POLITECNICO DI TORINO

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MASTER OF SCIENCE IN "NANOTECHNOLOGIES FOR ICTS"

MASTER'S DEGREE THESIS

Development of a *DNA extraction* microfluidic lab-on-a-chip device

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April 2018

Abstract

This Master's thesis project is based on the development of a microfluidic platform for the DNA extraction that joined with an on-chip Plasma extraction procedure and a PCR (Polymerase Chain Reaction) can provide a LOC (Lab-on-a-chip) for POCT (Point-of-care-testing) in Liquid Biopsy application.

Currently the cancer detection is based on the so called Tissue Biopsy, that thanks to a surgery is able to extract cells directly from the cancer tissue that can be then analysed providing the disease required informations. The main drawback of this technique is the high invasiveness at which the patient needs to be subjected just for a diagnostic purpose. Nowadays, in order to overcome this huge limitation, a lot of efforts are employed over the so called Liquid Biopsy, also known as cell-free DNA (cfDNA) analysis.
This technique takes as advantage the discovery of the precisely so called cfDNA, that showed up to be a non-encapsulated DNA in blood stream with a concentration that is higher in cancer patients.
According to studies Non-small cell lung cancer (NSCLC) is the leading cause of cancer related death for both men and women and the last cancer stage (IV) has less than 1% of five years survival rates. Because of these data it was chosen to focus the research over the Stage IV of NSCLC.
The steps to be followed in a Liquid Biopsy procedure start from a simple blood draw for sample collection, followed by a plasma extraction, the *cfDNA extraction*, the PCR (able to isolate and amplified the cfDNA fragments of interest) and then a final analysis to get the required informations of the sample.

The interest in implementing these operations over a LOC is related to many aspects as the portability, speed of diagnosis and treatment and sample consumption.
The DNA extraction characterization was first performed outside the device with two methods (Silica column and Silica magnetic beads) and then brought over the chip.
The achieved results showed an higher efficiency for the beads method, that's why it was the implemented one over the microfluidic platform where, instead, the lowest values were obtained. This is not meant to be surprising since the procedure was more difficult to be performed, in terms of handling and mixing of the solutions. However as the extraction has been carried on, in terms of time, the efficiency trend increased thanks to the higher achieved confidence in following the procedure and making each step properly.
This last consideration is very encouraging for the final application since the efficiency could become even higher with the enhancement of the experience achievable with time. Furthermore, if necessary, larger quantity of the starting sample can be employed to get the cfDNA amount required for the following steps.

Acknowledgements

Finally the end of this hard, long but gratifying path that gave me joy and sorrow, satisfaction and disappointment.
I'd like to thanks all the people that were by my side during these years and I feel lucky to say that there are many.

I'd like to start from Dr. Matteo Cocuzza, my thesis advisor, for his suggestions and his high availability that showed to me not just during this last project, but even before during all the exams I needed to attend with him.

Thanks to Dr. Samuel K.Sia that gave me the chance to make my own project following all the ideas came into my mind, thanks for your great support. I also want to thanks all the laboratory members in particular my tutor Nicole Blumenfeld, that helped me to be introduced in a contest that was new to me with great patient, and Rodrigo Chaves that was always ready to give his advises.

Then how not to start from my parents, that have always supported my studies morally and economically even with my attitude, very hard to deal with. I know it could have been easier to have a chill, kind and thankful daughter during these years but I reserved the greatest gratitude for now. You are the reason why I'm graduating now and why I will have the chance to build the future I'd like to. So thanks mum and dad!
Thanks to my sister, that is now one of my greatest advisor, seven years younger than me and much wiser though. You are my shoulder, so glad we get closer in the last years and I get one of my best friend under my roof.
Thanks to my larger family too, thanks to zio Massimo, zia Rosella, zio Sergio, my forever young cousin Alessandro, and least but not the last zia Anna that has always bet on me. I love you all.

I feel lucky to have so many friends I need to thanks that I don't know where to start from.

Thanks to my oldest friends ever, Giudi, Ele, Fra and Toros. We are together from the high school or even before and we have always found the time to see each other even if our lives are not so easy to fit. Special thanks to Elenina, you are the laziest and most introverted person I've met in my life, but I know I just need to pick up the phone and

you are gonna answer. The same words are meant to be for Carolina that has always been ready to listen to my problems and share together our messed up stories making us feel less strange.

Thanks to "I CALDI", I don't even know why I should thank you guys, you are the most whiny, counterproductive and stubborn persons I know but you are my friends, for better or worst, and I appreciated ,almost, all the time we had together. Thanks Luci, Matti and Edo for made your home almost mine too, and thanks Edo for getting crazy with me during all these years of study and thanks for worrying about me as not as many do.

Special thanks to Elisa and Claudia, my Bamboline, because with you I can always act as stupid as I am and I know you are gonna love me anyway, maybe you love me because of that actually.

Thanks to my Virgis, we met each other not so many time ago and you are my daily mate, I miss so much to have you here making our own improbable projects.

I still need to thanks all my university mates, that faced with me these hard two years, so thanks Tiziano, that deserves a special tribute for his first effort of making me give up but then he helped me in solving every doubt I got, and I got many, Fra, my tricks mate, Davide, Fede and Giuse.
Thanks to Daniela, my confidant, my bachelor study mate, my motivator.
Thanks to the other Daniela too for our laughs that sound like a free breath in that hell called opera.

The last people I need to thanks are the one that I still feel close to me after seven months in that crazy place that is NYC. Thanks to Yann, we partied, we talked, we shared our feelings and our thoughts, you became my best friend in that jungle, so thanks for that.
Thanks to Yahya, we went through a lot of things together but you showed me the city in a way I would have never seen.

I was going to end up these long Acknowledgements but I feel like I forgot to thanks someone that sounds like.. my oldest and best neighborhood mate, thanks Giacomino!

Thanks to the other people I forget to mention but the list would have become too long!

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List of Acronyms and Abbreviations

POCT	Point-Of-Care-Testing
LOC	Lab-On-a-Chip
μ-TAS	Micro-Total Analysis System
MEMS	Micro-Electro-Mechanical-System
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
dsDNA	Double-Stand DNA
ssDNA	Single-Strand DNA
NA	Nucleic Acids
cfDNA	Cell-Free DNA
ctDNA	Cell-free-Tumor DNA
gDNA	Genomic DNA
NSCLC	Non-Small-Cell Lung Cancer
SCLC	Small-Cell Lung Cancer
IC	Integrated Circuit
PDMS	Poly-DiMethyl-Siloxane
PMMA	Poly-Methyl-MethAcrylate
PC	Poly-Carbonate
SPE	Solid-Phase-Extraction
CNC	Computer Numerical Control
dH_2O	Deionized Water
ddH_2O	Purified Water
Na_2HPO_4	Sodium Hydrogen Phosphate
SiO_2	Silica
Fe_3O_4	Magnetite
G_uHCl	Guanidine Hydrochloride
C_3H_8O	Isopropanol
C_2H_6O	Ethanol
Tris-HCl	Tris-Hydrochloride
IPA	IsoPropyl Alcohol
3-APTES	3-AminoPropylTrieThoxySilane
μ	Magnetic Momentum
B_r	Remanent Magnetization
H_c	Coercitive Force
δ	Magnetic Domain Wall
J	Magnetic Exchange Constant
S	Total Spin Quantum Number

a	Inter-Atomic Spacing
K	Magnetic Anisotropy Constant
V	Potential
σ	External Particles Diameter
r	Distance between two particle
$\mathbf{F_m}$	Magnetic Force
$\mathbf{F_d}$	Hydrodynamic Drag Force
m	Magnetic Moment
V	Bead Volume
μ_0	Vacuum Permeability
χ	susceptibility
B	Magnetic Field
H	Magnetic Field Strength
η	Fluid Viscosity
VDW	Van Der Waals
bp	Base Pair
BB	Binding Buffer
WB	Wash Buffer
EB	Elution Buffer

Chapter 1

Introduction

1.1 Lab on a chip technology for POCT

The need of accomplish complex laboratory tests in areas in which a fully laboratory equipment is difficult to supply or providing a quick analysis test in loco, brought to the concept of **Point-of-Care-Testing (POCT)**.
POCT is defined as medical diagnostic testing able to move testing back closer to the patient.
This new way of testing has to integrate in a single chip multiple functions in order to achieve its goal and it has become possible due to the continuing advances in technology that have produced smaller devices that are more suitable for an outside laboratory application.
Nowadays always more attention has been paid over POCT due to the several advantages associated that can be summarized as follow.

Advantages

- **Portability**: it allows for more frequent and consistent testing since they can be performed by the patient even at their home. Furthermore they can also provide test results in location where clinical laboratories are not present
- **Speed of diagnosis and treatment**: there is no need of transport the sample in different laboratories
- **Sample handing by a single operator**: it decreases the probability of contaminations or errors due to sample handled by different people

- **Sample and reagents consumption**: POCT are usually implemented in miniaturized devices that lead to a lower amount of sample and reagents
- **Power consumption**: this is also a consequence of the miniaturization

Disadvantages

- **Limited number of available tests**
- **People training for the usage**: who is performing the test needs to follow carefully the instruction and if not it can lead to wrong results

Lab-on-a-chip (LOC) (or micro-total analysis systems (μ-TAS)) seems to be the perfect answer to the POCT requirements, being by definition a device that integrates one or several laboratory functions on a single integrated circuit [5].
These devices are a subset of the so called **micro-electro-mechanical-systems (MEMS)** that are tiny integrated devices or systems that combine sensors and/or actuators with electrical components. LOC seems to show great promises in different applications as in the field of proteomics (shorter time for protein analysis), cell biology (ability to control cells at single-cell level), chemistry (high efficiency in chemical reaction control) and molecular biology (DNA/RNA amplification and detection).
The Sia Lab at the Columbia University in the City of New York is working on the development of a *sample to answer* PCR platform, that is crucial in several testing such as HIV, viruses and fungi diagnosis, but in this case the focus is over the so called *Liquid Biopsy* also known as cell free DNA Analysis, that allows to diagnose cancer.
Lung cancer is the leading cause of cancer related death for both men and women. It is possible to differentiate lung cancer in Non-small cell lung cancer (NSCLC) and Small-cell lung cancer (SCLC). NSCLC comprises 80-85 % of all lung cancers with poor diagnostic at late stage diagnosis. Looking at the NSCLC five years survival rates the Stage I has 45-49 %, Stage II 30-31 %, Stage III 5-14 % and Stage IV < 1 % ([45]). According to these data the POCT platform development was centered on the Stage IV of NSCLC.

1.2 Microfluidic devices

When a LOC needs to work with fluids, as in this specific project, **microfluidic devices** are employed so a quick review over them is reported. Microfluidic devices allow to work with very small volumes of liquid within a fluid network, made of channels and chambers. In order to manipulate the fluids these devices are integrated with several components such as micro-pumps, micro-valves, micro-needles and many others.
The first idea that can arise is that the microfluidic is just a geometrical miniaturization of its macrofluidic counterpart, but the passage from macro to micro hides much more

details.
The main issue related to scaling is the higher surface/volume ratio that brings more attention in controlling the surface characteristic.
An important effect to point out is that the surface forces dominate over the volume ones at microscale, in fact reducing dimensions of a factor k implies that the volume forces are decreased of k^3, the surface forces of k^2 and the linear ones of k. Obviously this have a consequence on the pressure that has to be applied to drive a flow in a channel:

$$\Delta P = \frac{8\mu LQ}{\pi r^4} = \frac{8\mu LQ}{wh^3} \tag{1.1}$$

Where ΔP is the pressure drop between the two ends of the channels, μ is the viscosity, L is the channel length, Q is the volumetric flow rate and r is the channel radius (w the width and h the height for a rectangular channel). Thanks to this formula it's possible to evaluate the pressure across a channel according to the applied flow rate. As the geometrical dimensions are scaled down as the pressure dramatically increases, so it's important to find a balance between the flow rate needed and the pressure that can't be too high.
An other important change that has to be considered is related to the *Reynolds number* that is defined as following and according to its value determines if a liquid flow is laminar or turbulent :

$$Re = \frac{(\rho VD)}{\mu} \tag{1.2}$$

D is the characteristic length of the channel (length or diameter) that in microscale is reduced providing a lower *Re* and so to a flow that is always laminar. This affects the way how two liquids can be mixed, that in this case becomes just because of diffusion and so long channels are often required.
Also the thermal system is involved, in fact the heat is conducted out from a body more quickly leading to excellent thermally isolated or conductive structures.

1.2.1 Materials

Microfluidic chips can be fabricated with several materials but each time the choice is made in order to meet the requirements of the specific application. First of all the material does not have to change the fluid property and not interact with possible buffers, it has to be able to work at temperatures required in the process and also doesn't have to overcome the production cost available.
The last is also a point to be considered with attention since, while electronic devices are fabricated using IC (Integrated Circuit) technology, MEMS are fabricated by sophisticated manipulation techniques that depend on the kind of material used and the cost of fabrication can turn out to be very high.
Microfluidic devices can be divided in three categories materials: inorganic, polymeric, and paper.

Inorganic

Silicon has an high resistance to organic solvents, high thermo-conductivity and also has a quite high Young modulus (130- 180 GPa). But due to it's hardness is not easy to use for active components fabrication as valves or pumps.
Glass is an optically transparent and electrically insulated, amorphous material. It is compatible with biological sample but the main issue is related to its high fabrication cost.

Polymers

Elastomers consist of cross-linked polymer chains; they can stretch or compress when external force is exerted, and return to the original shape when the external force is removed. The main elastomer used in microfluidic chips is **Polydimethylsiloxane (PDMS)**.
This is one of the most common material due to its low cost, rapid fabrication and ease of implementation. It can be bonded to glass, Polymethylmethacrylate (PMMA) and PDMS. It is optically transparent, inert and non-toxic. When PDMS is produced it shows viscoelastic properties, that means it acts like a viscous liquid, but after a certain treatment at high temperature it becomes an elastic solid, very suitable to be used as membrane.
Thermoplastics are highly cross-linked polymers that can maintain their shape after cooling. Optically clear polymers, thermoplastics are resistant to permeation of small molecules, and stiffer than elastomers. The most common thermoplastic used are **Polycarbonate (PC)** and **Poly-methyl methacrylate (PMMA)**. PC has high impact resistance, good machining properties and it is PCR compatible but poor resistive to certain organic solvents. PMMA is the least hydrophobic plastic material, optically transparent and PCR compatible and it has rigid mechanical properties and most of all is very cheap.

Paper

Paper is a flexible, cellulose-based material. It is very cheap and easily chemically modified but due to its weak mechanical properties it can be used just in a limited number of applications.

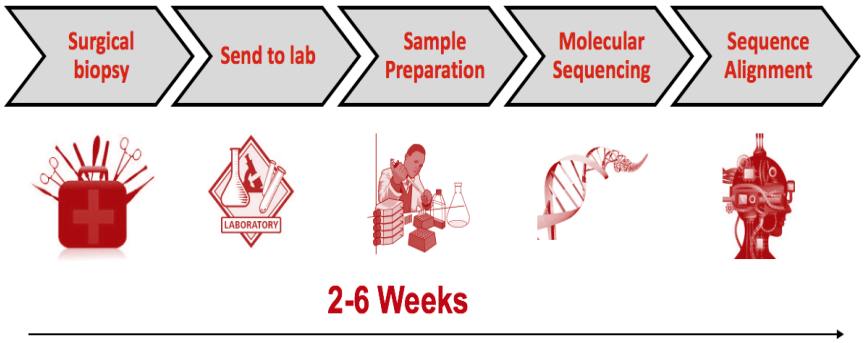
1.3 Liquid Biopsy

Liquid Biopsy is a blood test able to look for tumor DNA directly from a blood sample, allowing to get a much faster and safer sampling step and moreover a simpler monitoring of diseases progression or response to treatments. It is a very new method that hopefully

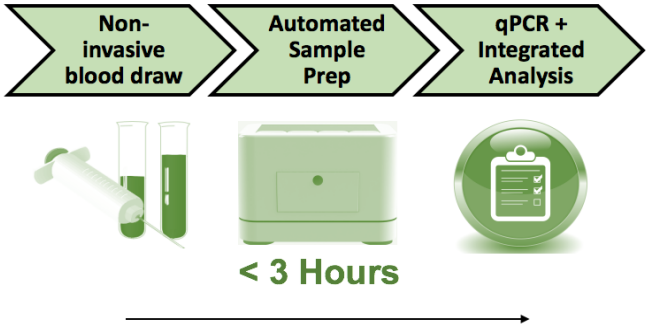
will replace the *Tissue Biopsy*[1] that has several drawbacks as being highly invasive and time consuming.
Liquid Biopsy is also known as cell free DNA (cfDNA) analysis since it is based on the discover of the the so called cell-free tumor DNA (ctDNA), that is a tumor-derived cfDNA.
cfDNA was described at first by Mandel and Metais in 1948 [12] as a non-encapsulated DNA in blood stream with a length that can vary from 100 to 170 bp. It was found just some years later that cancer patients showed higher levels of plasma cfDNA with respect to healthy people and this led to discover the release in the blood of ctDNA.
It is still not very clear how this DNA is released by the tumor but thanks to the ability of detecting low levels, it's possible to diagnose cancer up to 2 years prior to the initial diagnosis and, since it also reflects the entire tumor genome, it allows to monitor the disease.
cfDNA concentration has not a fixed value because it depends on several parameters, the patient itself, the age, the kind of tumor and especially the stage of the tumor.
The values found in literature are very variable so it's hard to define an exact range of cfDNA concentration, both for healthy and cancer people. According to Pérez-Barrios et al. ([13]) cancer patients can show a concentration that varies from 0.2 to 24 ng/μL and just taking into consideration the ones with lung cancer at III and IV stage a mean value of 1.25 ng/μL is found.
Szpechcinski et al.([14]) instead point out how the mean concentration (NSCLC) is 8.2 ng/mL, with a very wide range spreading from 1.12 to 40.89 ng/mL, but they don't make any distinction between the different stages.
A good summary of cfDNA concentration in healthy and cancer patients is provided by Fleischhacker et al. ([15]), where it's very clear how researchers need still to figure out a precise range. Just employing a different detection methods and a different number of NSCLC patients, cfDNA concentration changes from 3.7 to 318 ng/mL.
So until now it's almost impossibile to define an exact cutoff that defines the cancer stage but overall it's possible to say that the cfDNA is very low and cancer patients suppose to show it of the order of ng/mL. In order to take advantage of this analyte Liquid biopsy has to follow some steps.
At first the sample is collected by a simple blood draw, plasma is then extracted from the whole blood, starting from plasma sample the cfDNA is extracted providing a purified solution that can be used as starting sample for the PCR, able to isolate and amplified the cfDNA fragments of interest, that means the ctDNA. Thanks to the amplification the subsequent analysis is easier to implement having an higher amount of DNA available.
Obviously the implementation of these steps over a LOC platform allows to get all the advantages listed before (Fig.1.1).
The aim of this work will be to characterize the cfDNA extraction out-of-device with several methods and then to bring it over a microfluidic chip so to combine it with the PCR

[1]The removal of cells or tissues for examination by a pathologist. In order to get the cells or tissues different procedures can be exploited but all of them are invasive and require a surgery. [24]

platform developed in the laboratory by other members and obtain a unique LOC able to provide the extraction and the amplification of cfDNA for liquid biopsy analysis.



(a) *Tissue biopsy steps flow*



(b) *POC liquid biopsy steps flow*

Figure 1.1: Tissue and liquid biopsy steps [45].

1.4 PCR

Polymerase chain reaction (PCR) is a technique developed by Kary Mullis in the 1980s used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence [1].
PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA

complementary to the offered template strand and the goal is usually to provide enough of the target DNA so that it can be analysed or used in some other way as for sequencing or gel electrophoresis. The process is based on the mixing of several components (DNA template, pcr master mix, water, downstream and upstream primers able to bind to the dna extremities that contain the dna fragment to be amplified) then subjected to a thermal cycle that follows these steps (Fig.1.2):

- **Denaturation** 95°**C**: DNA strands are separated providing single-stranded template
- **Annealing** 55°**C**: primers bind to their complementary sequences on the single-stranded template
- **Extension** 72°**C**: the so called *Taq* polymerase extends the primers, synthesizing new strands DNA

This cycle is usually repeated 25-35 times generating up to billions copies.

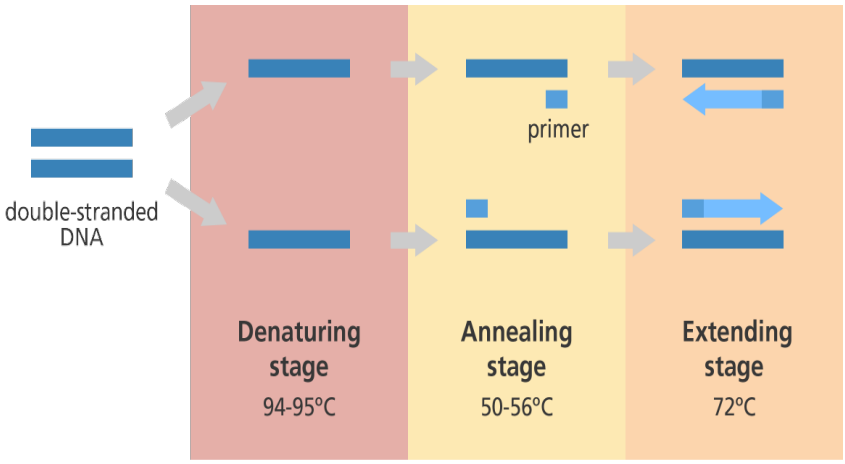


Figure 1.2: Polymerase chain reaction steps

1.5 State of art: DNA extraction

1.5.1 Solid phase extraction

To provide pure Nucleic Acid (NA) samples, free of contaminants, usually POC devices employ a separate extraction systems using solid phase extraction, most of all, Silica based.

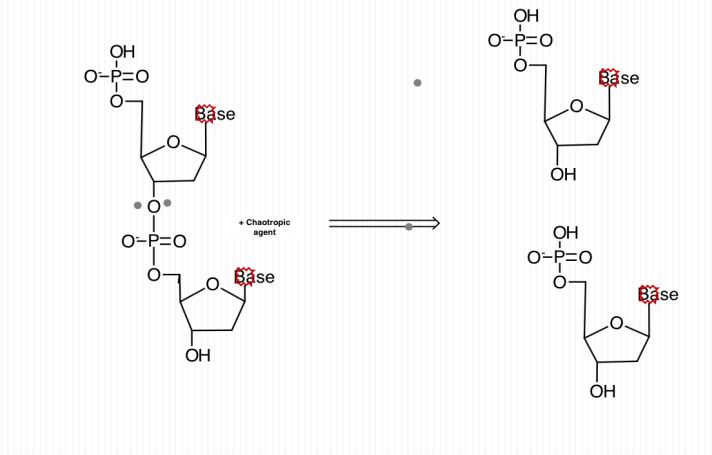
Solid-phase extraction (SPE) is a sample preparation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties [2]. In this case the liquid mixture is the sample and the compound to be isolated is the DNA. There are several SPE methods where the difference is embedded in the Silica form. It can be basically used as micro-nanoparticles, gel or membrane. All these techniques can be exploited both outside and inside the device (for detail see next section). Here the general workflow of silica based extraction method is presented, considering that all the methods are composed by the same main steps (Binding, Washing and Elution).

According to which kind of DNA wants to be extracted a first *sample preparation* has to be done. If the objective is the cfDNA a Plasma extraction is performed, for genomic DNA instead a cell lysis from the starting sample is done, in order to break the cell membrane and make the nucleic acids free in the sample.
The **Binding step** consists of mixing a binding buffer, usually a chaotropic agent, with the sample and the Silica. Chaotropic buffer is used since it's able to enhance the binding affinity between DNA and silica surface via hydrogen bonding and hydrophobic interactions. As can be seen in figure 1.3.a the chaotropic agent allows to make a phosphate group free from the DNA, and in the meanwhile the water present in the buffer protonates the Silica surface (Fig.1.3.b). These reactions make possible the binding between the silica and the dna (Fig.1.3.c) since the phosphate group becomes "exposed" and an hydrophobic interaction is possible.
The **Washing step** allows to remove other biological components bound to the silica surface and to get rid of the salts usually present in the binding buffer. Since the wash buffer is alchool based the silica is left air dried for a while, in order to make all the alcohol evaporates and not interferes with the next step.
The **Elution step** re-hydrates nucleic acids so that the they are again free inside the solution. The elution buffer can be just deionized water.

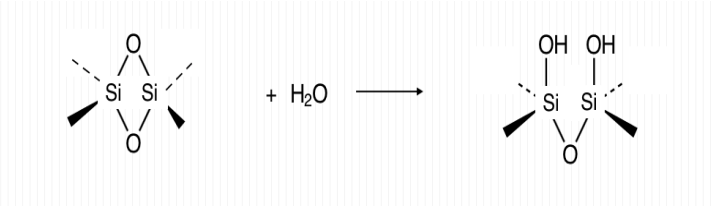
Whatever method used it will follow these main steps, just implemented in different ways according to the specific protocol.
Out-of-chip methods show some drawbacks as told before, starting from the high amount of sample and buffers, up to the need of a lot of laboratory technologies.

1.5.2 Lab-on-a-chip Solid phase extraction

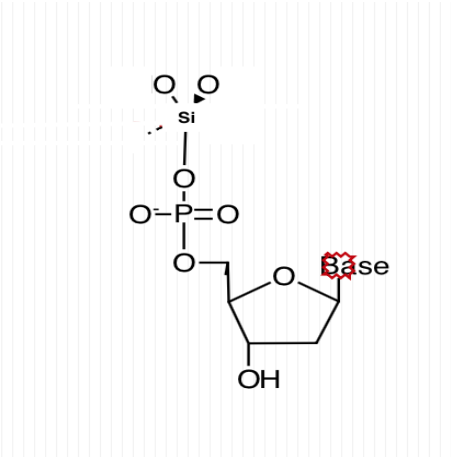
There are several SPE methods that can be implemented on a LOC and the choice depends on the specific application requirements, the device design, the laboratory equipments available and other parameters. In the following the main silica methods are briefly explained.



(a) *Chaotropic agent makes a phosphate group free from DNA*



(b) *Water protonates Silica surface*



(c) *Binding of the DNA to Silica surface*

Figure 1.3: Binding step reactions

It has to be noticed, as mentioned before, that regarding the silica phase chosen the procedure is always the same, in which the sample is bound, washed and then eluted. Basically the possible implementations are the following:

- Silica membrane
- Silica sol-gel matrix
- Silica beads
- Magnetic Silica beads

Silica membrane

Silica membrane is usually a porous silica layer through which the sample is made flowing in order to allow the dna to bond to it. Chen et al. ([6]) presented a microfluidic cassette that integrates a porous, glass fiber membrane able to capture DNA from a lysed blood sample mixed with a chaotropic agent. The membrane is 2 mm diameter and it's supported by a perforated disk of PC. In order to fix it molten paraffin was used ensuring sealing.
This method allows to get a further compounds separation taking advantage of the pores dimensions, this could become an interesting point for some applications. However the membrane is usually very brittle and its fabrication very challenging.

Silica sol-gel matrix

Silica sol-gel matrix is a continuous network structure constructed inside the channel allowing the sample flowing while it remains fixed in the channel. Kelley et al. ([8]) described a device in which the sol-gel [2] precursors are flowed into the chamber as a liquid and then gelled in place. The efficiency of this process was found to be not very satisfactory, probably because of the small pores size in the sol-gel (hundreds of nm) that may not allow a good flow of the sample. Other disadvantage is related to the low reproducibility of the experiment due to the low stability of the material.

Silica beads

Silica beads are solid phase micro or nano-particles. They need to be fixed inside the channel where the extraction takes place and this represents the main issue for which two ways has been found. The first one is to combine them with a sol-gel phase. Kelley et

[2]Sols are liquid colloidal suspensions which, when catalyzed, gel to form solid structures

al. ([8]) showed how silica beads can be added to the sol-gel precursor mixture to form a slurry. This approach was valid but is not well applied to a microfabricated device since the beads tended to settle out of the slurry and clog the channels, so to overcome this problem they first packed the particles into the system and then held them together by the sol-gel. The second way is to design a weir structure inside the channel with an heigh that is lower with respect to the beads diameter, so that they can't move outside the extraction bed and the sample can flow through.

Magnetic Silica beads

An other way to fix the particles inside the channel is to take advantage of an external magnetic field able to interact with magnetic micro or nano-beads. The principle is the same as before in which the fluids are made flowing over the particles bed, created this time by putting a permanente magnet, a magnetic stand or coils close to interested region.

Chapter 2

Silica beads

In this study a first attempt was done working with Silica beads, trapped in a bed extraction channel by the fabrication of two opposite weirs.
Silica beads (75 - 200 μm) were purchased from Sigma-Aldrich (MO,USA), the device design was done by SolidWorks® software and then CNC (Computer Numerical Control) micromilled with MILL XY [1] in a PMMA chip of 1.5 mm thickness. The first design was very simple in order to test the effectiveness of the weirs in blocking the beads. It was made up of a channel with the two sides weirs and a short channel in the middle for the beads insertion. Channels were 250 x 250 μm width x height while the weirs, that need to have an height lower then the beads diameter, were fabricated 25 μm tall. The inlet for the beads insertion was drilled manually and then connected to a tube with an inner diameter of 0.7 mm and an outer one of 1.3 mm. In order to provide a sealed connection between the tube and PMMA chip, they were glued with a two-component epoxy adhesive (Loctite FixmasterTM). Prior to the gluing both the surfaces of the tubes and the device were roughened with a 2000 grid sand paper to get better adhesion.

2.1 Loading

At first the beads were just mixed with dH_2O (1g in 20 mL) but they aggregated inside the solution providing an hard loading in which most of them clogged in the inlet, without being able to achieve the channel.
A way to increase their solubility was found by bringing water pH to 8 (adding Na_2HPO_4

[1] Milling is the machining process of using rotary cutters to remove material from a workpiece by advancing (or feeding) the cutter into the workpiece at a certain direction [17]

buffer) and then sonicating the solution for 10 minutes.
What has been observed was that they were well dispersed as soon as taken out from the Sonicator but after very short time they aggregated again. In order to keep the solution homogeneous during the loading it was put inside a tube, itself put inside the Sonicator and connected through tubing to the inlet of the device. In the meanwhile the outlet was connected to a syringe pump (Kent Scientific) that, applying a negative pressure, sucked the solution from the tube making it flowing inside the channel.
This was a quite long and tricky procedure but it worked to avoid the clogging. The beads were successfully loaded over the channel and observed through an optical microscope Leica DMI3000 B used in Bright field (Fig. 2.1). However once the loading has been

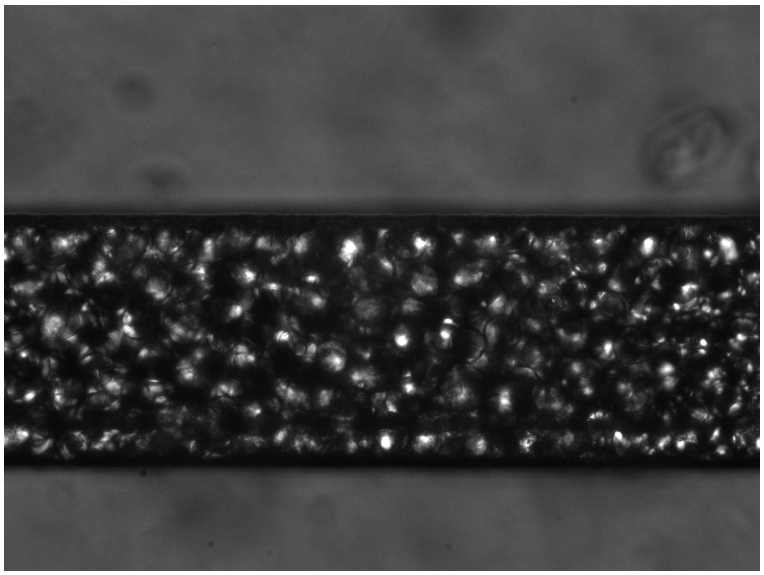


Figure 2.1: Silica beads trapped in the channel

achieved, what was observed is that the particles overcame the weirs. Since a profilometer, to check the weir height, was not available, an other way was found.

2.2 Weir measurement

A thin layer of PDMS was cast over the device, put in the oven for 10 minutes and then the PDMS was demoulded providing a mirror image of the channel. At this point a slice of PDMS was cut along the channel, including the weir, and observed at the microscope. Fig. 2.2 shows the height of the weir as 183.66 μm but it was overstimated considering that there was a thin layer of PDMS that increases the measured thickness. For this reason

a slice of that layer was also cut and measured turning out to be 51 μm. So the weir height ended up to be 132.66 μm. This provided an explanation of why the beads kept on overcoming the weir.

Since the problem was intrinsically embedded in the CNC mill machine and not soluble

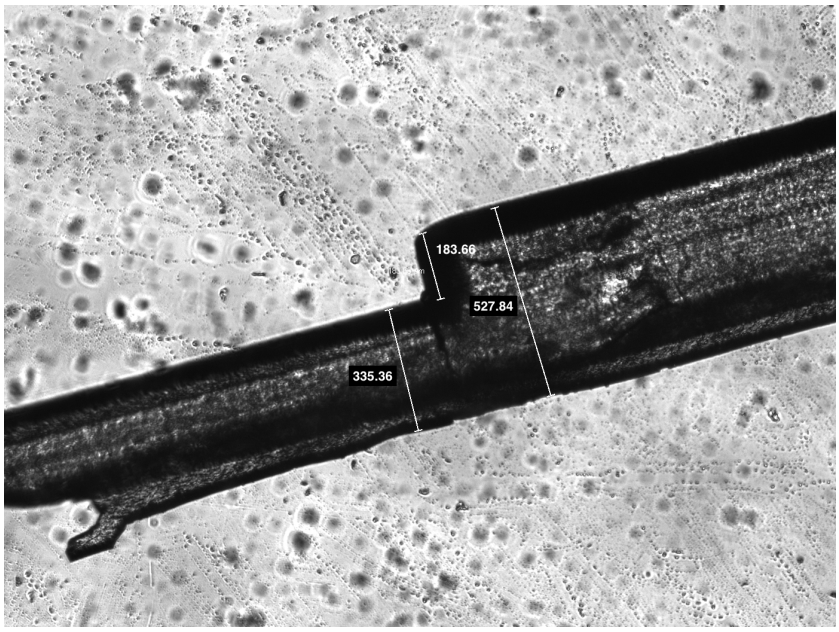


Figure 2.2: PDMS reverse image of the weir

in enough brief time an other method to achieve the DNA extraction was followed.

Chapter 3

Magnetic silica beads

In order to overcome the problem related to the weir fabrication, magnetic beads were chosen as new solution and fixed by the application of an external magnetic field.

3.1 Magnetic beads characterization

Fe_3O_4 magnetic beads coated with silicon dioxide (2.5 - 4.5 μm) were purchased from G-Biosciences, they were supplied in phosphate buffered saline, pH 7.4 with 0.09% Sodium Azide and 0.02% Tween-20.
There were no instruction of which kind of magnet should be used with them, so in order to get an idea of which magnet purchase able to block them inside the channel, a brief theoretical analysis has been made starting from a summary of the physic behind.

3.1.1 Magnetism Theoretical background

The magnetic properties of a material come from the orbital and spin motion of electrons, each one contributes to the so called *magnetic moment* μ. The momentum depends on the inner structure of the atom (number of electrons, their shell location and consequently their quantum number), and its interaction with an external magnetic field defines the magnetic behaviour of the material itself.
The different kind of behaviours can be summarized as :

Diamagnetic materials when subjected to a magnetic field generate an induced magnetic field inside in the opposite direction with respect to the applied one, generating repulsive

force. They are basically pushed away from the magnet. This behaviour disappears as soon as the external force is removed. It has to be said that all materials show at least a small diamagnetic effect.

Paramagnetic materials are composed by atoms that possess a permanent magnetic moment but due to the thermal motion the overall moment of the material is zero. Anyway when magnetic field is applied, magnetic moments of each atom aligns themselves according to the field. As soon as it is removed, the thermal motion becomes too strong and the material magnetization vanishes.

Ferromagnetic materials show the more interesting properties. They are composed by the so called *magnetic domains*, that are regions in which the magnetization is in a uniform direction. When no external field is present they are randomly oriented and so no net magnetic field is produced, but as soon as the the field is applied they all line up in the same direction causing an enhancement of the external magnetic field. Even when the field is removed the material maintains its magnetization due to the tendency of the dipoles to dispose themselves parallel with respect to each other, minimizing their energy and achieving a stable state. To better understand this phenomena is useful to take a look at the so called *Hysteresis loop* (Fig.3.1) where the x axis refers to the external magnetic field and the y to the material magnetization. During the first magnetization a ferromagnetic material follows the dashed line as the applied magnetic field increases up to the point *3*, where almost all domains are aligned (magnetic saturation). When the magnetic field is brought to zero (point *4*) a *remanent magnetization* is still present, in order to bring it to zero an opposite magnetic field (*coercitive force*)has to be applied (point *5*). At this point the same behaviour as before is observed, keep forcing a negative magnetic field the internal magnetic domains align (opposite direction than before) until the saturation occurs (point *6*). Removing the field a remanent magnetization is obtained again, and then forced to be zero applying a positive field. As can be seen the plot does not come back to the origin but to the point 3. This is the physical explanation of the memory owns by ferromagnetic materials. Iron, Nickel, Cobalt and Manganese are the most common ferromagnet.

Antiferromagnetic materials have atoms or molecules that show anti-parallel moments between them so that the magnetism generated by dipoles oriented in one direction is canceled out by dipoles aligned in the reverse direction. Overall the material has a zero net magnetization.

Ferrimagnetic materials are composed by atoms that, as for ferrimagnetic, own opposite magnetic moments. However in this case they are not equal in magnitude so a net magnetization is present. Materials as magnetite and ferrite are ferrimagnetic.

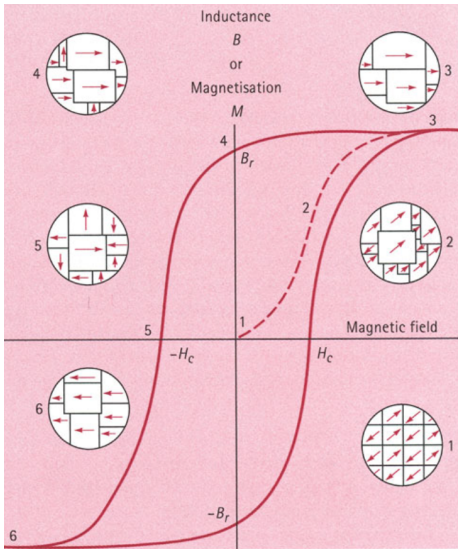


Figure 3.1: Hysteresis loop

3.1.2 From macro to micro

Moving from a macro-scale to a micro-scale material leads some different features in the magnetic analysis.
Generally nanoparticles are mono-domain since their dimension is typically of the same order of magnitude of the magnetic domain wall ($\delta = \sqrt{\frac{JS^2\pi^2}{Ka}}$ being J the magnetic exchange constant, S the total spin quantum number of each atom, a the inter-atomic spacing, and K the magnetic anisotropy constant) instead microparticles show multiple magnetic domains.
In this work the diameter is between 2.5-4.5 μm (the calculations will be done considering 3.5 μm) and being the magnetite domain size of 30 nm, the second case is presented.
Magnetic particles in the range of micrometers (typically 0.5-5 μm) can be composed by a single magnetic core or by multiple magnetic nanoparticles in a non-magnetic matrix (Fig. 3.2), in the following the first case will be consider for simplicity and since the results are valid also for the second.

The aim of this analysis is to get the maximum flow rate that the particles will be able to support without moving in the channel, according to the magnetic force and the channel dimensions. In this way the choice of the magnet to purchase can be done, and also the

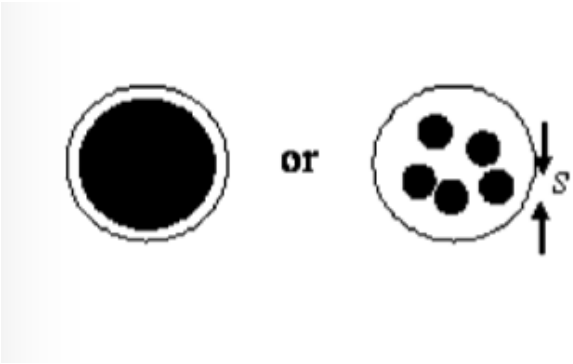


Figure 3.2: Magnetic microparticles internal core

dimensions and geometry of the bed extraction chamber can be optimized.
The starting point is to consider all the forces acting on the particles, and they can be evaluated taking into account that two different diameters are actually present, the one of the magnetic core and the one that also includes the external silicon dioxide layer.
It's possible to describe the interaction potential between the particles as function of the diameter of the magnetic core d, the external diameter σ and the distance r between the center of the two beads

$$V(r) = \begin{cases} \infty, & 0 \leq r < \sigma \\ \frac{-\alpha d^6}{r^3}, & r \geq \sigma \end{cases} \tag{3.1}$$

where α is a material-dependent parameter. As can be seen the potential starts decreasing for $r = \sigma$.
Moreover also a Van Der Waals attraction potential between the two external silicon dioxide layer will be generated and it can be defined as following:

$$V_w(r) = \frac{-\sigma A}{24(r-\sigma)} \tag{3.2}$$

where A is the Hamaker constant. This means that as σ increases as the VDW attraction becomes important.
The last force is the magnetic force acting on them by the magnet itself:

$$F_m = \frac{1}{\mu_0}(\mathbf{m}\cdot\nabla)\mathbf{B} \tag{3.3}$$

This force is the one acting over a single magnetic bead, treated as a point-like magnetic dipole. Considering that $\mathbf{m}$ = Vμ0$\chi\mathbf{H}$ and $\mathbf{B}$=μ0$\mathbf{H}$ an easier form of the equation is achieved:

$$F_m = \frac{V\chi}{\mu_0}(\mathbf{B}\cdot\nabla)\mathbf{B} \tag{3.4}$$

When the particles are trapped inside the channel and a fluid is made flowing, they are subjected to the hydrodynamic drag force

$$F_d = 6\pi\eta r\Delta\mathbf{v} \tag{3.5}$$

Two possible magnet fields strength have been considered, one typical of a *magnetic tape* (B_m = 24 μT) and the other of a *magnetic stand* (B_m = 0.5 T). The pair and VDW forces have been calculated by multiplying the potential value (evaluated with the appropriate boundary conditions) for the beads distance, that was considered as their external diameter, assuming they are stacked. What has been obtained is that the pair and VDW forces are very irrelevant with respect to the magnet force applied by the magnet (as presumable), so they were neglected. Equalizing eq. 3.3 and 3.5 the maximum velocity that a particle can support without moving, when subjected to an F_m magnetic force, is evaluated

$$\Delta\mathbf{v} = \frac{2r^2\chi(\mathbf{B}\cdot\Delta)\mathbf{B}}{9\mu_0\eta} \tag{3.6}$$

Then multiplying it for section of the bed extraction chamber the maximum flow rate was extrapolated. The chamber that was taken into consideration for the calculation has the geometry shown in Fig. 5.1 (5 x 1 mm). It turned out that the maximum flow rate using a magnetic tape is around 1.2 x 10^{-6} μL/min, with a magnetic stand 12000 μL/min. Since the flow rate imposed by the magnetic tape is not achievable a magnetic stand was purchased and used in the extraction procedure.

Chapter 4

DNA SPE tube

In this section the DNA extraction was done outside the device, in order to better characterize the protocol to be followed and the associated efficiency.
The starting sample for the extraction procedure should be plasma, as mentioned before, in order to improve the sensitivity towards cfDNA thanks to the previous removal of useless material from the rough whole blood sample. However, since plasma samples are quite expensive (about 1 mL 70 - 160 $), the choice was to use a PCR product that, starting from gDNA, provides DNA fragment comparable to cfDNA length. In this way large amount of sample was available and ready to use. Before each experiment a PCR was performed using a Mastercycler pro machine (eppendorf); the thermal cycle run by the machine has been previously optimized by other members of the laboratory.
In detail the employed one was:

- 95°**C**: 2 minutes
- 94°**C**: 30 seconds
- 54°**C**: 30 seconds
- 72°**C**: 5 minutes

repeated 30 times.
A mixture of 25 μL PCR master mix 2X , 0.5 μL upstream primer 100 μM, 0.5 μL downstream primer 100 μM, 21.5 μL nuclease free water and 2.5 μL Jurkat gDNA template 0.1 mg/mL was prepared and the PCR was run.
To check the success of the amplification the sample was submitted to electrophoresis by 2% agarose gel, followed by observation under UV light.
Electrophoresis is a technique that allows to detect the length of charged molecules by applying an external electric field. Regarding the specific case of DNA its negatively charged

backbone makes the molecules move in the opposite direction of the field and longer is the chain longer the needed time to travel along the membrane. So the gel, a permeable matrix in which molecules can move, is inserted inside an apposite electrophoresis equipment for the field application and at the end (circa 15 minutes) a set of bands can be observed by UV light, where the shortest fragments will be at the bottom and the longest at the top of the matrix. In order to understand which length the bands correspond a molecular-wight size marker (ladder) was also made running together.
In the first column 10 μL of nuclease free water and 10 μL of 50 bp DNA Ladder were inserted, in the others columns the sample was put instead of the ladder. As can be observed in Fig.4.1 the bands of the samples show to be in between the second and the third line of the ladder, this means that the DNA fragments were in between 100 and 150 bp, that was the expected result.

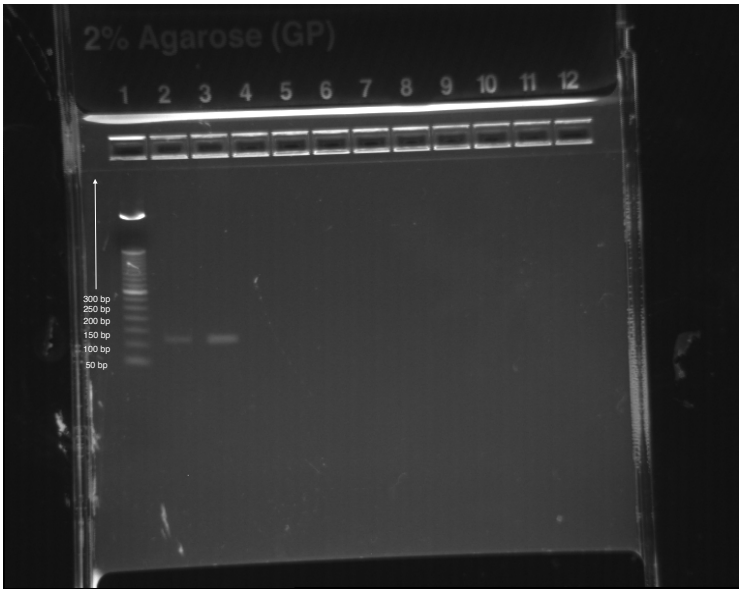


Figure 4.1: Electrophoresis apparatus

Once the sample has been obtained the extraction was performed both with silica magnetic beads and silica column in order to obtain a comparison of the two methods.

4.1 Silica magnetic beads

The first step consists of a pre-treatment of the silica magnetic beads in order to isolate them from the supernatant they are provided with. After that the purification protocol is explained and the results shown and interpreted.

4.1.1 Reagents

The reagents used were purchased from QIAGEN where the Binding Buffer was a Guanidine hydrochloride (GuHCl) and Isopropanol (C_3H_8O) based, the Wash Buffer composition was not declared but 220 mL of Ethanol (C_2H_6O) were added manually to the whole vial, finally the Elution Buffer was 10 mM Tris hyrochloride (Tris-HCl), pH 8.5.

4.1.2 Preparation of Silica beads

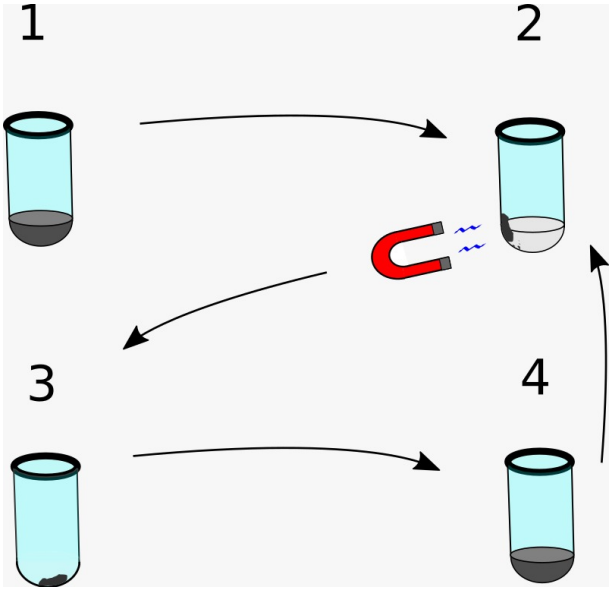


Figure 4.2: Silica beads preparation

At first the whole beads vial was vortex for 30 seconds (Fisher Scientific Analog Vortex Mixer), 50 μL of solution were transferred inside a 1.5 mL tube, placed on the magnetic stand (GE Healthcare MagRack 6) for 30 seconds to immobilize the beads at the tube wall and the supernatant was removed by aspiration with a pipette.
The tube was removed from the stand, 100 μL of Elution buffer were added and the beads resuspended by vortex until an homogeneous solution has been obtained again. The tube was put again on the rack and the Elution buffer aspirated. The last step involving the Elution buffer was repeated twice (see Fig.4.2).

4.1.3 Purification of Nucleic Acid protocol 1

A standard protocol was provided by the G-Biosciences together with the Silica beads:

1. Mix 40 μL of sample and 90 μL of Binding buffer with magnetic beads and thoroughly by pipetting
2. Incubate with tilt rotation for 2 minutes at room temperature
3. Place the tube on the magnetic stand for 30-60 seconds to immobilize the beads at tube wall
4. Discard (or collect) the supernatant as unbound substances by aspiration with a pipette, and then remove the tube from the magnetic stand
5. Add 100 μL Wash Buffer and re-suspend the beads by pipetting
6. Place the tube on the magnetic stand for 30-60 seconds to immobilize the beads at tube wall
7. Discard (or collect) the supernatant as unbound substances, and then remove the tube from the magnetic stand
8. Repeat steps 5-7 twice
9. Air-dry for 5-20 min
10. Add 10-100 μL Elution Buffer (or ddH_2O) and re-suspend the beads complex by vortex
11. Incubate with tilt rotation for 3 minutes at room temperature
12. Place the tube on the magnetic stand for 30 seconds and collect the supernatant to a clean tube

When the extraction has been completed the sample was tested using a NanoDrop Spectrophotometers (Thermo Scientific Fig. 4.3) that allows to quantify and assess the purity of the sample.

Nanodrop working principle

Nucleic acids strongly absorb UV light with wavelengths of 260 nm, due to the resonance structure of the purine and pyrimidine bases.

Figure 4.3: Nanodrop machine

Thus, enlightening the sample over the UV light range and then measuring the intensity of the transmitted light, it's possible to extrapolate the Absorbance and connect it to the DNA concentration through the *Lambert-Beer equation*:

$$\frac{I_1}{I_0} = e^{-k_\lambda l} = T = e^{-A} \tag{4.1}$$

where :

I_1: Transmitted light intensity

I_0:Impinging light intensity

k_λ: Attenuation Coefficient (light wavelength dependent and typical of the material)

l: Path-Length, basically the thickness of the sample

T: Transmittance

A: Absorbance

Defining the Transmittance as the ration I_1 / I_0 and the Absorbance as the opposite of the

logarithm of T, the equation assumes a much easier form:

$$A = k_\lambda l = \epsilon_\lambda l c \tag{4.2}$$

where :

ϵ_λ: Wavelength dependent extinction coefficient

c: NA concentration

Following Eq. 4.2 the DNA concentration value is calculated and it is possible to get an Absorbance spectrum, in which higher is the peak over 260 nm higher the NA concentration.
The special feature of the nanodrop technology is that it takes advantage of the surface tension of the sample to create a column between the ends two optical fibers, so that the optical path is formed with a small amount (few μL) of sample.
In order to perform the computation an initial cleaning of both measurement surfaces (optical fibers) was done putting 1.5 μL of deionized water and then dried with a clean, lint-free lab wipe.
A drop of Elution buffer was then pipetted on the bottom surface, the sampling arm lowered and the *Blank* button clicked from the software on the PC. During this time the light from Xenon flashlamp passes through the top optical fiber, down through the liquid column and is detected by the internal spectrometer. This step was done in order to store the Elution buffer signal as reference spectrum, that means removing its absorption signal from the final measurement (as a matter of fact the provided solution is ideally composed by the DNA and the Elution buffer only).
Both surfaces were dried again, a drop of sample was pipetted and the *Measure* button clicked.

4.1.4 Results

The Nanodrop Spectrophotometer provides the absorption curve, the DNA concentration (ng/μL), the absorbance at 260 and 280 nm and the absorbance ratio 260/280 and 260/230. These are the necessary figures of merit for a correct evaluation of the measurement.

The **Absorbance at 260 nm** is the parameter directly correlated with the NA concentration, so it is evaluated in order to solve the Lambert-Beer equation.

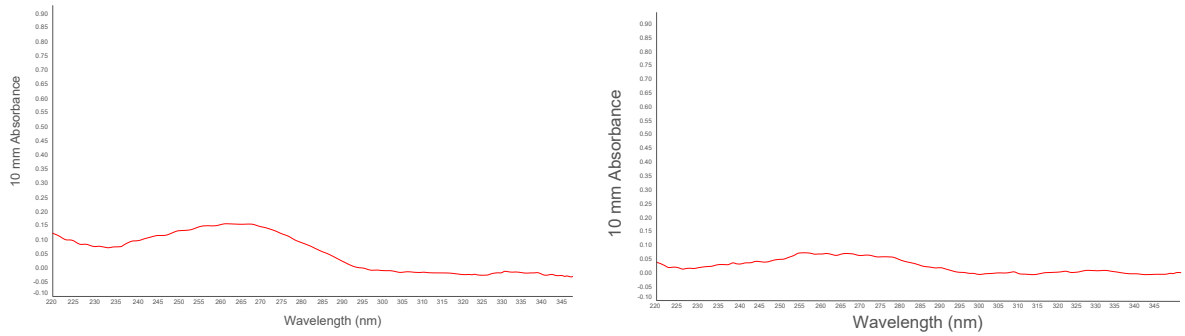
The **Absorbance at 280 nm** is measured since it represents the wavelength typically absorbed by proteins and phenolic compound (often used in buffer).

The **Absorbance at 230 nm** is usually related to organic compounds presence.

The 260/280 ratio is expected to be around 1.8 to consider the DNA sample as "pure", if lower the sample could be contaminated by proteins.
The 260/230 is usually higher and around 1.8 - 2.2, if lower Phenol, Chaotropic salts or other Aromatic compounds contamination could be present.
The obtained result shows a quite low peak over 260 nm, as can be seen in Fig. 4.9, that is associated with a concentration of 8.2 ng/μl and furthermore the absorbance ratios indicate a not pure sample (table 4.1).

Table 4.1: NA concentration extracted with magnetic beads (Protocol 1)

SAMPLE	NUCLEIC ACID	UNIT	260/280	260/230
first elution	8.2	ng/μl	1.64	2.11
second elution	3.4	ng/μl	1.55	3.62



(a) *Absorption curve after the first elution* (b) *Absorption curve after the second elution*

Figure 4.4: Absorption spectrum magnetic beads tube (Protocol 1)

In order to figure out which step of the extraction procedure has been the more troublesome, each waste solution was collected (after the binding and washing steps), a second elution step was performed and all of them run over the E-gel, together with a beads drop itself collected by the tube after the purification.
What has been observed is that some DNA was still present in the waste after the binding, in the second eluted solution and on the beads themselves. This led to understand that the elution and binding steps have not been successfully accomplished and so the protocol was modified a little bit.

4.1.5 Purification of Nucleic Acid protocol 2

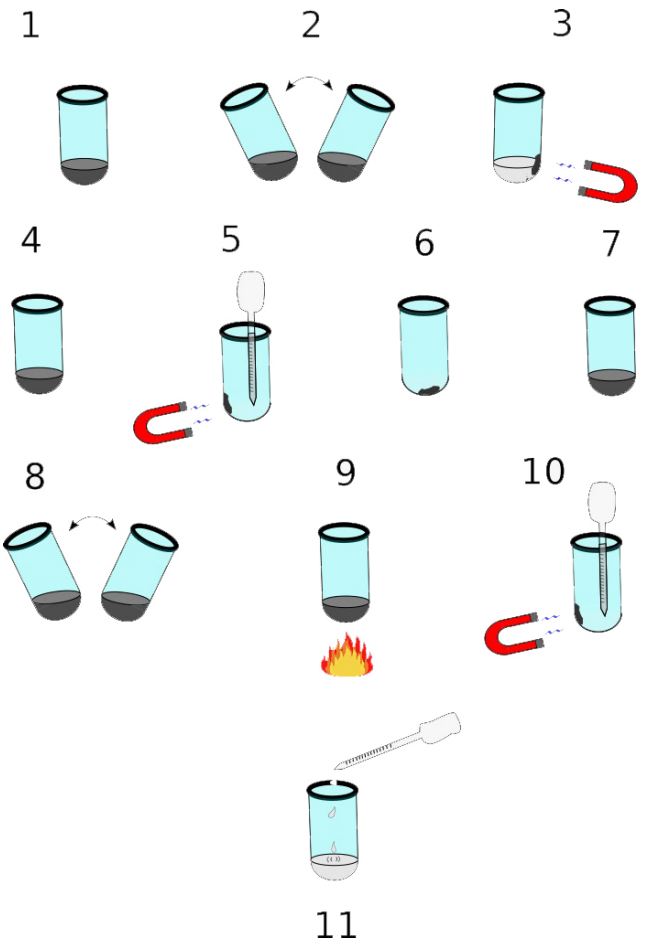


Figure 4.5: Silica magnetic beads extraction protocol

The modified protocol is here provided (Fig.4.5) :

1. Mix 40 μL of sample and 90 μL of Binding buffer with magnetic beads by pipetting
2. Incubate with tilt rotation for 2 minutes at room temperature and wait 10 minutes
3. Place the tube on the magnetic stand for 30-60 seconds to immobilize the beads at tube wall and discard (or collect) the supernatant as unbound substances by aspiration with a pipette. Remove the tube from the magnetic stand
4. Add 100 μL Wash Buffer, re-suspend the beads by pipetting and wait 2 minutes
5. Place the tube on the magnetic stand for 30-60 seconds to immobilize the beads at

tube wall and discard (or collect) the supernatant as unbound substances by aspiration with a pipette. Remove the tube from the magnetic stand.Repeat steps 4-5 twice

6. Air-dry for 10 min to make the possible alcohol traces evaporate
7. Add 10-100 μL of pre-heated Elution Buffer (or ddH_2O) and re-suspend the beads complex by vortex
8. Incubate with tilt rotation for 3 minutes at room temperature
9. Incubate at 65°C for 10-15 minutes
10. Place the tube on the magnetic stand for 30 seconds
11. Collect the supernatant into a clean tube

4.1.6 Results

In Fig. 4.6 a typical extraction curve is reported and as can be seen much more interesting results were obtained (Table 4.2 shows the associated data), even if some DNA was still present in the second elution solution. The 260/280 and 260/230 ratios point out satisfying values, usually associated to a pure sample.

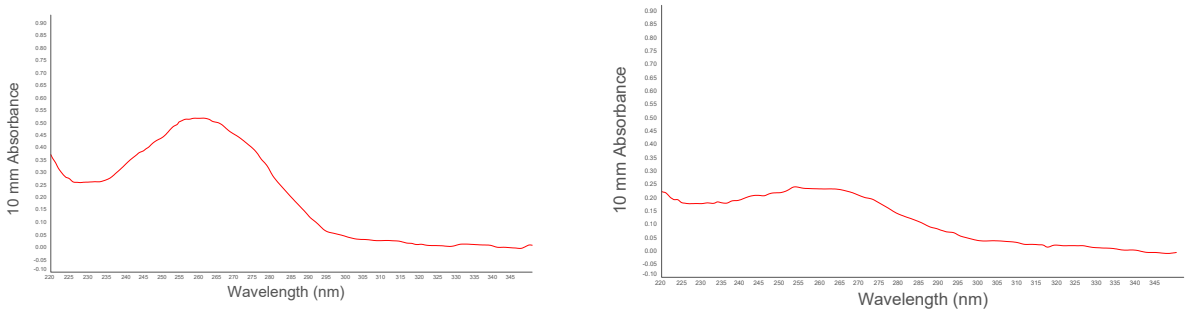
Table 4.2: NA concentration extracted with magnetic beads (Protocol 2)

SAMPLE	NUCLEIC ACID	UNIT	260/280	260/230
first elution	25.8	ng/μl	1.79	2.00
second elution	11.5	ng/μl	1.69	1.31

Repeating the measurements more times the mean value of the NA concentration, the standard deviation and the coefficient of variance were evaluated and reported in table 4.3. The data distribution is also shown in Fig. 4.7, where *2s* represents twice the standard deviation and it's associated to the range in which 68% of the data falls in. Both the standard deviation and the coefficient of variance points out quite reproducible results.

Table 4.3: Mean value, standard deviation and coefficient of variance of NA concentration extracted with magnetic beads and 40 μL of sample (Protocol 2)

NUCLEIC ACID (ng/μL)	STANDARD DEVIATION (ng/μL)	COEFFICIENT OF VARIANCE (%)
25.87	1.77	6.8



(a) *Absorption curve after the first elution* (b) *Absorption curve after the second elution*

Figure 4.6: Absorption spectrum magnetic beads tube (Protocol 2)

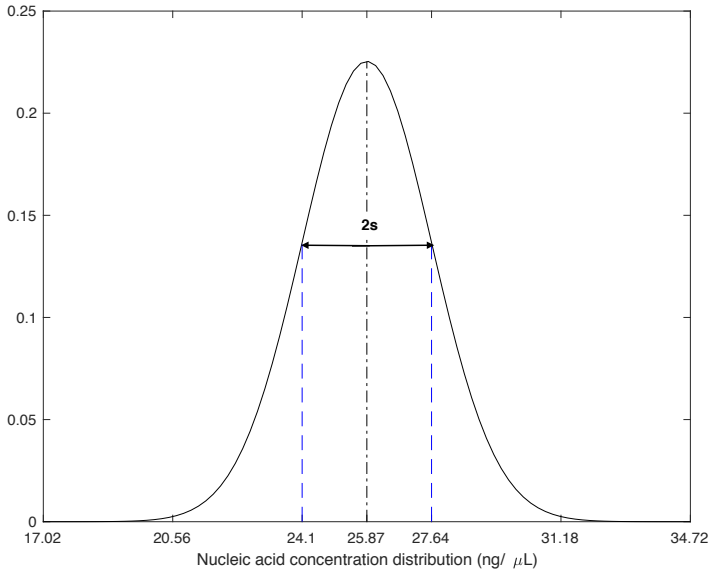


Figure 4.7: NA concentration distribution for magnetic beads extraction (40 μL sample)

The experiment was also repeated using just 10 μL of sample (Table 4.4) in order to get an idea of how the amount of sample affects the purification procedure. This point was done since a low sample consumption is one of the LOC devices requirements. The obtained concentration was lower than the one achieved with higher sample amount, as predictable. Anyway the result is interesting since the employed sample was 1/4 of the previous one (from 40 μL to 10 μL) but the extracted DNA was more than half (from 25.43 ng/μL to 13.4 ng/μL). So for applications in which an high final concentration is not required magnetic beads allow to employ also a low starting sample quantity.

Table 4.4: Mean value, standard deviation and coefficient of variance of NA concentration extracted with magnetic beads and 10 μL of sample (Protocol 2)

NUCLEIC ACID (ng/μl)	STANDARD DEVIATION (ng/μl)	COEFFICIENT OF VARIANCE (%)
13.4	2.5	18.6 %

4.2 Silica column

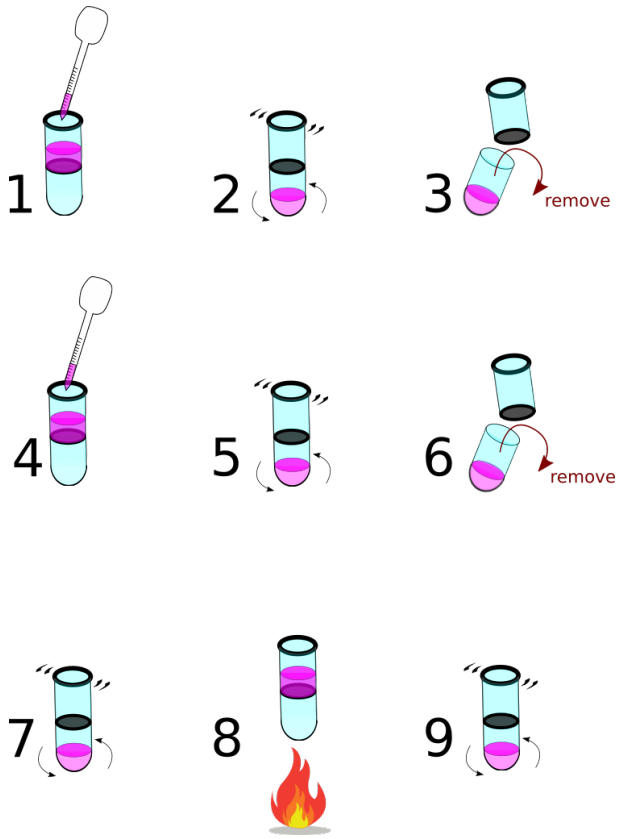


Figure 4.8: Silica column extraction protocol

An other method to perform the DNA extraction was used in order to make a comparison with the magnetic beads in terms of efficiency, time consumption, cost.
The working principle is the same, based on the interaction between DNA and silica in presence of a chaotropic agent. In this case the silica phase is a membrane able to capture the sample during the binding step and make the unwanted materials flow, while releases the DNA in presence of the elution buffer. Also the buffers employed were the same of the magnetic beads. The followed protocol was the one provided with the silica column kit but some changes has been made to optimize the process (Fig.4.8)

4.2.1 Purification of Nucleic Acid protocol

1. Add 5 volumes of Binding buffer to 1 volume of sample and mix. Then add 10μL 3 M sodium acetate and wait at least 1 minute
2. Centrifuge the column for 60 seconds
3. Discard the fluid that has passed through the membrane
4. Add 750 μL of Wash buffer
5. Centrifuge the column for 60 seconds
6. Discard the fluid that has passed through the membrane and repeat 4-5-6 steps twice
7. Centrifuge one time more to get rid of all the Wash buffer
8. Add 50 μL of pre-heated Elution buffer and incubate at 65°C for 10-15 minutes
9. Put the column inside a clean tube and centrifuge again for 60 minutes

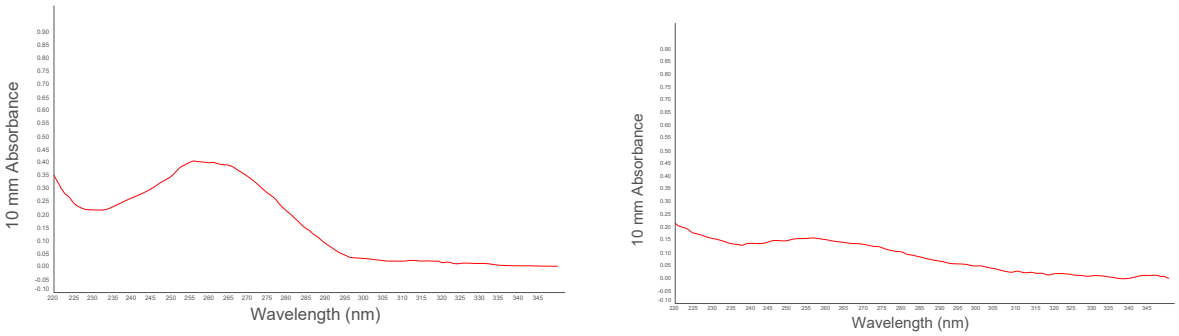
4.2.2 Results

As done for the magnetic beads the extraction was made using the PCR product as sample. The first extraction was made using 40 μL of sample and in Fig. 4.9 a typical absorption curve measured through the Nanodrop system is reported with the associated values in table 4.5.

Table 4.5: NA concentration extracted with silica column

SAMPLE	NUCLEIC ACID	UNIT	260/280	260/230
first elution	19.7	ng/μl	1.86	1.82
second elution	7.2	ng/μl	1.47	0.95

More measurements were done in order to calculate the average concentration value, standard deviation and coefficient of variance (table 4.6, Fig. 5.6).
The extracted DNA concentration is lower than the one achieved with the beads, the standard deviation is still not high that means that the results are quite stable and the coefficient of variance is very similar to the one obtained with the beads, so both the methods give enough good reproducible results.



(a) *Absorption curve after the first elution* (b) *Absorption curve after the second elution*

Figure 4.9: Absorption spectrum silica column

Table 4.6: Mean value, standard deviation and coefficient of variance of NA concentration extracted with silica column and 40 μL of sample

NUCLEIC ACID (ng/μl)	STANDARD DEVIATION (ng/μl)	COEFFICIENT OF VARIANCE (%)
22.43	1.93	8.6 %

A point that need to be highlighted is that, as before, the experiment was repeated using 10 μL of sample but in this case the Nanodrop curve and the E-gel showed the absence of the analyte.

4.3 Silica magnetic beads - Silica column results comparison

The obtained results do not point out the efficiency of the methods since the initial concentration of the sample was not known, and impossible to deduce. In order to get this information, essential to provide a characterization of the extraction procedure, the obtained purified sample was mixed with Nuclease free water and then extracted again.
In this way the concentration of the purified solution was known, measured with the Nanodrop, and after the dilution step it was manually evaluated knowing that the initial and final concentration of the solute doesn't change ($M_1V_1 = M_2V_2$).
According to the concentration achieved with the first extraction different starting concentrations have been used for the second purification ranging from 20 to 27 ng/μL.

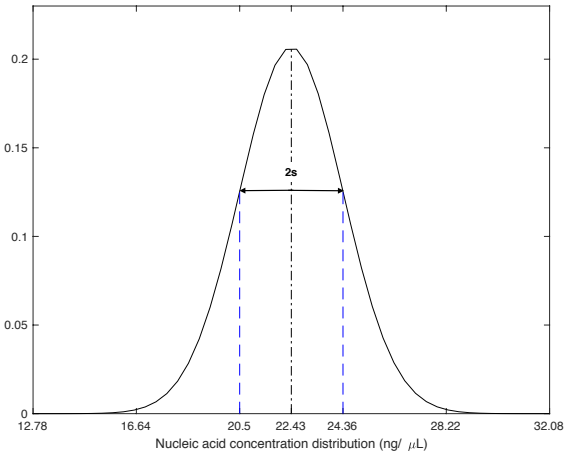


Figure 4.10: NA concentration distribution for Silica column extraction (40 μL sample)

Table 4.7: Comparison of efficiency, standard deviation and coefficient of variance between beads and column method

	Efficiency (%)	Standard deviation (%)	Coefficient of variance (%)
Magnetic beads	66.13	2.3	3.4
Silica column	42.7	7.71	18

In table 4.7 a comparison of the efficiencies, standard deviation and coefficient of variance between the two methods is shown.

In order to have the confirm that the results are consistent, meaning the 260 nm peak is really related to the DNA presence and not due to other contaminants able to absorb light at the same frequency, the sample before the extraction and the one after were run over the agarose gel.
As can be seen from Fig. 4.11 the lines are the expected ones, showing a DNA size around 150 bp, where the line on the second column is brighter since it comes from the starting pre-purified sample, meaning higher concentration.

Summarizing it's possible to affirm that the magnetic beads exhibit more advantages than the column. As shown in the table they provide higher efficiency, with also more reproducible results, having a coefficient of variance much lower.
Furthermore beads are overall cheaper because of their cost itself and the lower buffer consumption. Regarding the buffer consumption, working with the same sample amount (40 μL), beads allow to use 90 μL of BB against the 250 μL of the column and 200 μL of

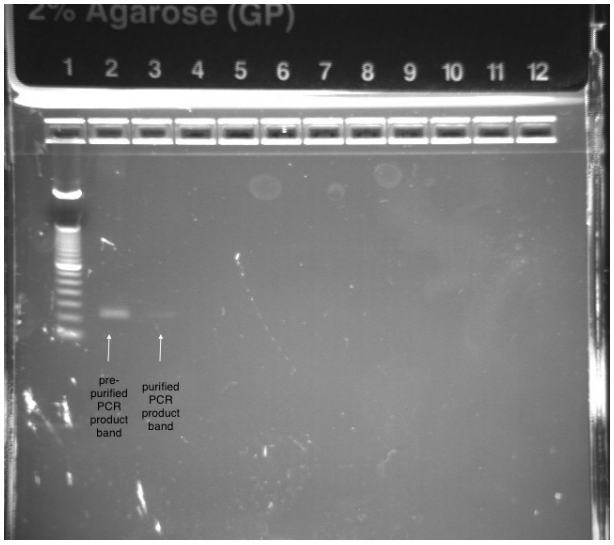


Figure 4.11: E-gel (Ladder column 1, starting sample column 2, final sample column 3)

Table 4.8: Magnetic beads and silica column advantages comparison

Magnetic beads	Silica column
Efficiency	Time
Price	

WB against 1500 μL.
Moreover the cost of one 5 mL vial of beads is 73$ and it allows to do 100 extractions, instead it's not possible to purchase just the Silica column without the buffers but one single kit (115$) enables 50 extractions.
However Silica column method is less time consuming, an extraction can be done in around 25 minutes against the 50 of the beads (Table 4.8).

Chapter 5

DNA SPE device

After the beads characterization inside the tube the same process was accomplished over a microfluidic device. Several design were tested in order to optimize the beads loading and assure their immobilization inside the channels.
The best configuration, also according to the calculation made before in Chapter 3, was found to be an hexagonal chamber 11 x 5 x 1 mm, connected to the inlet and the outlet through two valved channels 280 x 250 μm (width x depth) (Fig. 5.1) that allows to have better control of the flow.

5.1 Microfabrication

The device design was done with Solidworks® (see Fig. 5.2), that allows to define all the geometrical dimensions, then the project was directly sent to the machine used for the actual fabrication.
The device feature was manufactured with a CNC Micro-milling machine in a PMMA chip (25 x 34 x 1.5 mm), as described in Chapter 2, while the inlet and outlet drilled manually and connected to external tubes to insert and remove fluids.
In this configuration valves technology was implemented so to be able to get a good control of the flow. This choice implied that the top side of the device was bound to a PDMS layer, that constitutes the valve membrane, allowing to stop the fluid flow when pushed down by a solenoid (see section 5.4).
The other side needed to be bound too in order to seal the channels on both sides and avoid leakages.
At first the choice was a 0.2 mm PMMA layer that assured perfect sealing due to the high bonding force (PMMA-PMMA). Later it was substituted by an adhesive film since it equally provided good sealing (the back-pressure generated by the fluid motion was low

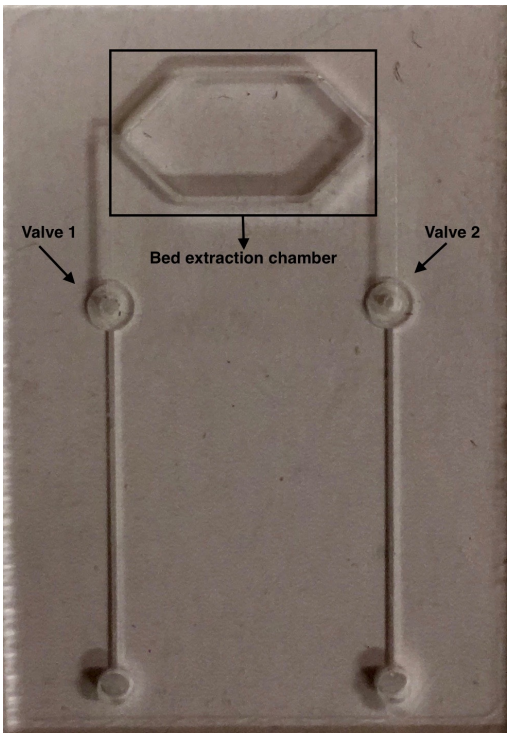
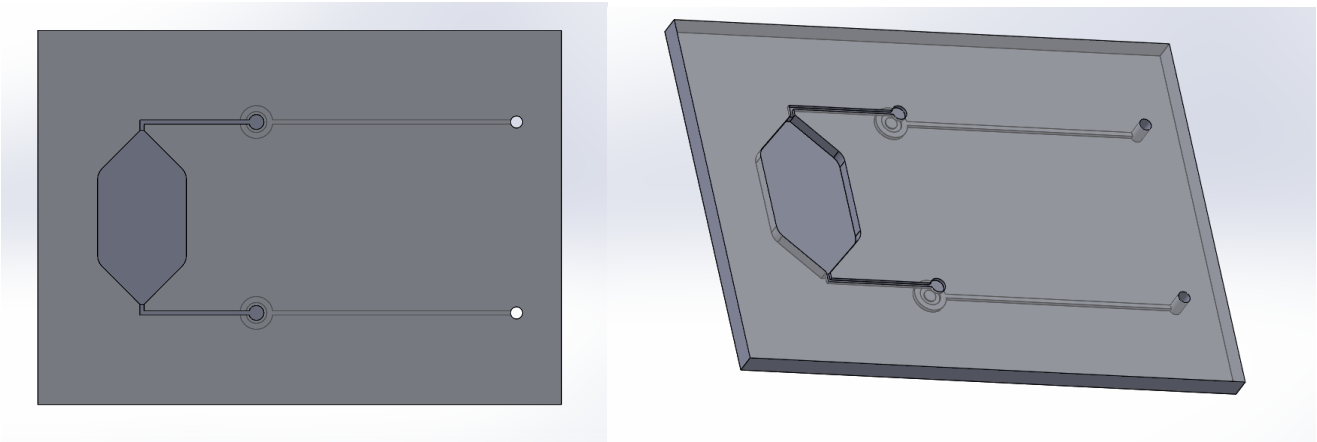


Figure 5.1: PMMA microfluidic device for DNA SPE



(a) (b)

Figure 5.2: Solidworks® valved chip design

Figure 5.3: 3D printed valves holder

enough to not break the bonding, even if the adhesion force was lower than the previous one), but the bonding procedure was much faster and cheaper.
To make the bonding with the adhesive film the device was previously washed with IPA and blench, dried with N_2 and then the layer was simply laid over, paying attention not to form air bubbles.
For PDMS-PMMA and PMMA-PMMA bonding see sections 5.4.3 and 5.4.2.
To exploit the valves function, an holder for the solenoids (Fig. 5.3) was 3D printed and the device inserted before each extraction.

5.2 SPE procedure

The protocol to implement the extraction on the microfluidic platform was not defined yet, so several experiments have been done in order to get satisfying results and get a well defined extraction procedure to follow.
The device was inserted in the solenoids holder, tubes connected to the inlet and outlet and the magnetic stand placed under the chamber, then the following steps were done.

1. 43 μL of beads are inserted through the inlet, holding the valve 1 open and valve 2 closed
2. The supernatant is sucked away by inspiration through a pipette by the outlet (both valves open)
3. 76 μL of dH_2O are made flowing (V_1 and V_2 open)
4. The remaining water is pumped out

5. 40 μL of sample and 90 μL of Binding buffer are mixed in a tube and then 38 μL inserted
6. The mixing is done by moving the magnet under the chamber (Fig.5.4) and then the solution is left there for a couple of minutes (both valves close)
7. The fluid is pumped out
8. Repeat 5-6-7 until the whole sample amount is consumed
9. 38 μL of Wash buffer are inserted and pumped out
10. Repeat step 9 twice
11. 30 μL of pre-heated Elution buffer are loaded, mixing is done as before and kept there for 10 minutes
12. The eluted sample is collected by sucking it out through a pipette

(a) (b) (c)

Figure 5.4: Mixing of solution inside the microfluidic device

5.3 Results

In this section the results are reported and, as in the previous chapters, some extractions were made starting from the PCR product so to provide a comparison with the other methods. The mean value achieved was calculated and shown in table 5.2 . A typical extraction curve is also reported in Fig. 5.5 with its related data in table 5.1. The ratios point out that the sample is not completely pure, some contaminants are probably still present, but they are satisfying results since they are not far from acceptable values and further optimization still need to be done.

Table 5.1: NA concentration extracted with microfluidic device (Fig.5.5)

NUCLEIC ACID	UNIT	260/280	260/230
11.5	ng/μl	1.60	1.79

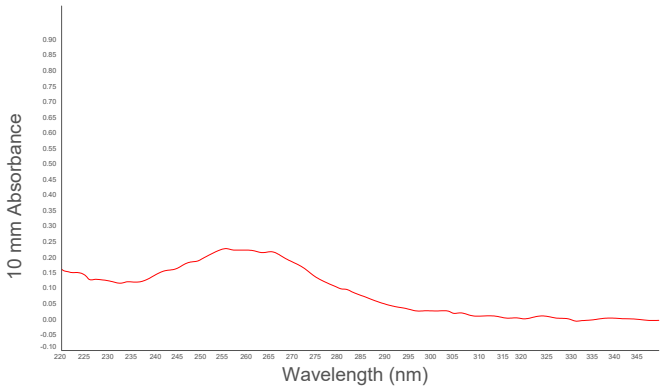


Figure 5.5: Absorption spectrum microfluidic device

Table 5.2: Mean value, standard deviation and coefficient of variance of NA concentration extracted on the device

NUCLEIC ACID (ng/μl)	STANDARD DEVIATION (ng/μl)	COEFFICIENT OF VARIANCE (%)
14	1.89	13.5

In order to analyse which step was not well accomplished, samples after the Binding and Washing steps were collected and run over the Agarose gel together with a beads sample

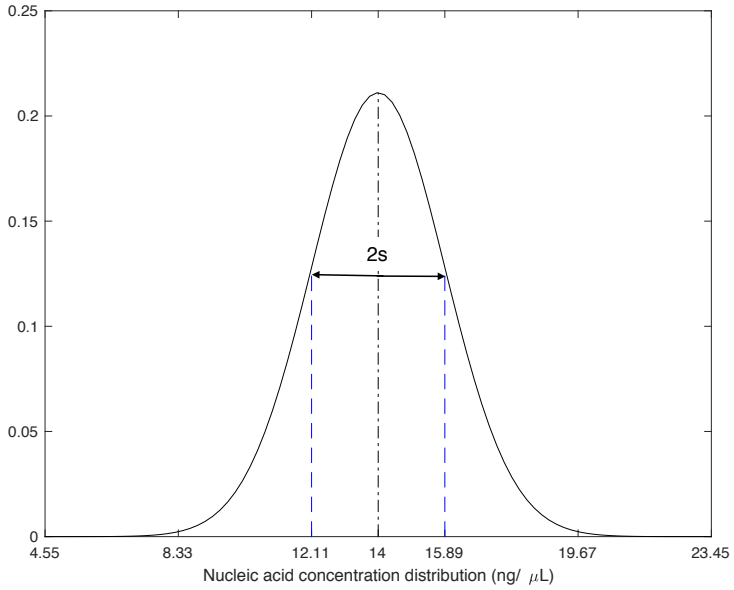


Figure 5.6: NA concentration distribution for device extraction (40 μL sample)

too (Fig.5.7). As can be seen the binding and washing steps were successfully done, in fact no residual DNA was present, just the elution seems to be hard to be efficiently done. This step was challenging also in the extraction tube procedure though, so it's not surprising that it remains the most difficult phase.

As done for the extraction tube the experiments were then performed starting with a pre-purified PCR sample, so to evaluate the efficiency achievable with the microfluidic device and in table 5.3 the results are reported.

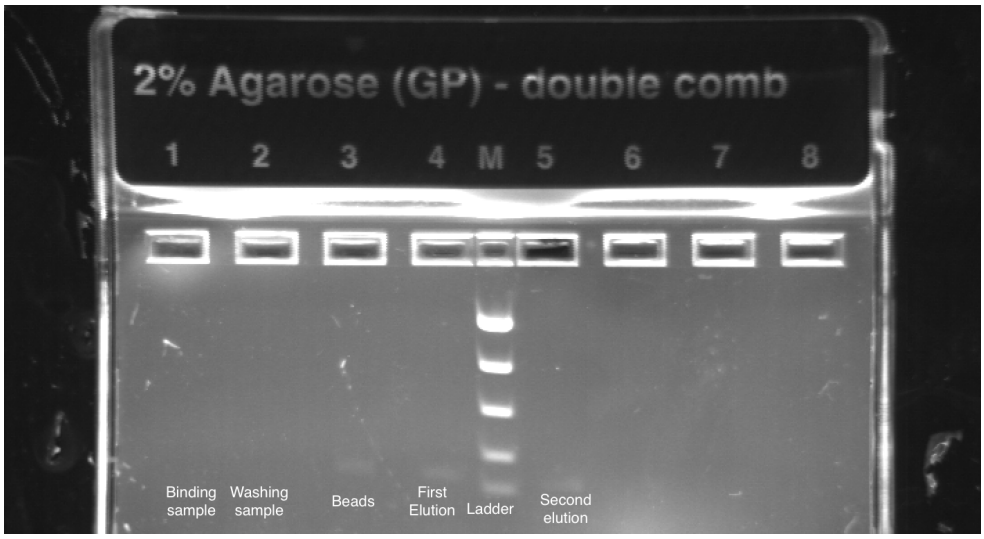


Figure 5.7: E-gel for DNA SPE device samples

Table 5.3: Efficiency, standard deviation and coefficient of variance achieved with DNA SPE on microfluidic device (mean starting sample concentration 20 ng/μL)

EFFICIENCY (%)	Standard deviation (%)	Coefficient of variance (%)
8.8	3.8	43

A summary is shown in Fig.5.8 that indicates how the magnetic beads have always provided better results in the tube but moving towards the device the performances decreases. This can be due to several reasons that together contribute to get lower efficiency. At first it was hard to collect the eluted sample directly from the outlet and moreover if the outlet tube didn't fit exactly the hole, some leakages would be observed. As it will be explained later, a sample collection chamber where the PCR could be directly run over, could reduce this difficulty.
In addition using just one inlet and one outlet all the buffers and both the waste and eluted samples flowed in the same channels, this may have led to an unwanted mixing and so a not pure sample.
Overall all the steps turned out to be more difficult to be implemented on the device with respect to the tube. It was hard to get rid of all the solution from the chamber and understand when the chamber was completely empty from a certain buffer. Also the mixing was not trivial to realize as in the tube procedure, as predictable.

Furthermore the standard deviation came to be quite high, which is obviously an unwanted aspect. This was presumably due to the same reasons just mentioned, that also made more difficult to reproduce the same protocol each time.

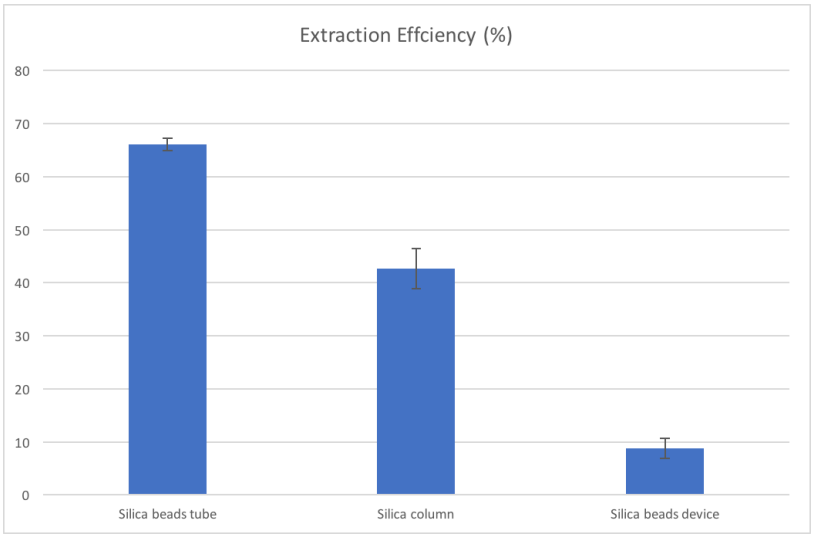
Moving towards the real final application of the microfluidic lab-on-a-chip device means to take into account the effective cfDNA concentration in a real sample that, as mentioned before, is not a well defined quantity but can be considered of the order of 100 ng/mL. However this concentration was not even detectable by the Nanodrop 2000, that shows a detection limit of 2 ng/μL for dsDNA.
So, being impossibile to measure such a low value, following experiments were done with a starting concentration by the order of 10 ng/μL, to indentify a possible trend of how the performances change by decreasing the DNA quantity.

Table 5.4: Efficiency, standard deviation and coefficient of variance achieved with DNA SPE on microfluidic device (mean starting sample concentration 10 ng/μL)

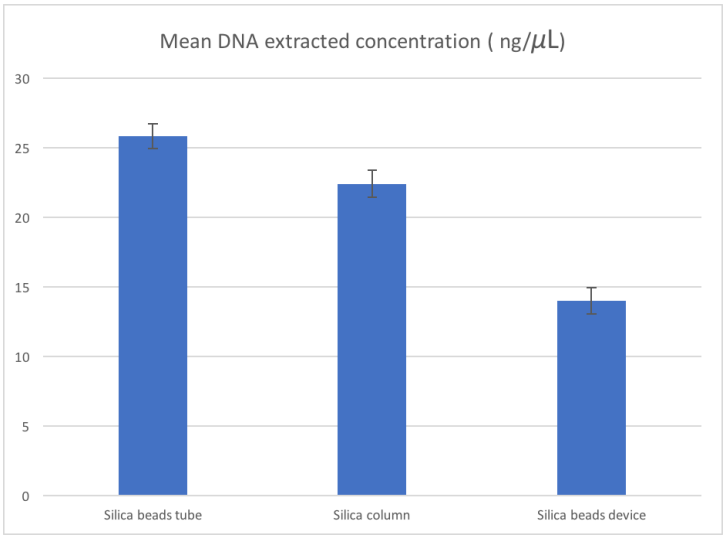
EFFICIENCY (%)	Standard deviation (%)	Coefficient of variance (%)
11.9	4.9	41

The results (Table 5.4) are quite unexpected since they furnish higher efficiency than the one achieved with an higher starting sample concentration, but still with a very high standard deviation.
The reason of this behaviour is probably due to the fact that the experiments with lower concentration have been made later in time, so more confidence has been taken in following the procedure and making each step properly. In Fig. 5.9 the efficiency trend is shown with respect to the extractions in time progression, where the higher sample concentrations were employed before. As can be seen going on with the experiments the efficiency values increase thanks to the better handling of the procedure.
This last consideration is very encouraging for the final application since the efficiency could become even higher with the enhancement of the experience achievable with time. Furthermore the amount of sample used was 40 μL so, even with a not very high efficiency, an higher quantity can be made flowing through the chamber to get more extracted DNA. Obviously a right trade off should be found between the efficiency and the amount of sample used.

After the extraction procedure has been optimized, a PCR was run with the purified sample, in order to understand if the two steps could actually be implemented one after the other in a final LOC platform.
As a matter of fact the main issue could have been related to the chaotropic agent present in the Binding buffer and organic solvent in the Wash buffer that are known inhibitors of PCR (new studies are in fact made in order to avoid the chaotropic agent as binding buffer [16]).



(a) *Efficiency comparison Silica magnetic beads tube, Silica column and Silica magnetic beads device and relative standard deviation*



(b) *NA extracted concentration comparison Silica magnetic beads tube, Silica column and Silica magnetic beads device and relative standard deviation*

Figure 5.8: Results comparison (Silica beads, Silica column, Device)

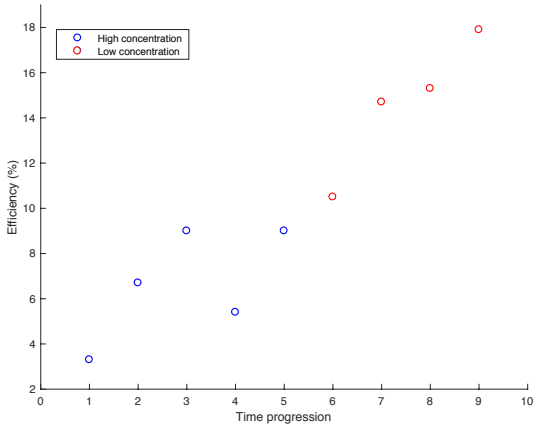


Figure 5.9: Extraction efficiency trend with respect to the performed attempts in time progression

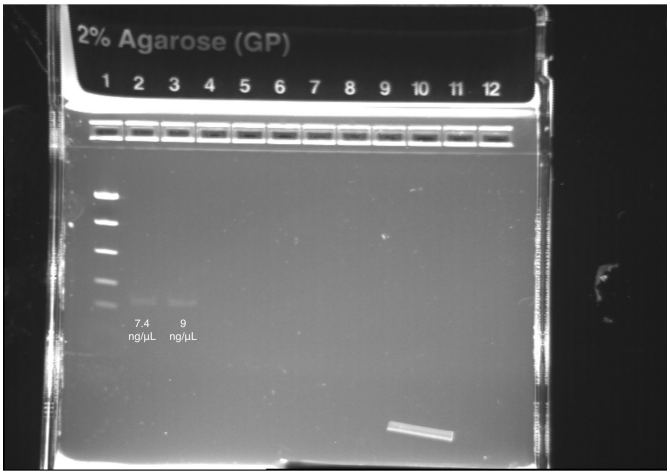


Figure 5.10: E-gel for DNA SPE samples after PCR

So if the extraction were successfully accomplished, the same band achieved with the DNA template is supposed to be found when the PCR product is run over the agarose gel.
Two different samples are shown in Fig.5.10, one with an initial concentration of 9 ng/μL and the other of 7.4 ng/μL. As can be seen both samples are correctly amplified and no unexpected bands are present.
Even though to understand if the efficiency of the PCR has been compromised by some buffers inhibitors it would be necessary to measure the amount of DNA in the final sample. This step couldn't be done in the laboratory since the Nanodrop technology can't detect the DNA amount in a non purified sample, as explained before.

5.4 Valves

In this section a short overview of the valves used in this work is presented.
Vertical valves actuated with a solenoid were chosen taking advantage of a first characterization previously done by an other member of the Laboratory (Fig. 5.11). The basic idea

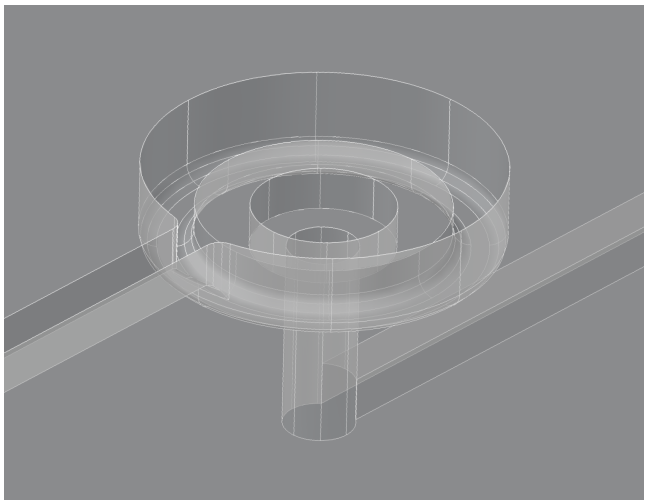
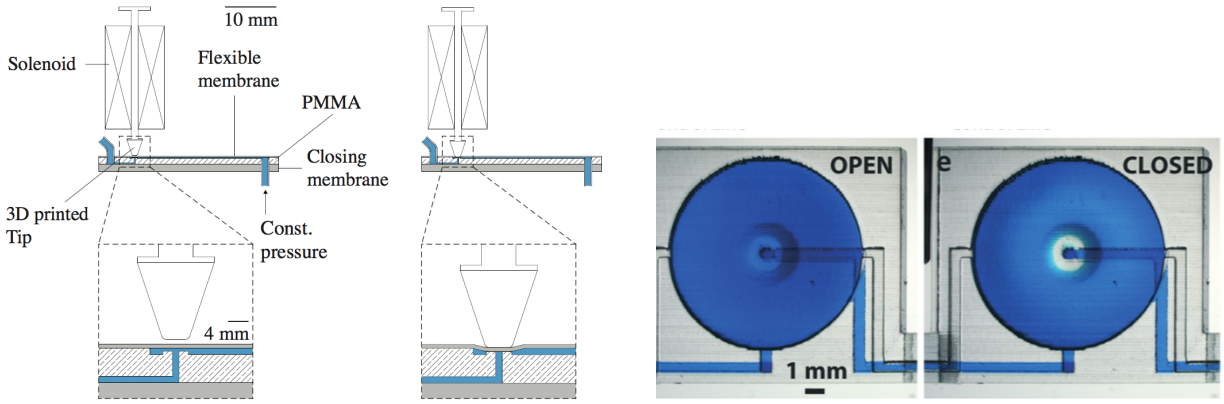


Figure 5.11: Valve Autocad® sketch

is to bond a membrane to the device top side and thanks to the pressure applied through a 3D-printed tip, glued to the solenoid, it is deflected and does not allow the fluid to flow (see Fig.5.12 [9]).

A solenoid actuator (see Fig.5.13) is a kind of electromagnetic actuator able to convert an electrical signal into a magnetic field producing a linear motion, that in this case is the mechanical movement of the plunger. The applied force is an attractive one that leads



(a) (b)

Figure 5.12: Vertical valves working principle. *(a)* On the left the open state is shown where the plunger is pulled up allowing the fluid flow. On the right the closed state is guaranteed by the pushed down plunger *(b)* A 3D drawing of the main body of the valve

to the closure of the valve, then thanks to a coil the plunger moves back to the original position and the valve opens. The setup for the valves actuation is based on an electrical circuit connected to the solenoids and to an Arduino Uno platform, itself connected to a personal computer able to send the appropriate commands (Arduino® 1.8.3 software) to control the solenoids movements and so the opening and closure of the valves. The electronic circuit is composed by a NPN transistor (TIP102) in which the Base leg is connected to a 220 Ω resistor and the Collector to a diode (1N4004) (see Fig.5.14), everything obviously connected to the solenoid and the power is supplied by the Arduino Uno itself. This kind of valves can be employed both with a PMMA and a PDMS membrane and for

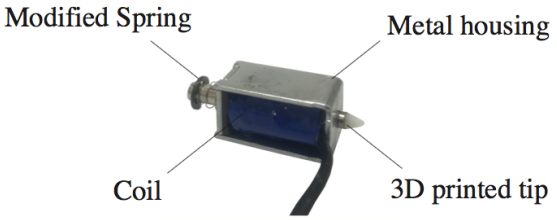


Figure 5.13: A solenoid actuator, with e ferromagnetic plunger, a coil, a metal housing and a pull back spring.

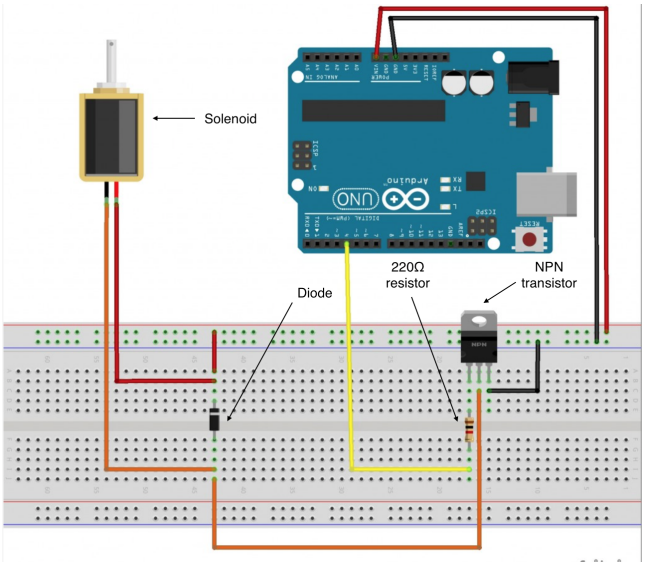


Figure 5.14: Electronic circuit for valves actuation scheme [21].

this project the second one was chosen due to advantages in terms of leakages and power (for further details see [9]).

5.4.1 PDMS membrane

The process to create PDMS is very easy and consists of mixing silicone elastomer curing agent and silicone elastomer base (1:10) and then degassing the solution for at least an hour to remove all the air bubbles, this step is especially important for later membrane fabrication.
Usually to fabricate a PDMS membrane a spin-coater is used but not having this tool available in the laboratory a different way has been followed [22].

1. Immerse two glass layers (50 x 75 mm) in a water-hand soap mix (40:1) for 10/15 min
2. Blow dry the slides with N_2
3. Put tape layers at the two borders of one slide (N.B. thickness of the tape will be the thickness of the membrane, in this case 1.2 mm)
4. Make the PDMS flowing along the slide starting from one edge, close to the tape
5. Put the second slide over the PDMS layer

6. Clamp with four binder clips the glass-PDMS-glass sandwich
7. Put in the oven at 65°C for 2 hours
8. Separates the two plates using a thin box-cutter and the PDMS will remain on one of the two
9. Release very gently the PDMS film with the use of tweezer

5.4.2 PDMS-PMMA bonding

In order to bond the PDMS membrane to the PMMA chip the following procedure is pursued.

1. Clean the PMMA device with IPA (Isopropyl Alcohol) and 10 % blench for 10 minutes
2. Blow dry it with N_2
3. Activate it with Corona discharge for 40 seconds
4. Prepare a solution of H_2O and 3-APTES (3-Aminopropyltriethoxysilane) (20:1) and put in the oven at 85°C until the temperature is achieved
5. Immerse the device inside the solution for 1 minute
6. Blow dry it with N_2
7. Put in the oven at 65°C for 10 minutes
8. Activate both the chip and the PDMS with Corona discharge for 40 seconds
9. Lay the PDMS layer over the device, making attention not to have air bubbles between them
10. Put the PDMS-PMMA sandwich inside the oven at 65°C for 2 hours

The chemistry behind the bonding is not completely clear yet, possible explanation are provided by [18, 19, 20] and summarized in the scheme (Figure 5.15).

5.4.3 PMMA-PMMA bonding

A possible PMMA-PMMA bonding technique is the Hot embossing but it requires very high temperature (i.e-99-104°C) and an high load needs to be applied ([20]). This procedure was tried in the laboratory but even with a good control of the temperature and the

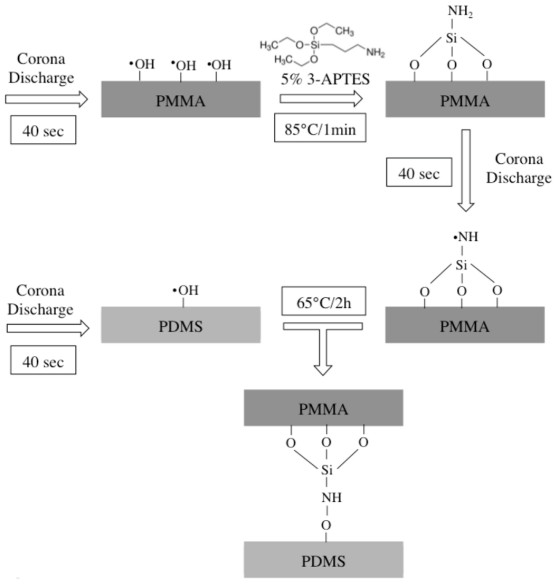


Figure 5.15: PDMS-PMMA bonding scheme [18, 19, 20]

load the results showed up to be not very satisfying [9].
The following protocol was then followed ([9, 20]) :

1. The two layer are washed with 10 % bench and then put in Ethanol for 30 minutes (the standard procedure predicts to sonicate them in Ethanol for 10 minutes, but due to the big size of this chip it was difficult to find a sealed container to put inside the sonicator)
2. Both washed with IPA and with a thin layer of that still in between they are put together
3. Clamp the PMMA-PMMA films together with four paperclips between two glass layers
4. With the use of a syringe the IPA remained inside the channels is sucked away
5. Put in the oven at 70°C for 10 minutes

Chapter 6

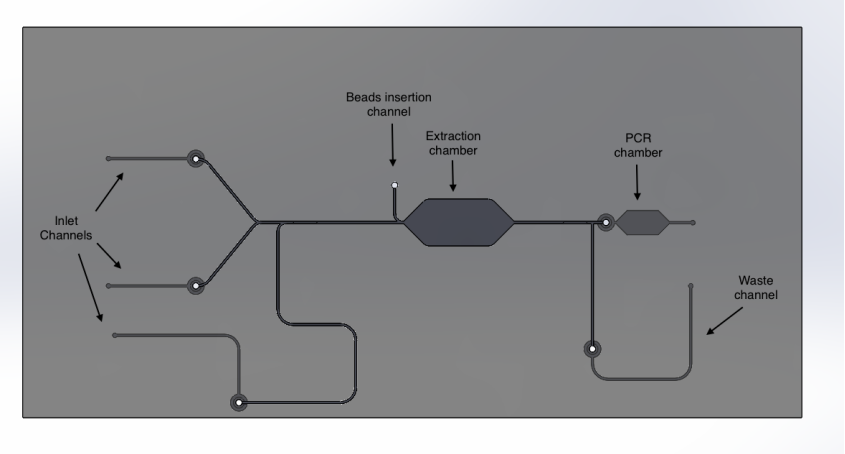
Work in progress

In this chapter the next steps to be done in order to optimize the extraction procedure and furthermore join it with the PCR over a unique device are explained.

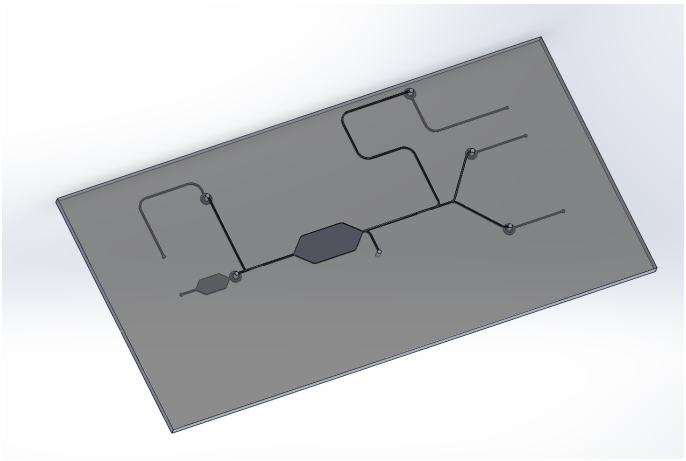
At first a new chip feature has been designed to overcome the limitation related to the old geometry. The Solidworks® file is shown in Fig. 6.1 where the extraction chamber is at the center of the chip, a short channel close to it was made for the beads insertion and three inlets that allow to introduce the buffers separately so to avoid an unwanted mixing of them. Then a PCR chamber was added so to run it directly on the microfluidic platform after the sample purification. Finally an extra outlet was put to make flow over there the waste products that can be discarded. A further step could also be to replace one of the inlet with a long serpentine channel connected to two inlets that would allow to get the mixing of the sample and the binding buffer directly over the chip (remember that in micro-scale the mixing of solutions is achieved just by diffusion so a long channel is necessary, and a serpentine geometry enables to occupy less space as possible).

As can be seen the whole devise is valved and in this case the correct functioning of the valves is very important, otherwise the solutions will be able to follow randomly the different channels. A new holder has been 3D printed as in the previous case (Fig. 6.2). The electronic circuit configuration is the same as before but since five solenoids were controlled, the power supplied by the Arduino Uno was not enough so a 9V battery was connected to the bread-board (Fig. 6.2).

The working principle is based on the loading of the sample and BB (previously mixed) through one of the inlet leaving the related valve and the one of the waste channel open, in this way the DNA will bind to the beads while the unwanted supernatant keep flowing and discaded. The washing step works in the same way but the inlet will be a different one. At the elution step the EB is made flowing but the waste channel valve is kept close

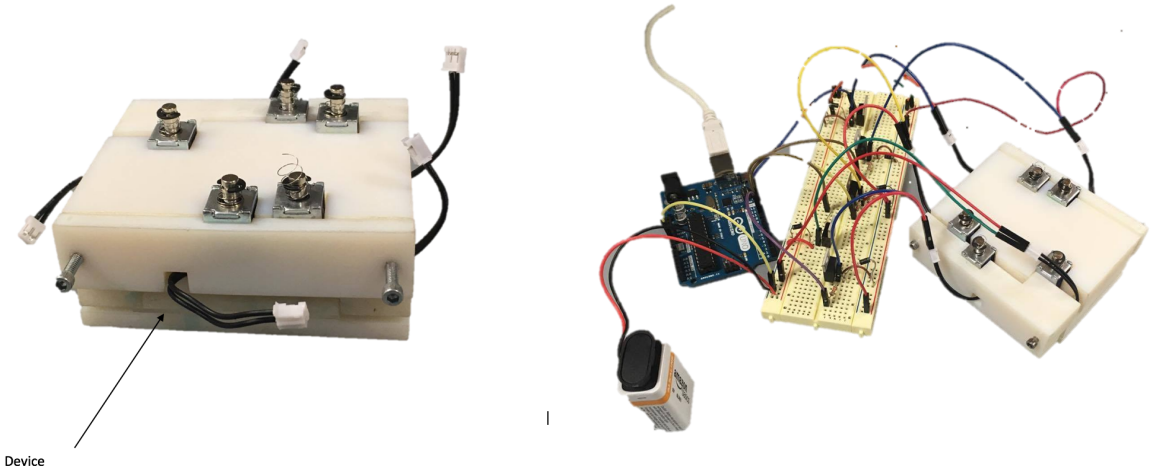


(a)



(b)

Figure 6.1: Solidworks® design of the new microfluidic chip. Three different inlets are present to allow the loading of the different solutions, the extraction chamber, the PCR chamber and the waste channel.



(a) *3D printed new microfluidic chip holder* (b) *Electronic circuit for multiple valves actuation picture*

Figure 6.2: Valves holder and electronic circuit

and the one of the PCR chamber open, so that the purified sample is collected and the PCR can be ideally run directly over there.
However this mechanism couldn't be tested since no time was available anymore.
The other step that would need to be made is to make the purification with a starting concentration that is of the same order of magnitude of a real sample. In order to be able to measure such a low concentration value a new instrument has to be used and a possible solution is *Qubit fluorometer*, that has a dsDNA sensitivity cutoff of 0.01 ng/μL. As before no time was available to try this possibility.

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