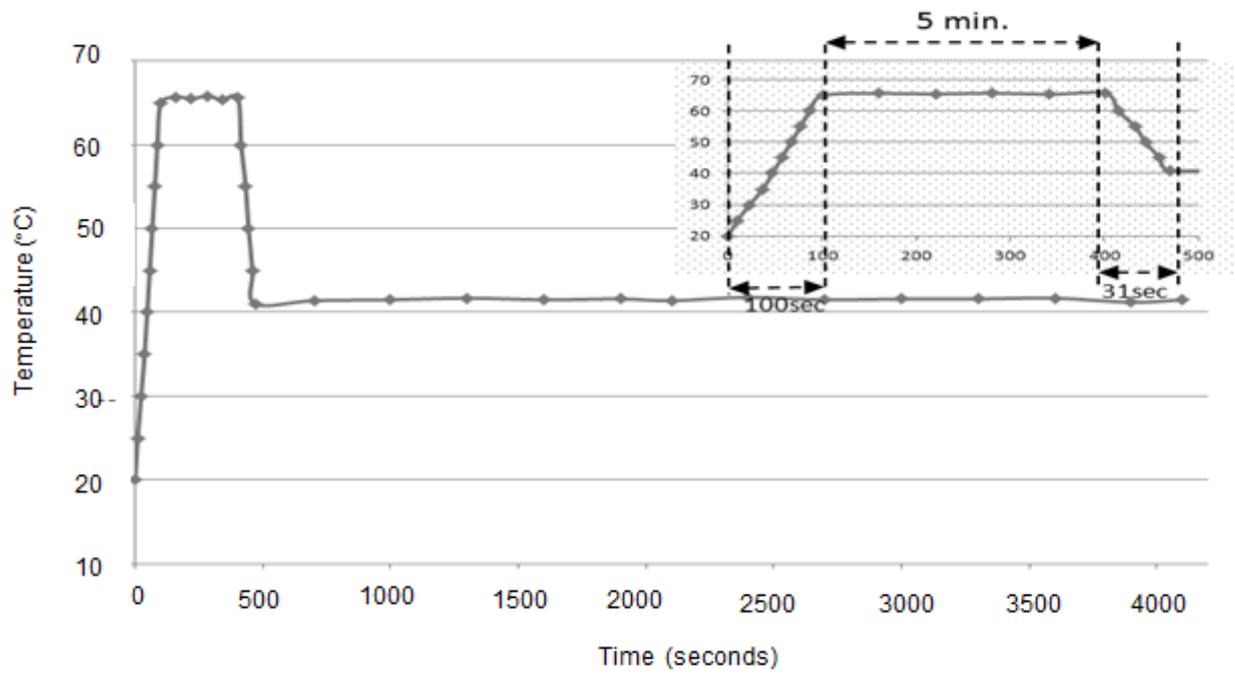
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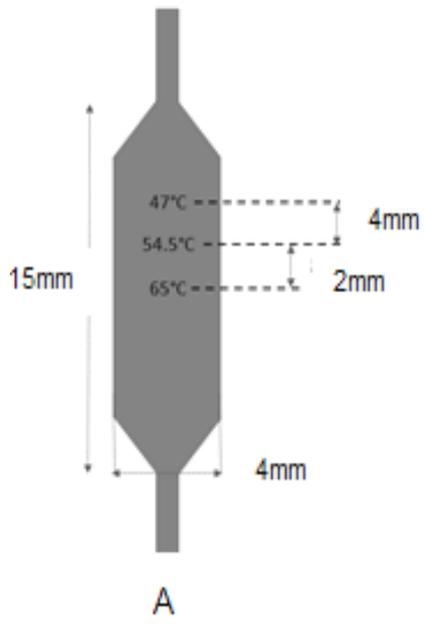




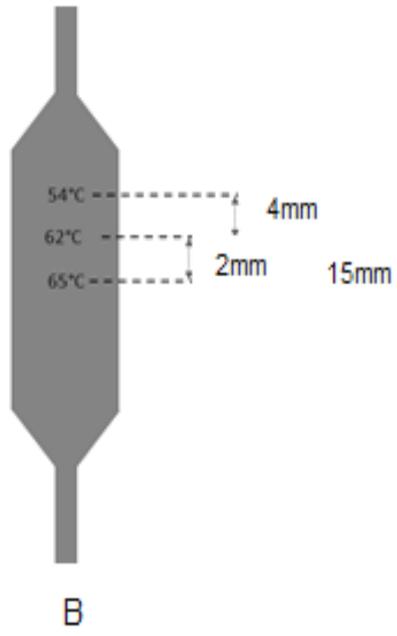
Heating using NIR emitter (10.8 Watt)



At focus laser power 2W



Defocused 4mm & increase
Laser power to 3.8W



Heating profile with IR heater

