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DEVELOPMENT OF MICROFLUIDIC DEVICE FOR CELL LYSIS

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Development of Microfluidic Device for Cell Lysis

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Supervisor: Prof. Dr. Paulo Ricardo da Silva Pereira

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“If we understood the world, we would realize that
there is a logic of harmony underlying its
manifold apparent dissonances."
(Jean Sibelius)

ABSTRACT

The use of certain protocols to diagnose diseases requires specific tools and highly qualified professionals. This high-end scenario has leveraged the development of new mechanisms and processes on biomolecular analyses. In this context, recent discussions regarding new portable and low-cost devices which enable faster results have been receiving attention due to the possibility of speeding up physician decision making. The speed of decision-making has garnered attention even prior to the 2020 pandemic, and has proved to be an important factor in successful treatments. The process of disrupting cell membrane is known as cell lysis. This process is a fundamental step in several molecular analyses such as Polymerase Chain Reaction (PCR) standard analysis to diagnose diseases such as Sars CoV 2 (COVID-19) and many others. The standard protocol to perform PCR uses specific chemicals to rupture cell membrane, which increases the costs of tests. Since these materials are not currently available in the Brazilian market, it results in a long lead time. In addition, the protocols used to run PCR require specific training for staff, adding extra cost and time into the process. Therefore, this study presents the development of a microdevice for cell lysis. The proposed device was made using microfabrication techniques developed by the microelectronics industry. The microdevices have shown some advantages to conventional technologies such as portability, faster results, and a decrease of contamination risk when the samples are handled in a closed system. To prove the efficiency of the device, two analyses were performed: RNA concentration measured by a spectrophotometer and amplification of genetic material using PCR methodology. Based on results obtained through this technology, it was possible to establish the great prospective brought out by this method on health services reducing cost and speeding up results. A microdevice with 15 µm electrodes got promising results when compared to the ones from standard techniques. A significant time reduction on sample preparation, aside from portability and friendly user operation are some of benefits provide by the proposed device.

Key-words: Cell lysis. Diagnostic. Microfabrication. DNA amplification.

RESUMO

O uso de protocolos ágeis para diagnóstico, que exigem laboratórios bem equipados e profissionais altamente especializados, tem estimulado o desenvolvimento de novos mecanismos e processos para análises biológicas. Neste contexto, as discussões recentes sobre dispositivos compactos e inovadores para diagnóstico de doenças, que permitam análises rápidas e com baixo custo, têm ganhado destaque por mostrarem a possibilidade de antecipação da tomada de decisões pelos profissionais da saúde no cenário corrente de pandemia. Atualmente, o processo de rompimento da membrana celular, conhecido como lise celular, está na base de diversas técnicas de análise do material genético, dentre elas, o processo de amplificação de segmentos de código genético (PCR, *Polymerase Chain Reaction*), utilizado, por exemplo, na detecção do vírus Sars CoV 2, causador da COVID-19. O protocolo padronizado deste processo utiliza substâncias químicas específicas para realizar o rompimento da membrana celular, o que contribui para o aumento do custo do teste, pois é necessário importar estes insumos especiais. Além disto, ele demanda a operação por técnicos com treinamento específico. O presente trabalho explora o desenvolvimento de um microdispositivo para lise celular. O microdispositivo proposto foi construído com base em técnicas de microfabricação oriundas da indústria de microeletrônica, apresentando várias vantagens sobre os equipamentos convencionais, tais como: rapidez na realização da lise celular (em comparação com o método de lise atualmente empregado), dispensa do uso de reagentes e diminuição da probabilidade de contaminação da amostra por tratar se de sistema fechado. Para comprovar a funcionalidade do dispositivo, são realizados dois métodos de análise da lise celular: quantificação de RNA disponível na amostra por Nanodrop e amplificação do material genético após lise por técnica de PCR. Com base nos resultados do desenvolvimento desta tecnologia, através da prova de conceito, pode-se determinar o potencial de inclusão do método nos serviços de saúde com os objetivos de reduzir custos e agilizar o diagnóstico. O dispositivo microfabricado contendo eletrodos de 15 μm, obteve resultados promissores, de forma que foi observado comportamento compatível aos protocolos de extração já existentes. Havendo uma redução significativa no tempo de preparação para análise, o que mostra o potencial do dispositivo, outros potenciais do dispositivo incluem portabilidade e fácil utilização.

Palavras-chaves: Lise Celular, Diagnóstico, Microfabricação, Amplificação RNA.

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

DI	Deionized Wa
DNA	Deoxyribonucleic acid
FEA	Finete Element Analysis
NMP	N-metilpirrolidone
ITO	Indium Titanium Oxide
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffer Solution
PDMS	Polydimethylsiloxane
PET	Polyethylene terephthalate
PGMEA	Propylene glycol methyl ether acetate
PoC	Proof of Concept
RNA	Ribonucleic acid
RNAseP	Ribonuclease P
RTqPCR	Real Time Quantittive Polymerase Chain Reaction
TCLE	Informed Consent Form (*Termo de Consentimento Livre e Esclarecido*)
TMAH	Tetramethylammonium Hydroxide

CONTENTS

1 INTRODUCTION

The global biosensor market is estimated to reach 36 billion dollars in 2027, according to a study published by the Grand View Research company in March 2021. The genesis of this market was distinguished by the development of the rapid glucose test and is currently growing both in revenue and in the diversity of the tests, including the pregnancy test and the (OH, 2017) oximeter.

The concept of biosensor refers to a device able to measure a biological or chemical reaction and transmit its response by electrical or colorimetric way. There are several biosensors available on market and new ones are created every year. Some of the characteristics that are common to biosensors are the following ones: response time, price, sensitivity, and specificity. Regarding response time, this is a feature that strongly affects the early clinical decision-making in health scenarios, often directly impacting the reduction of mortality (DINCER et al., 2019).

During the 90s, some devices and concepts specific to the biosensors' market emerged, such as the "lab-on-a-chip" (LOC). This concept refers to the miniaturization of a clinical analysis laboratory in a small portable structure that contains one or several steps of a laboratory process. In the scientific literature, there is a wide variety of publications suggesting different assemblies, materials, methodologies, and measurements to assess markers of health or disease conditions. Their common point is the usage of some microfabrication techniques originally developed by the microelectronic industry for manufacturing devices that can process biological samples, such as the microfluidics devices (REYES et al., 2002).

Biosensors have important advantages when compared to current laboratory analytical instruments, such as: reduced volume of reagents, small sample volume, short analysis time, low price per test, possibility of integrating different tests on the same device, portability, and an increased sensitivity of the analysis. The possibility of combining several processes in the same device contributes to the reduction of both the probability of contamination and the risk of human error, since all the analyte processing is confined to a single device (J.; D.; R., 2020).

In 2020, a historical fact showed the importance of developing this technology. The pandemic of the respiratory virus Coronavirus SARS-CoV-2, the etiologic agent of the coronavirus disease (COVID-19), proved the need for rapid advances in the identification of microorganisms. Currently, there are two main tests for identifying this virus: the rapid test (by the detection of antibodies) and the Polymerase Chain Reaction (PCR) test. Both tests have significant limitations. The rapid test assesses the presence of antibodies generated by the host's immune system, which can happen 7 to 10 days after infection. PCR (the gold standard diagnostic technique in this case) detects the presence of the virus in the host, but it is limited in carrying out large-scale tests because it depends on complex intermediate steps, such as sample preparation (this step is

known as extraction) and DNA amplification. At this stage, the genetic material held inside the cell is released (cell lysis), which is usually done by chemical methods. Briefly, the detection of the virus by the PCR technique is expensive to the health system, in addition to requiring qualified professionals, specialized equipment, and laboratories with a reliable infrastructure (ZHANG et al., 2020).

Considering these issues, this work aims to develop an electronic and microfluidic device capable of performing cell lysis in a short time, based on the premise that a sufficiently intense electric field is capable of breaking the cell membrane (cell lysis). The proposed device can be used as part of a biosensor to release genetic material for further analysis, but also as a platform to streamline tests with conventional laboratory analysis equipment. The biological sample is deposited in a microfluidic channel where a high electric field is generated through the application of a voltage difference to microfabricated electrodes. The parameters to be determined during the research work are the speed of passage of cells along the channel and the magnitude of the electric field required for ensuring the cell lysis.

In order to test the efficacy of the device, a comparison of conventional extraction methods with the proposed method will be carried out using the same biological sample. For this, experiments will be carried out with a sample containing genetic material to evaluate the amplification by PCR and concentration measurement by Nanodrop, which will allow the determination of the best set of parameters to perform the electrical lysis.

1.1 Motivation and scope

The need for rapid tests that speed up the decision-making process by health professionals has been the object of study by several research groups over the decade. The current pandemic scenario has made this requirement even more evident, since tests for the detection of Sars-CoV 2 virus still use complex and time-consuming protocols such as PCR. Moreover, they require a costly laboratory infrastructure for their execution. For instance, to perform the extraction of 30 samples by the chemical method, at least 3 hours of work by health professionals are required.

Therefore, a technology that enables faster cell lysis can improve the diagnosis time and ensure faster interventions by the medical team, especially in the context of a pandemic. In addition, the availability of portable and cheaper diagnostic tests allows for an expansion of the population's access to the test.

This work is limited to a microfruidic device employing planar electrodes. It is not part of this work to study three-dimensional structures nor other electrode geometries. Concerning biological analysis, this thesis will only use endogenous gene as sample meant to observe molecular aspects.

1.2 Objectives

1.2.1 Main Objective

This thesis aims to develop a device capable of performing cell lysis by the application of an electric field to a microfuidic channel manufactured with microfabrication techniques.

1.2.2 Specific Objectives

- Develop a process to manufacture electrodes using the lift-off technique;
- Develop a manufacturing process of microfluidic channels using the soft lithography technique;
- Develop a proof-of-concept method for the proposed device;
- Evaluate the parameters associated with the electrical lysis process and assess the impact of each of them on the process effectiveness;
- Compare the proposed device with the gold standard cell lysis technique;

1.3 Work Structure

This work comprises 5 chapters, including the introductory one. It brings a context of cell lysis in the literature review chapter, followed by a brief description of concepts linked to the understanding of the proposed work, such as: electric field and microfluidic devices. This chapter ends with a description of the state of the art regarding cell lysis performed on microdevices. Next, in the materials and methods chapter, the manufacturing of the projected microdevice is described. The thesis ends with the exhibit and discussion of the results obtained, followed by the conclusion and and future prospects.

2 BASIC CONCEPTS

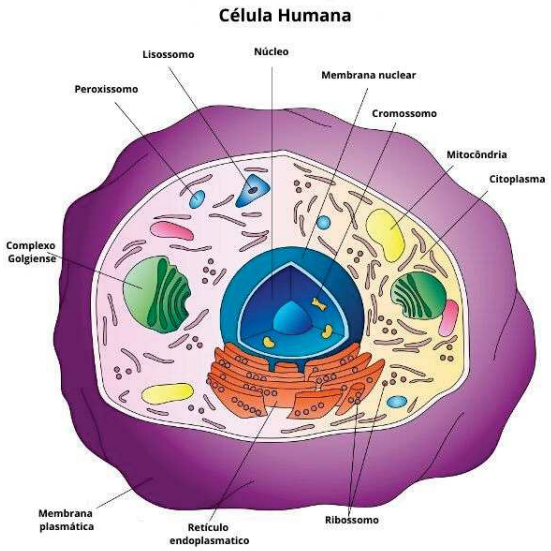
This chapter describes the basic concepts underlying the work proposal. Initially, a contextualization of the cell lysis concept is shown, followed by its applications. Next, there is a brief description of the microfluidic devices and finally a survey of those devices used for performing cell lysis.

2.1 Cell Lysis and Extraction Protocols

The analysis of genetic materials is commonly done using the PCR technique, which is based on the replication of specific DNA or RNA sequences with the help of a thermal cycler. To perform this technique, sample preparation steps are necessary, such as: collection of the biological material, cell lysis, and purification of the genetic material. At the collection stage, a biological sample is taken from the patient, usually blood, urine, saliva, or secretion. Next, the cell lysis is performed, releasing the genetic material originally confined inside the cell nucleus. The final extraction process is the purification of the genetic material, done by removing other intracellular components (lysosome and endoplasmic reticulum). Once all steps are completed, only the genetic material remains. (BITENCOURT et al., 2007).

The lysis process is required by the PCR technique because of the cellular organization. Figure 1 shows the arrangement of a typical cell, where the genetic material is kept within the nucleus, surrounded by cytoplasm, and protected by the cell membrane.

Figure 1 – Cell Structure.



Source: GONI

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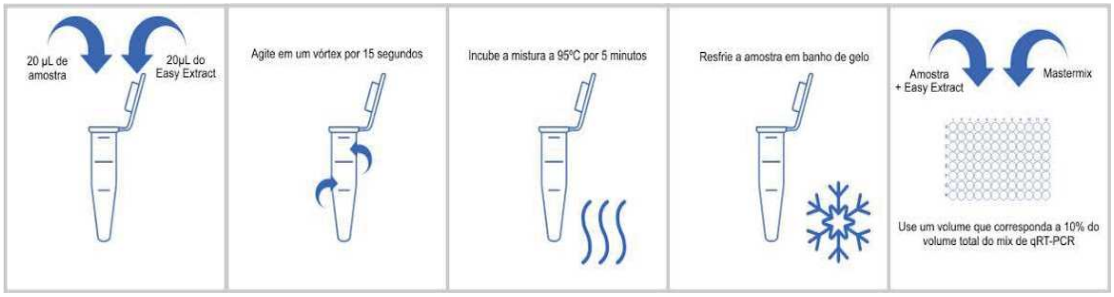
Hence, the release of genetic material from the nucleus can only happen by the disruption

of the cell membrane. The most popular methods for this purpose involve thermal, electromagnetic, or chemical processes. (ISLAM; ARYASOMAYAJULA; SELVAGANAPATHY, 2017)

The thermal method is done by exposing the biological sample to a specific temperature. There are commercial kits available for this, like EasyExtract, that simplify the extraction. Initially, a vortex is performed to mix the reagent and the sample, followed by the temperature cell lysis step at 95 °C for 5 minutes. Finally, the sample is cooled down and ready for the amplification process. Figure 2 shows the steps of the thermal lysis procedure.

Figure 2 – Thermal Extraction.



Source: Interprise.com

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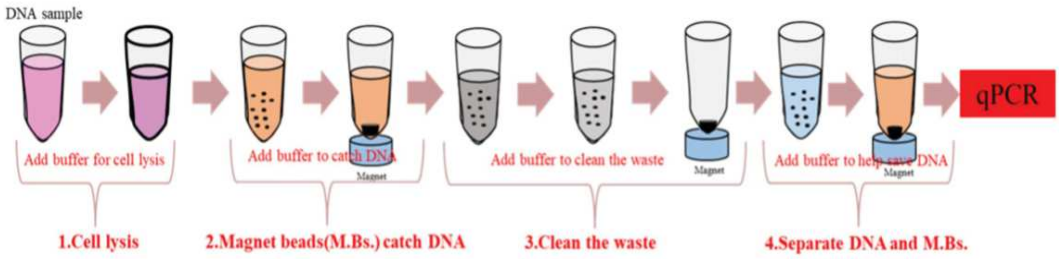
The electromagnetic technique uses magnetic beads to release the genetic material within the cells. According to the work developed by Chiang et al. (2020), solid particles (magnetic beads) were used to extract DNA from an embryo culture media. The authors justify the choice of this technique due to the low amount of genetic material present in the sample. In the final stages, cleaning with buffer solutions is performed and finally the genetic material is sundered from the magnetic particles, the Figure 3 shows the steps required to perform this kind of extraction.

Figure 3 – Magnet Extraction using magnetic beads .



Source: available on (CHIANG et al., 2020)

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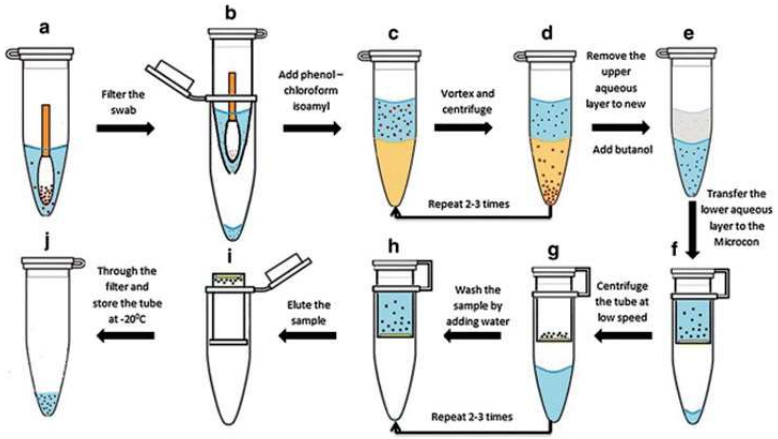
The chemical method is the most widely used and depends on imported commercial reagents, laboratory infrastructure (exhaust hood, centrifuge, tube shaker, water bath) and qualified personal. The steps of the chemical extraction method are shown in Figure 4. A biological sample is deposited in an eppendorf by a qualified professional, then a buffer is added to release the genetic material. Afterwards, washing steps for sample purification are performed

using alcohol and filter paper. The paper retains genetic material during the washing steps and only releases it using a a chemical reagent of negative charge. (AZIMI et al., 2011).

Figure 4 – Chemical Extraction.



Source: (ARYAL,2018)

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Even though this technique is the most commonly used for extraction, it ends up limiting the number of diagnostics highlighted during the SARS-CoV-2 pandemic due to high execution time (approximately 3 hours considering 30 samples) and cost of the chemical reagents (estimated approximately at R$1,100.00 for 50 samples), besides expenses related to the infrastructure and labor required to perform it. The main advantage of this technique is its standardization due to the degree of purity obtained in the extraction due to elimination of biological components (mosty proteins) not part of genetic material.

Table 1 – Commercial available kits.

Method	**Kit Comercial**	**Time**	**Pros**	**Cons**	**Cost**
Chemical	Pure Link RNA	20 minutes	High Purity	Equipments Complicated Contamination Risks	R$ 22,00
Magnetic	Norgen Isolation Kit	40 minutes	High Purity	Equipments Runtime	R$ 16,00
Thermal	easy extraction	5 minutes	Low Runtime	Equipments Complicated	R$ 14,00

Source: Autor.

2.2 Genetic Material Analysis

With the improvements of the molecular biology techniques, it is possible not only to identify genes related to physical characteristics, but pathogens as well. One of the most used molecular biology techniques for this was developed by Mullis et al. (1992) - Polymerase Chain

Reaction. This technique is the gold standard for identification of viral, bacterial and parasitic pathogen genes. (RAHMAN et al., 2013).

The PCR technique uses primers which are small DNA fragments designed to find a specific region of DNA. Two primers are used: one to indicate the beginning of the region to be amplified and another to indicate where the amplification finishes. Molecular diagnosis techniques are evaluated according to the presence of specific gene in the region delimited by the primers. The identification of this region is signaled by the fluorescence emitted by probes previously added to the primers. To perform the PCR, a DNA or RNA sample is used, hence extracting genetic material from a biological sample becomes mandatory to use it. Once the extraction procedure is done, the extracted material is mixed with the primers and probes and finally the solution is deposited onto a transparent plate that will be inserted in a thermal cycling equipment - thermocycler.

In the thermocycler, the plate containing the components necessary for the reaction is submitted to three temperature stages, usually in the range of: 95 °C, 56 °C e 72 °C, respectively. In each cycle, if the gene delimited by the primers is located, a couple of probes release fluorescent molecules. Due to the duplication of the material accomplished by thermal cycling, the emission level of the fluorescence also doubles during the cycling. The time and temperatures of each step depend on several factors, including the kit used and the type of sample. This result represents the presence or absence of a specific genetic sequence assigned by the primers. The interpretation of the result obtained in this technique is based upon the number of cycles needed to start the amplification in addition to fluorescence level reached at the last thermal cycling. The steps to perform thermal cycling are shown in figure 5.

Figure 5 – Steps to perform DNA/RNA amplification.

Source: Autor.

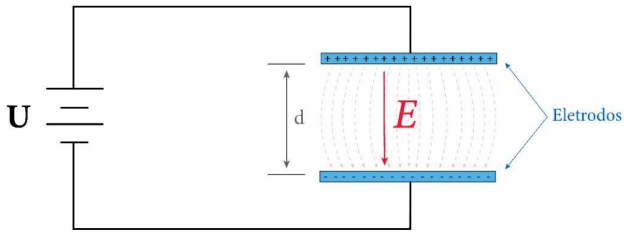
To ensure good performance on PCR, it is possible to quantify and assess the purity of DNA/RNA. This analysis can be done through spectrophotometry. In this technique a small volume of the sample already extracted (in the order of microliters) is deposited in the equipment

which emits ultraviolet (UV) radiation at a wavelength of 260 nm (characteristic of DNA/RNA) and then measures the amount of light absorbed by the sample. In addition to this wavelength, the equipment measures the absorbance at 280 nm. This wavelength represents the amount of protein available in the sample. It is worth to mention that the 280/260 nm ratio represents the amount of the genetic material in ration of the available protein in the sample. Therefore, the lower the concentration from this measurement, the greater the purification, and lower the availability of protein.

2.3 Electric Field

The electric field (E) is defined by the potential difference between two points. The difference of potential is generated by the accumulation of electric charges. Based on Coulomb's law, all electric charges exerts electric field on each other. However, the accumulation of charge can be calculated from the vector sum of these (SCHMITT, 2002). Figure 6 shows a model of the electric field.

Figure 6 – Eletric Field Model.



Source: Autor

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The potential (U) is an electrical quantity which is linked to the charge difference between two points. The electric potential and the electric field are related by the separation between the charges (d) according to equation 1:

$$E = \frac{U}{d} \tag{1}$$

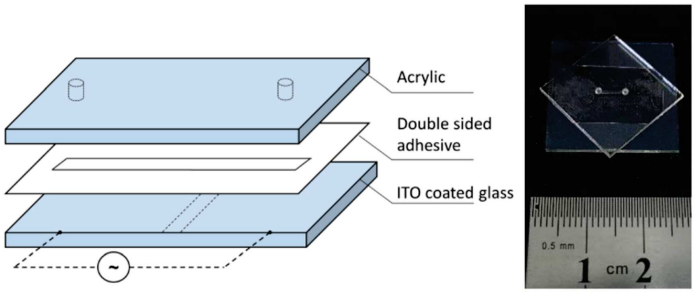
According to equation 1 (taken from Schmitt (2002)) it is possible to observe that smaller the distance between two points, the greater the electric field generated by the same amount of charge. Likewise, the greater the electrical potential, the greater the field generated by the same distance. Estabilished by International System of Units (SI) the electric field is expressed in Volt per meter (V/m), the distance in meters (m) and the electrical potential in Volt (V) (MAHMOUD et al., 2010).

2.4 Microfluidic Devices

The manipulation of small volumes of fluid has been used by the most diverse branches of science. In the context of engineering applied to healthcare devices, this became a concept called lab-on-a-chip, which has a number of advantages related to handling in small scale, process integration, among others (ZHU et al., 2019).

In the article published by Wei et al. (2019) a microfluidic device was manufactured (figure 7) with ITO (Indium Tin Oxide) electrodes. The authors justified the choice of this material because the material is transparent and, thus allowing the visualization of the cells during the lysis process. The microfluidic channel was made using a double-sided tape cut by laser 500 µm in width, 5 mm in length and 1 mm in height. The electrodes were made from a glass slide with a 180 nm layer of ITO. A separation on ITO layer was made by laser with a width of 50 um. The tape was placed on top of electrodes, follow by an acrylic sheet containing two holes (for fluid inlet and outlet).

Figure 7 – Paralell Electrodes.



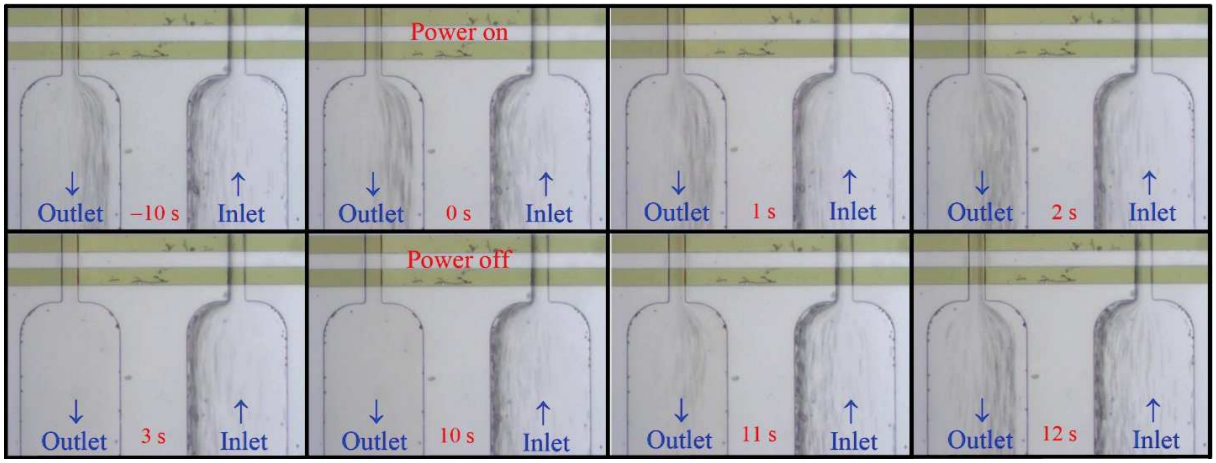
Source: Taken from Wei et al. (2019)

The results observed by the authors demonstrate the possibility of a simple device for the cell lysis process. To show the operation of the device, a 36V (peak to peak) potencial was applied . The sample flow rate was from 0 to 0.3 µL/min during the experiment. As a result, an efficiency of 100% of cell lysis was achieved using a signal frequency of 45 kHz.

In the work of Lo e Lei (2019) a similar device, as the one proposed, was manufactured in their work. It describes a continuous flow microfluidic device for cell lysis using planar electrodes. The influence of the sample flow was evaluated in the cell lysis process. A temperature sensor was also developed to evaluate the impacts of temperature on the lysis process. The microfluidic channel ("U" -shaped) was manufactured using soft lithography as a way of optimizing the device area. the width of the channel was 50 µm, height 43 µm and length 1.07 cm. The potential applied in the study was 20 Vpp and 1 MHz frequency.

In figure 8 it is possible to see one of the results presented in the article. The authors show device operation on a timeline. The flow rate was 50 µL/h. Noteworthy that within 3 seconds the cells do not appear on the device output which means that cells were lysed. The authors used a

Figure 8 – Cell Lysis on Microfluidic device.

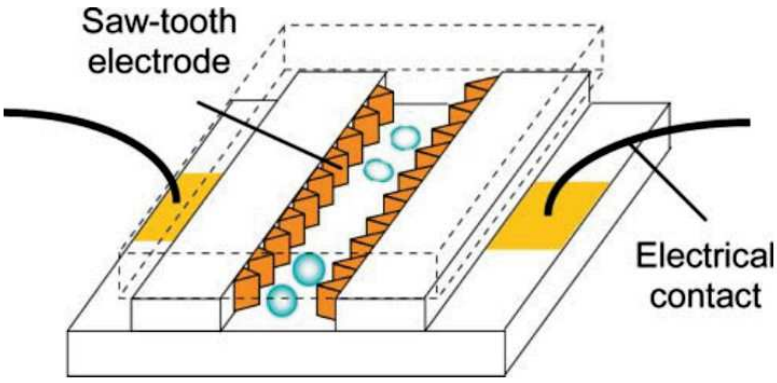


Source:Taken from Lo e Lei (2019).

minimum electric field of 1.2 x 10^5 V/m, considering red blood cells. Regarding temperature, it was concluded that flows less than 300 µL/h correlate with temperatures below 55 °C. This temperature indicates the start of the thermal lysis process.

Lu, Schmidt e Jensen (2005) and collaborators developed a device with three-dimensional structures for electrical lysis. Gold walls were manufactured as structures for electrodes.The main advantage of this type of structure is the uniformity of the electric field (better than that the one obtained with planar electrodes). As a sample material, the authors used HT-29 cells, applying a peak voltage of 6 V with a frequency of 5 kHz. The sample was inserted using a syringe pump with a flow rate of 0.25 µL/min. Figure 9 shows the device proposed by authors.

Figure 9 – 3D Electrodes.



Source: Taken from Lu, Schmidt e Jensen (2005).

In this work, the authors mention that in lysis process the electric field needs to be in the order of kV/m, which can be accomplished by increasing the voltage applied to the electrodes or reducing the distance between them. Another factor brought up by the authors, concerns the use of alternating voltage on the electrodes. According to them, a DC voltage greater than 1 V would

be above the maximum limit of water electrolysis, which would result in bubbles and raise the pH of the solution in the nearby regions to the electrodes(LU; SCHMIDT; JENSEN, 2005).

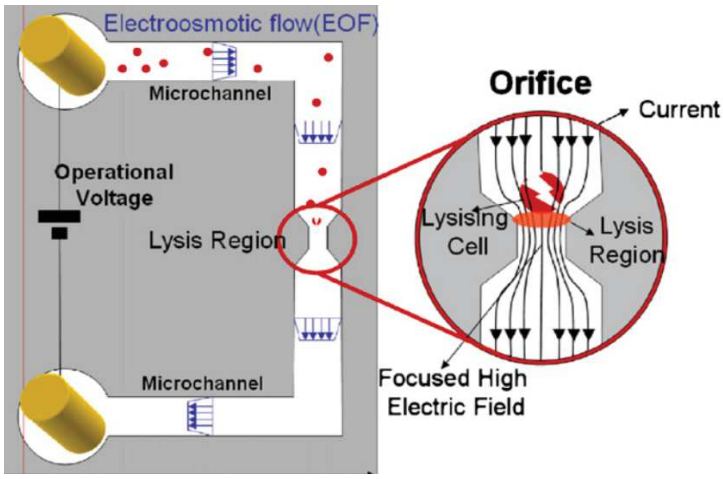
In regard of three-dimensional structures, the work published by Mernier et al. (2010) uses carbon electrodes for cell lysis. The authors present an equation of transmembrane potential, which is related to cell membrane rupture voltage (equation 2):

$$\Delta\phi = 1.5RE_0 cos\frac{\theta}{\sqrt{(1+(\omega\tau)^2)}} \tag{2}$$

This equation works when the potential applied to the electrodes is alternated. The transmembrane potential ($\Delta\phi$) depends on the diameter of the cell R, the electric field (E_0), the time constant τ, which is associated with the cell membrane capacitance and finally ω which depends on the frequency of the applied voltage. An outcome from this work is that, in addition to the cell lysis effect, the use of alternating voltage also generates the dielectrophoresis effect, which means that in regions close to the electrodes the cells are repelled (MERNIER et al., 2010).

In the work brought out by Fox et al. (2006) the concept of transmembrane potential is studied for different types of cells. The authors define this value as the difference in charge between intra and extracellular regions separated by the cell membrane composed of a double layer of phospholipids. This potential is formed mainly due to the difference between the negative charges from proteins and the DNA of the mostly negative intracellular part with the positive charges from the extracellular part that contains Sodium and Calcium.

Figure 10 – Focused Electrical Field .



Source: Retirado de Lee e Cho (2007).

For the purpose of assess the amount of cells ruptured by lysis process Zhou et al. (2016) developed a fluidic micro-device where impedance spectroscopy was performed to correlate cell

concentration and impedance. Lysis was done with sucrose and dextrose solution, the samples were injected into the microfluidic channel at a flow rate of 20 µL/min, and after a period to reach equilibrium along the channel, three measurements were performed with a spectrum of 100 Hz to 1 MHz with applied potential of 250 mV. The authors showed the relationship of low-frequency impedance with the cellular concentration of the sample, which was previously measured by the Neubauer method.

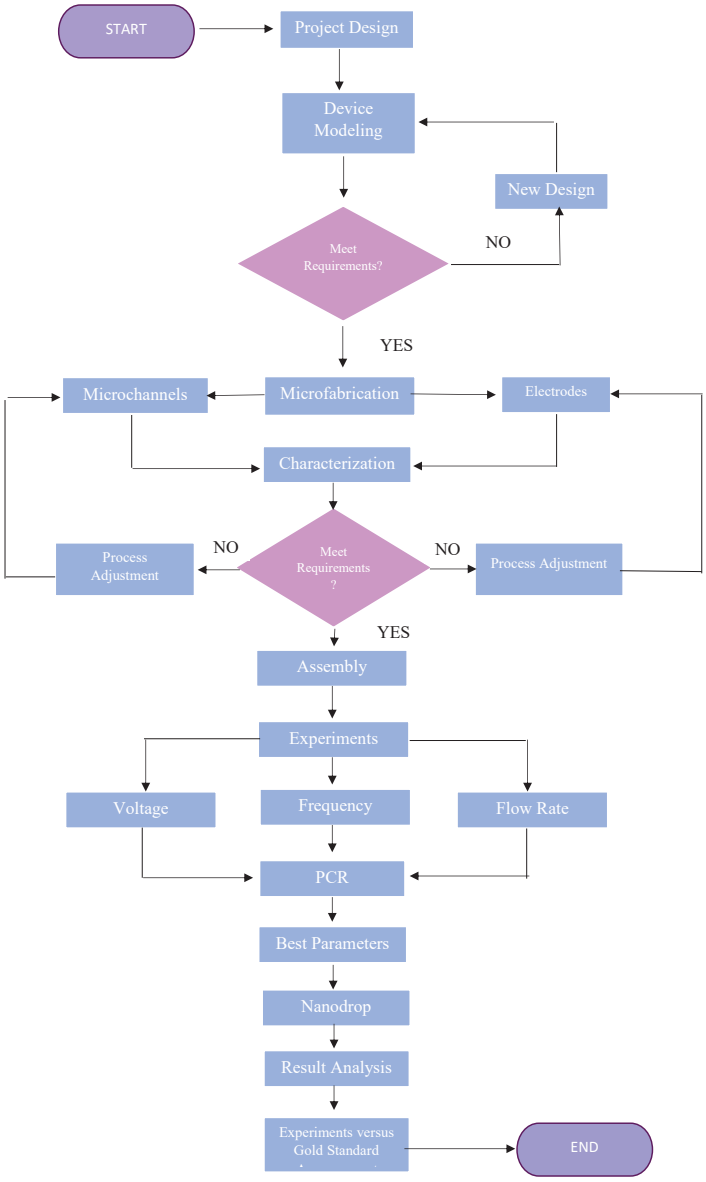
The work of Fox et al. (2006) also evaluates different electrode geometries for cell lysis. The authors emphasize that planar interdigitated electrodes have a greater uniformity in the electric field compared to other shapes such as sawtooth. In the same work, the authors describe a device that has a narrowing in the channel (figure 10 is able to amplify electric field in a section of the channel. This type of device was addressed in the work Lee e Cho (2007) which uses a DC voltage of 50V to reach an electric field of 1.2 kV / cm according to simulations presented in the paper.

Another feature highlighted by Lee e Cho (2007) is the flow created by electroosmosis which exempts from the use of a pump. The authors showed that this type of device was able to lysis red cells. Despite this, in the study published by Morshed, Shams e Mussivand (2013) shows as a disadvantage of this type of device the fact that the channel width in the lysis region must be smaller than the cell diameter (20 µm in the case of red cells) for the device to be efficient.

3 MATERIALS AND METHODS

This chapter describes the research proposal for manufacturing the device designed in this study. The manufacturing methods used and the description of the equipment and materials required are detailed. The chapter ends with a description of the test procedures that will be adopted. The flowchart shown in figure 11 describe the sequence of steps that will be carried out.

Figure 11 – Development Structure.

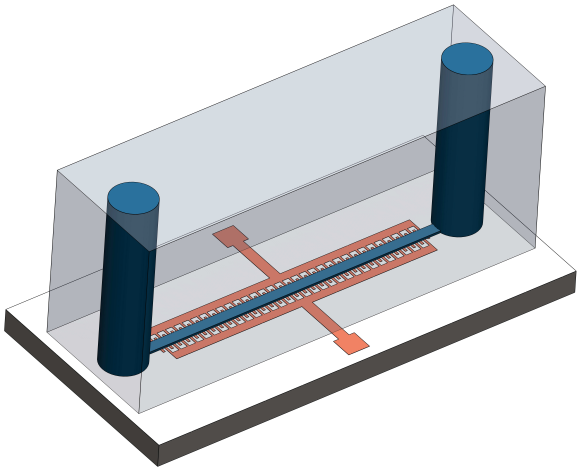


Fonte:Autor

3.1 Research Design

For the fabrication of cell lysis microdevice as bring up in this project,it was considered two microfabrication techniques: lift off and soft lithography. The first one involves manufacturing the electrodes using a silicon wafer with a thin oxide layer as a substrate. The second technique contemplates the fabrication of a microfluidic channel through the polymer polydimethylsiloxane (PDMS). For proof of concept, nasopharyngeal secretion samples will be used and cell lysis performance will be compared to the gold standard method. The setup for testing also includes a syringe pump to control the flow rate of sample fluid along the device and a signal generator to apply potential to the electrodes.

Figure 12 – Design.



Source:Autor.

The figure 12 shows an overview of the device design. The manufacturing methods mentioned, as well as the materials and equipment required will be described below. The methodology finishes with a section containing an investigation on the tests carried out.

3.2 Electrodes Design

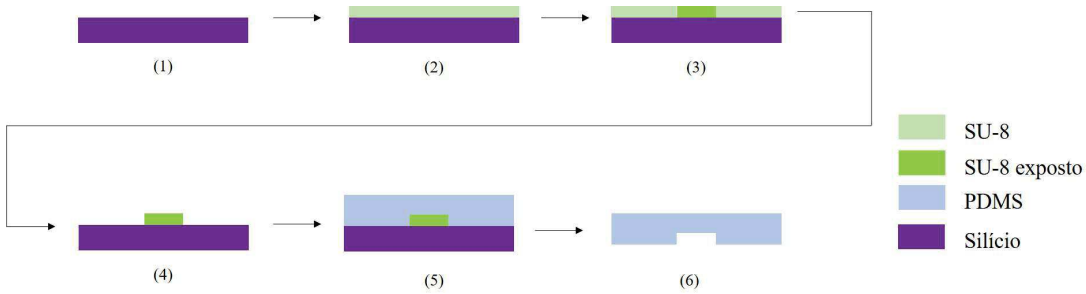
The electrodes were constructed in a planar way as described in the methodology. The geometry of the electrodes was chosen based on shapes used in the literature. The same criteria was applied to selecting flow rate, which was chosen in the same order of the ones mentioned in correlated works cited on section 2.4. Furthermore, a simulation was performed to determine the voltage to be applied in the electrodes, which generates the necessary electric field capable of rupturing the cells.

The applied voltage will be alternating and not continuous, to avoid the phenomenon of electrolysis, which generates bubbles along the microfluidic channel and also damages the genetic material, preventing further analysis. This remedy has already been used in some works like Morshed, Shams e Mussivand (2013) and Wei et al. (2019). Another parameter tested is the frequency of the signal, which was range aiming to analysis its effect on cell lysis. It should be noted that, in addition to the electric field be intense enough to break the cell membrane, it is necessary that cells are exposed long enough in this field. Thus, it is clear that cell lysis performed using the proposed device rely on a number of factors not being restricted just to the magnitude of electric field.

3.3 Microchannels

Regarding the material selection for manufacturing the microchannels, it is necessary to take into account some properties of interest, which include biocompatibility and transparency. Hence, it was chosen to use PDMS. The method developed in 1998 by Tang e Whitesides (2010) uses an inverse mold to the structure of interest. The elastomer material is casting on the mold, cured and removed. This process is known as soft lithography and its main advantage is low cost compared to other lithography techniques.

Figure 13 – Soft Lithography Fabrication.



Source: Autor.

The process, like most lithography techniques, begins with substrate cleaning. Due to the ease handling in the equipment and flat surface, a silicon wafer can be used as substrate. Cleaning is performed according to the protocol established by Kern e Puotinen (1970), usually referred to as RCA cleaning. The protocol is carried out in three steps: removal of organic contaminants, removal of metallic contaminants and removal of native oxide. The first cleaning is done with a solution of H_2O:H_2O_2:NH_4OH (5:1:1), the second step is done with a solution of HCl:H_2O_2:H_2O (1:1:6), and finally the removal of native silicon oxide is done with HF:H_2O (1:10).

Thereafter, SU-8 negative photoresist (Gersteltec) is deposited using a spin coater. This photoresist is normally used in this process due to its large range of thickness, an important aspect

in this process, since the thickness of the photoresist determines the height of the microchannel.

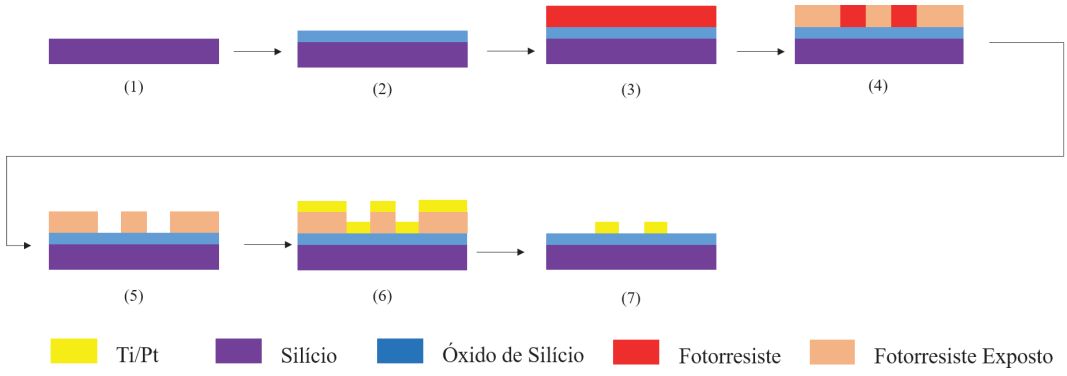
Once deposited, soft bake is done in two stages: 65 °C e 95 °C, according to manufacturer's specification. Step (3) is the exposure of the photoresist that will be carried out using the MicroWriter direct imaging equipment. Once exposure is finished, another baking (post exposure bake) will be done. Developing (4) is made using the solvent Propylene Glycol Ether Methyl Acetate (PGMEA), recommended by the photoresist manufacturer. Develop will be performed using a glass container, where the substrate with photoresist will be immersed in the solvent. Developing time will be determined according to experiments and tests that would be done previously.

The mold process ends with rinsing after development, which is done with isopropyl alcohol and deionized water. In the molding step (5) the substrate alongside the mold is placed in a petri dish, then PDMS is deposited, with sufficient volume to cover the mold. The set is taken to an oven where PDMS is cured for 4 hours at 60 °C. The final step (6) is peeling of cured PDMS from the mold.

3.4 Electrodes

The electrodes will be made using a microfabrication technique widely used by semiconductor industry, called lift off. In this technique, openings are patterned in photoresist according to the desired geometry, then the metal is deposited. The process finishes with photoresist removal. Figure 14 shows the steps of lift off process in detail. As substrate, a silicon water

Figure 14 – Lift Off Fabrication



Source: Autor.

coated with a thin layer of silicon dioxide. This layer is required to insulate the silicion from the electrodes. The process begins with substrate cleaning (1) according to the RCA procedure proposed by Kern e Puotinen (1970), as described in section 3.3, with the exception of the oxide removal step, since oxide layer will act as a dielectric between the electrodes and the silicon wafer used as substrate for the device. After cleaning, the photoresist is deposited using a spin coater (2). The rotation and time parameters will be chosen according to the photoresist's

technical data sheet. According to Voigt et al. (2005), negative type photoresists are indicated for lift off due to their thermal stability and wall resolution.

Prior to the exposure process, the photoresist is pre-cured (soft bake) for few minutes to evaporate solvents and also for the photoresist to adhere to the substrate. Soft bake time is selected according to the manufacturer's specification. Considering the infrastructure available in the laboratories, the exposure process (3) uses a direct (maskless) imaging equipment with a wavelength of 385 nm. On technical information available about photoresist, the dose for the above mentioned wavelength of the equipment is not available. Hence, a dose test will be carried out to determine the proper dose to be applied. Afterwards another baking (post exposure bake) is done. This process helps the adhesion of photoresist to the substrate and also reduces the stress generated by the photolithography process. (VESTERS; SIMONE; GENDT, 2017)

The areas not exposed (4) by light are dissolved through a chemical process. In order to accomplish that, a solution (developer) is used to dissolve the unexposed photoresist. Developing time changes according to the thickness of the photoresist layer, thus it will be defined based on the information available on the photoresist and also based on experimental tests.

Metallization step (5) is the deposition of a thin layer of metal by sputtering. The deposition will be done in two steps. First, an intermediate layer of Titanium (10 nm) will be deposited between Silicon Dioxide and Platinum, due to Platinum poor adhesion to the oxide. Finally, a layer in order of 100 nm of Platinum will be deposited. The deposition process parameters (time, power and gas flow) will be defined based on previous experiments.

The process is completed with fully removal of the photoresist (6), also carried out by a chemical process with a solution indicated by the photoresist manufacturer. Once the electrodes are ready, PDMS structure with the microchannel is bonded onto electrodes. For this process, the electrodes and PDMS structures are cleaned again with isopropyl alcohol and acetone. The structures are rinsed with deionized water to remove solvents, and finally dried with nitrogen.

Subsequently, the surface of the microchannel and electrodes are exposed to oxygen plasma for activation process. This process removes hydroxyl groups (-OH) from the PDMS surface, which when superimposed on the surface of the electrodes forms covalent bonds, making the bonding irreversible. After overlapping, the set is cured once more using an oven.

3.5 Proof of Concept - PoC

This is a feasibility study of excluding purification steps along with speed up cell lysis procedures to PCR analysis. The hypothesis is that the device for cell lysis grants sufficient genetic material necessary to perform the PCR technique. The microdevice fabrication described in the methodology was conducted at Semiconductors Research Facility at Unisinos (itt CHIP) in São Leopoldo and proof of concept through amplification of RNA (PCR) and spectrophotometry were performed in molecular biology laboratories at the same institution. In order to test the

proof of concept, a secretion sample was included nasopharynx and oropharynx. The sample was obtained from a laboratory in the Vale do Rio dos Sinos at HEMOCORD which has a library of negative secretion samples for SARS-CoV-2.

Nasopharynx and oropharynx secretion sampling was performed by laboratory professionals partners to the study, which includes a tube containing three swabs with secretion in 4 mL of saline solution. From this sample, 500 µL will be reserved for electrical lysis.

The column based extraction method is currently the gold standard for RNA extraction for biological samples. This method uses detergent solutions and centrifuges to purify the genetic material, these steps are performed in an airflow control environment (laminar Flow hood). For testing on the purposed device, the patient sample is injected in the microfluidic channel through a syringe pump to control flow rate and an electrical potencial which is applied to the electrodes. A volume of 100 µL of the secretion sample is injected to the microfluidic channel, thus the cells are subjectef to electric field generated by the electrodes. Finally, the sample is removed from the device and storage for analysis. Nanodrop and PCR were chosen to assess the performance of each set of parameters, besides each condition was performed in triplicate.

3.6 Equipments

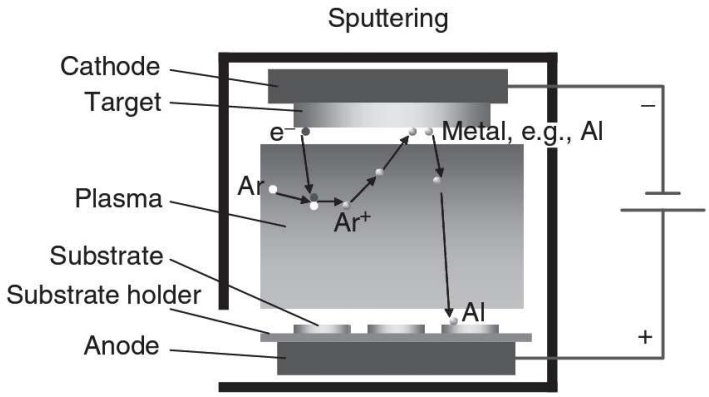
To develop techniques mentioned in electrode fabrication and fabrication of the microchannels it is necessary an appropriate environment such as clean room and proper equipment for microfabrication. Therefore, all the chosen techniques were selected based on the structure of the Unisinos Semiconductor Institute - itt Chip partner of Graduate Program in Electrical Engineering - PPGEE.

3.6.1 Sputtering

Physical deposition of materials is essencial for manufacturing microdevices. The foremost techniques used to physical deposition of metals and dielectrics are: evaporation and sputtering. The last one main advantages rely on thickness control and repeatability.

Sputtering process uses ion bombardment to remove atoms from a selected target. Initially, a vacuum is generated inside the chamber, afterwards, an inert gas (usually Argon) is injected. The gas is ionized and accelerated towards the target due to the high electric field created by a high voltage source. The collision of the ionized gas molecules on the target removes atoms from the target. As result, the material on the target surface is deposited on the substrate. Figure 15 shows the schematic of sputtering equipment. The deposition thickness depends on a set of parameters, such as: process time, power and gas flow. Some additional parameters can also interfere with the characteristics of the material being deposited. For instance, in the work of Ponmudi, Sivakumar e Sanjeeviraja (2019), deposition is performed with heating on the substrate to control grain size. In the work of Alfonso, Torres e Marco (2006), the roughness of

Figure 15 – Equipment Schematic.



Source: (GENG, 2005).

the deposited film is evaluated by applying a polarization voltage between the anode-cathode terminals. In this teshis, two metals are deposited using sputtering: Titanium and Platinum

3.6.2 Plasma Cleaning

Plasma removal is a process in which a material is exposed to ionic bombardment to eliminate impurities, remove materials or activate surfaces. In practice, plasma is generated inside a chamber at low pressure where a gas is injected. The gas used depends on the application, typically Argon is used for cleaning, Oxygen is used for removal and surface activation, and Chlorine is used for removal of metallic materials. Inside the chamber, the atoms or molecules of injected gas are ionized by a high electric field and accelerated towards the surface of the sample (FRANKLIN; BRAITHWAITE, 2009).

In the plasma process, the relevant variables are: cycle time, power and gas flow. The potential applied to the electrodes impacts on the speed at which the gas ions will reach the sample, higher the potential, greater the collision energy, making cleaning or removal of material more effective. The gas flow impacts the amount of ions that will be accelerated thus, it has influence on material removal rate.

3.6.3 Laser Writer

The photolithography process is used for engraving patterns using light. This process is widely used in the electronics industry to define patterns in silicon wafers and also in printed circuit boards. In this process an ultraviolet light sensitive material (photoresist) is etched onto the material where the pattern will be defined.

The photosensitive material (photoresist) exposure is limited by a mask which contains patterns of interest to be engraved on the base material. After exposure is done, the non-exposed

part of the photoresist (in case of negative photoresist) is solubilized by a specific chemical solution for developing the photoresist. This process is quick and effective, despite the manufacture of the mask is time-consuming and expensive.

In research laboratories, due to the need for several prototypes and tests, direct engraving on the photoresist (maskless lithography) is used instead of the mask. The equipment used for direct exposuring on the photoresist is called MicroWriter. This equipment has a light source that exposures the photoresist according to the design loaded in the equipment software. The substrate is positioned on a mobile table that moves according to the inserted design. This process allows the fabrication of structures smaller than 1 micrometer. As a disadvantage, this process has a longer recording time, compared to the process using a mask (RHEE, 2010).

3.6.4 Rapid Thermal Processing

The rapid processing furnace is used for heat treatments, such as: in the annealing of metals after deposition, in the annealing of silicon after impurities implantation, oxidation of materials, among others. The rapid treatment oven (RTP) has a high temperature ramp due to heating by infrared lamps, however, it can only process one wafer at time. When used for Silicon Oxide growth the process can be named Rapid Thermal Oxidation.

Silicon oxidation is considered one of the great discovery in the manufacture of integrated circuits (chips), having outstanding electrical and optical properties. Silicon Oxide is often used as a dielectric in the manufacture of transistors and also as a protective layer (passivation) on the chips. In practice, thermal oxidation can be divided into two categories: dry and wet. Briefly, wet thermal oxidation uses an oven where the wafers are placed in a quartz tube heated up to 1200 °C in the presence of water vapor. The great advantage of this method is the oxide growth rate and also the maximum growth thickness up to 2.5 µm. (LINDROOS et al., 2009)

Dry oxidation can be performed in convection ovens or rapid thermal processing (RTP) ovens. The main difference is the atmosphere in which the oxidation takes place, in this process an atmosphere of oxygen is used. The great advantage of the RTP process is its high ramp of temperature rise and fall, which reduces the thermal load of the process, in addition, the grown oxide has superior electrical properties compared to wet oxidation because of superior oxide density. In contrast, the oxidation thickness obtained in rapid thermal processing ovens are generally lower up to 500 nm.

3.7 Ethical Aspects

The project respects the resolutions of normative 466/2012, which governs clinical research carried out on human beings in Brazil and it has a letter of consent from HEMOCORD. The project was forwarded to UNISINOS Research Ethics Committee and was only carried out after approval by CAAE 50502221.2.0000.5344. The TCLE was also applied to the participants.

This study was limited to the standardization and validation of the technique. For the validation of the technique, a nasopharyngeal and oropharyngeal secretion sample was used, but there was no additional collection of biological material, using the same material analyzed by the laboratory. The anticipated risks for the study were minimal, such as possible situations of low volume of collected samples. To avoid them, the researchers will exclude from the experiment patient who has a sample quantity below 4 mL. There was also no risk of contamination from researchers and associates, as there was no manipulation of a positive sample for SarsCov-2. The expected benefit after the development of the research is the creation of a device able to perform cell lysis faster compared to the gold standard method, thus speeding up the diagnosis of patients through molecular biology techniques (BRASIL. . . , 2012)

4 RESULTS AND DISCUSSION

This chapter shows the devices manufactured according to the methodology. It also explores the results obtained from the tests performed.

Based on the methodology presented, the research's first stage was the definition of voltage, frequency and flow parameters to be tested. Three voltage levels, three frequencies and two flows were chose. All combinations between parameters were executed, and each combination was performed in triplicate. Table 2 shows the tested parameters.

Table 2 – Parameters

Voltage (V)	Frequency (Hz)	Flow Rate (µL/min)
5	1k	100
10	100k	300
20	1M	-

Source: Autor.

Thus, the number of devices needed to perform the proposed tests was 54 devices. The choice of voltage, frequency and flow rate levels were made according to the works cited in the Microfluidic Devices section. Once the parameter were defined, the geometry of the electrodes was chosen through simulation in order to assess the magnitude of electric field reached with the chosen voltage values. Furthermore, the simulation made it possible to evaluate electrical field uniformity's. The selected design was interdigitated type as the one used by Krueger e Stieglitz (2008).

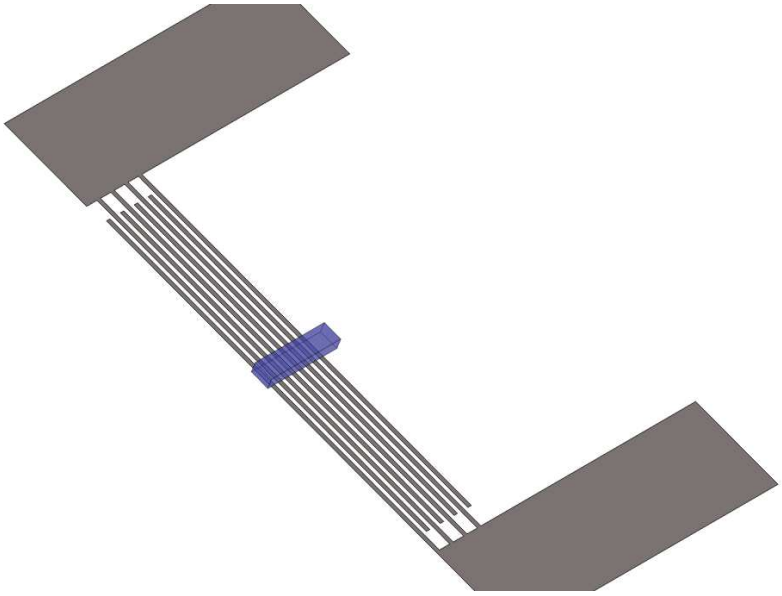
4.1 Finite Element Analysis

Finite element analysis (FEA) was done using COMSOL physical modeling software. For generate an electric field with high magnitude without the need to apply a high potential, a distance of 15 µm was chosen, with each electrode having a width of 25 µm. The microfluidic channel was designed with 10 mm in length, with rectangular geometry with 60 µm in height and 100 µm in width, resulting in a volume of 0.06 µL.

The electrode along with the microfluidic channel model is shown in Figure 16. For simplification and optimization only a part of the device was used, with a smaller number of electrodes (4 pairs) and only a part of 200 µm microfluidic channel.

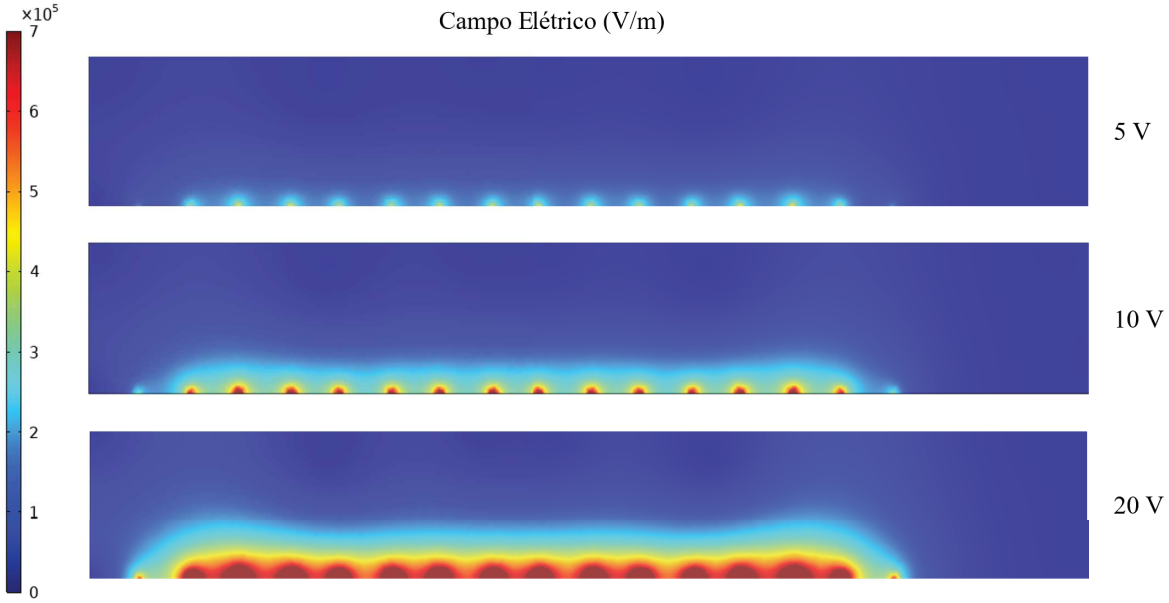
An important parameter of the simulation is the mesh which defines the number of points where the electric field is calculated , the more refined the mesh more points are calculated along the model, thus higher the resolution of the result obtained. For this analysis, a customized 2 µm mesh was used. After designing the model, three voltage amplitudes as shown in the table 2 were

Figure 16 – Device 3D model.



Source: Autor.

Figure 17 – Electric Field resulting from FEA.



Source: Autor.

studied. The results are shown in figure 17, the maximum magnitude of the electric field happens at the peak voltage of the applied signal.

As expected, the highest potential (20V) achieved the highest electric field magnitude. It is worth mentioning that in the upper part of the channel the electric field is null regardless of the applied potential and approaches its maximum values in the lower regions close to the electrode. (5V) the maximum magnitude of the field corresponds to $3x10^5$ V/m, at the intermediate potential (10V) the maximum value reaches $5x10^5$ V/m and at the highest potential this value reaches $7x10^5$ V/m.

4.2 Microdevice Fabrication Process

The next step after FEA was the fabrication of microchannels. The mold was manufactured employing soft lithography process described in the methodology in section 3.3. On a 100 mm wafer 20 molds were manufactured. Tests were carried out to achieve desired thickness of 60 µm. As result, a rotation of 350 rpm was used for 40 seconds in the step of photoresist deposition. After spin coating, the photoresist wafer was kept on the spinner for 15 minutes for relaxation. The first bake process (soft bake) was done in two steps: 65 °C for 10 minutes and 95 °C for 1 hour.

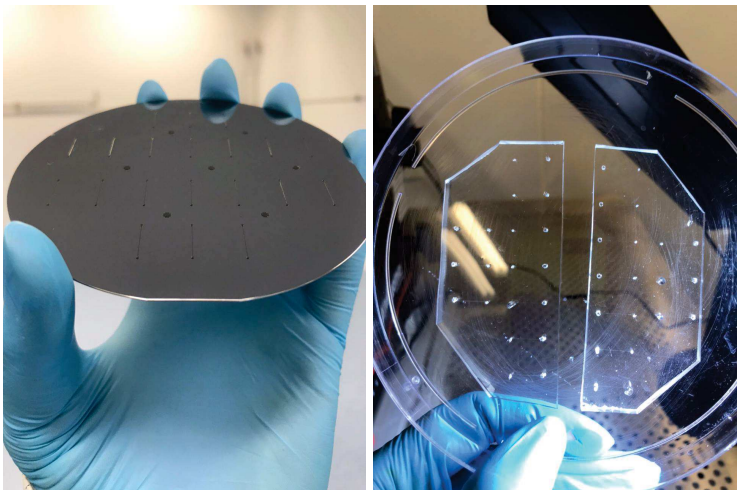
The exposure process was done on the MicroWriter equipment (Durham Magneto Optics) using a dose of 2500 mJ/cm^2. Once the exposure finished, another bake was carried out (post exposure bake) with the same parameters as the previous bake. The development was done with Propylene Glycol Methyl Ether Acetate (PGMEA) for 8 minutes, divided into two steps of 4 minutes with 300 mL of solution at each step. The wafer was rinsed with Isopropyl Alcohol and deionized water. Mold fabrication ends with a final bake (hard bake) of 135 °C for 2 hours.

The mold thickness was measured with a profilometer (KLA- Tencor D500) resulting in 64 µm. Due to demoulding, to manufacture all the necessary devices two molds were made in order to avoid variations in the channel geometry. The mold used in the soft lithography process along with the finished microchannels are shown in figure 18a.

The microchannels manufacturing finishes by doing the inlet and outlet of the channel. For the inlet a 23G needle (0.6mm outer diameter) and 30G needle (0.3 mm outer diameter) for the outlet were used.

Once the microchannels were finished, the next step was the electrodes manufacturing. The photoresist selected was AZ 2070 from Microchemicals. A 7 µm thick photoresist layer, using a rotation of 3000 rpm was spun on the wafer. The soft bake was performed using a hot plate at 95 °C for 7 minutes. The exposure process used a dose 1500 mJ/cm^2. After the exposure was finished, the post exposure bake was carried out using the same soft bake parameters, as instructed by the photoresist manufacture. The developing process was performed using 100 mL of tetramethylammonium hydroxide (TMAH) at room temperature for 3,5 minutes. After

Figure 18 – Microchannels made using Soft Lithography.



(a) SU-8 mold (b) PDMS microchannels.

Source: Autor

developing, a final bake (hard bake) was performed: 110 °C for 5 minutes.

After photolithography , Titanium deposition was carried out, followed by Platinum. The deposition was done by sputtering with the parameters according to table 3. Next, ending the electrode fabrication process, the photoresist was removed after metals deposition, for this purpose was used N-Methyl Pyrrolidone (NMP) solvent for 6 hours at 50 °C. Also, it was made ultrasound with 200 W of power in deionized water for another 2 hours, the result of fabrication of one of the wafers is shown in figure .

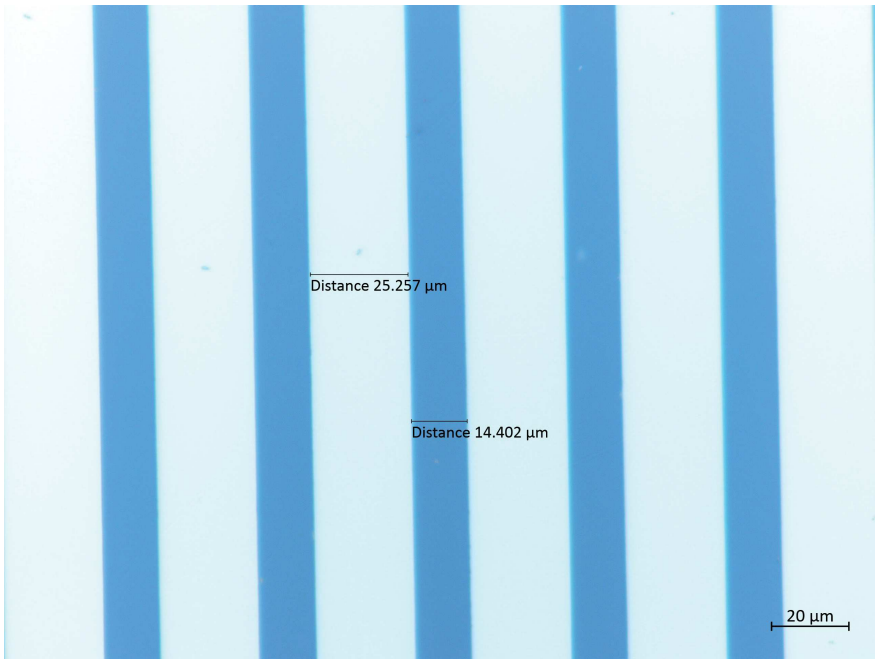
4.3 Experimental Details

For assembly the microchannels on the wafer containing the electrodes, the microchannel and the wafer were cleaning, for 3 minutes in acetone and Isopropanl. Next, they were rinsed with deionized water (DI) and dried with Nitrogen. In the last step, wafer and the microchannels were placed in oxygen plasma for 1 minute, with 100 W power and 100 sccm flow. In this process, the microchannel's surface is activated. The plasma removes hydroxyl groups -OH from PDMS which is superimposed on the wafer surface thourgh covalent bonds. To optimize the assembly, after plasma, the microchannels already bonded to the wafer are cured in oven for 2 hours at 80 °C. The final result of device assembly is shown in figure 20.

Once the assembly was finished, prior to the tests, a test bench was set up. For the test bench (shown in figure 21) a MPI TS200 Probe Station (MTI Corporation), a Fusion 4000 syringe pump (Chemyx) and a 33210A signal generator (Agilent) were used.

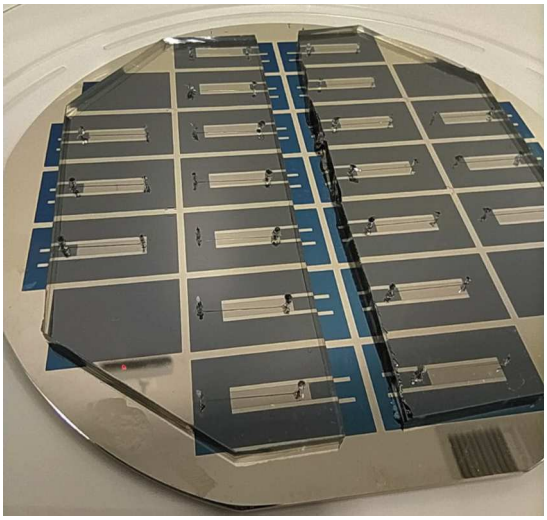
The sample was transferred to a 5 mL syringe, which was then attached to the pump. The devices were kept at 25 °C during the tests using the temperature control on microprobe. The

Figure 19 – Microlectrodes.



Fonte: Autor.

Figure 20 – Microdevices.

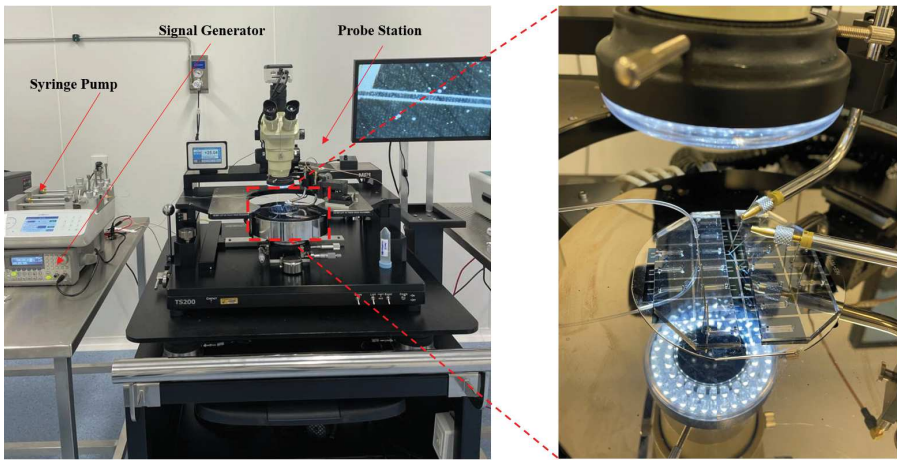


Source: Autor.

connection from the signal generator to the devices was made through the probe station. During the tests, the devices were held by vacuum to the table. Finally, the syringe pump is connected on devices by using a Tygon tube (diameter of 0,5 mm).

For each test, a volume of 100 µL was injected into the channel with the syringe pump, and after passing the sample through the microfluidic channel, a volume of 20 µL was taken for PCR analysis. Four 100 mm wafers containing 20 electrodes each were made. It was observed a percentage of defects (20%) due to short circuited electrodes (cause by defects in lithography)

Figure 21 – Experiment Setup.



Source: Autor.

verified by optical microscopy and mostly due to sample leakage in the inlet of microfluidic channel.

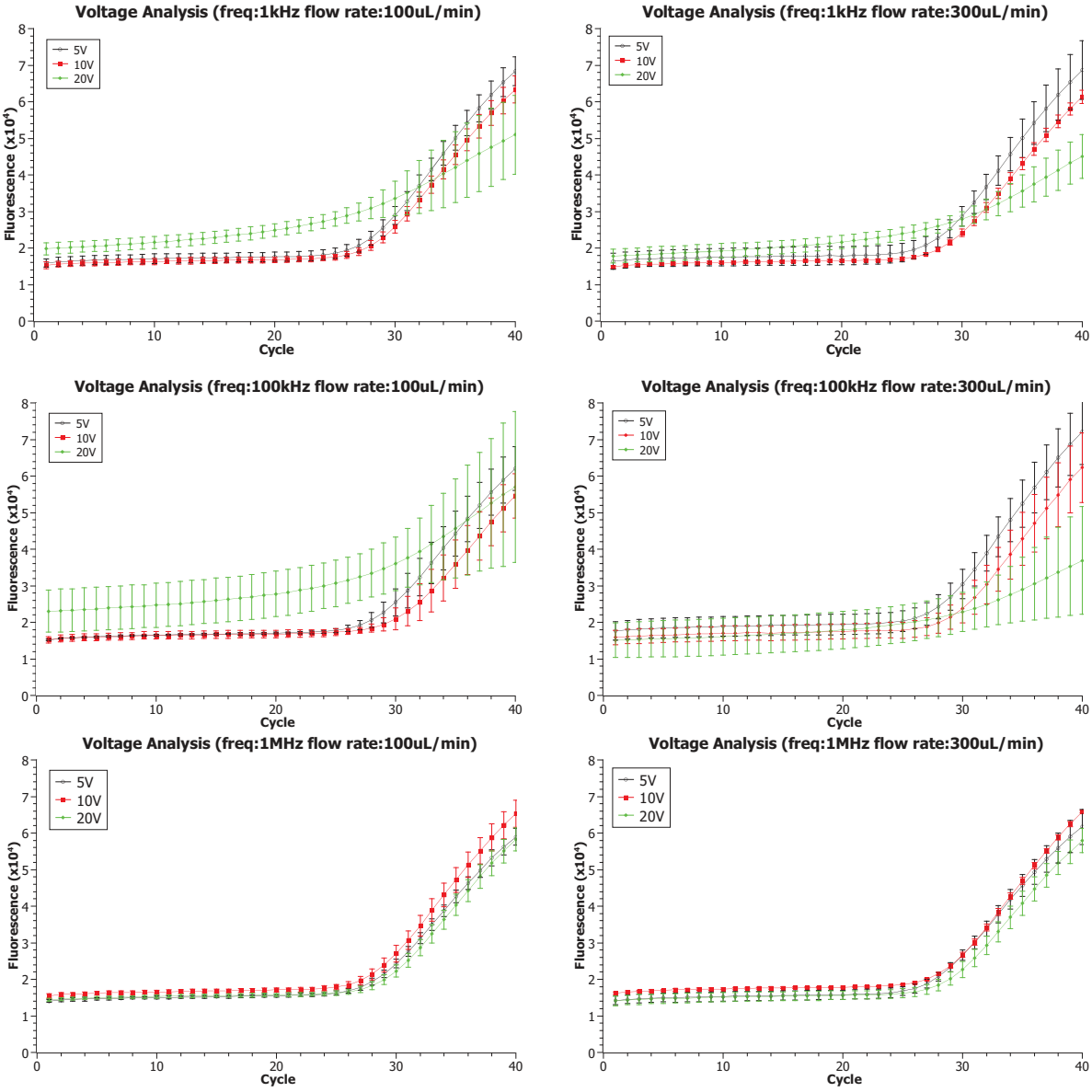
4.4 Experimental Results

A RT-qPCR (Real Time Quantitave Polymerase Chain Reaction) assay was done for each of 54 samples to assess the presence of genetic material. In order to do so, 10 µL of Master Mix was added to a 96-well plate containing a probe and primer for detection of the RNAseP gene. Finally,a small volume of 2.5 µL sample taken after cell lysis was also added. Then, the plate prepared with the reaction was coupled to a StepOne Plus ® thermocycler set to run a quantification analysis through TaqMan probes. In the equipment, 40 cycles were configured to reach temperatures of 50 °C, 95 °C and 60 °C respectively to perform the denaturation, annealing and extension of DNA strain. During each cycle a fluorescence is generated through the probe (from Master Mix reagent) if the primer finds its complementary nucleotide sequence sought in the reaction. Thereby,it is possible to detect and quantify the gene RNAseP.

Based on the results obtained by the amplification, the group of samples that used a voltage of 10 V was selected to spectrophotometry analysis. Again, all measurements were made in triplicate. In addition to the samples lysed by the method proposed in this work, the analysis of the pure sample (which is to say sample just collected from the donor) or without any kind of cell lysis (AM) was also performed, along with extracted sample by gold standard method which has purification steps (GS) and by thermal extraction (ET) in order to compare the performance of mentioned methods. The results of the spectrophotometry measurements are shown in the bar graph shown in the figure 24. The result refers to the mean and standard deviation obtained from absorption ratio (proteins over genetic material) given by spectrophotometer.

Regarding the amplification of genetic material, all experiments showed amplification

Figure 22 – Voltage Analysis.

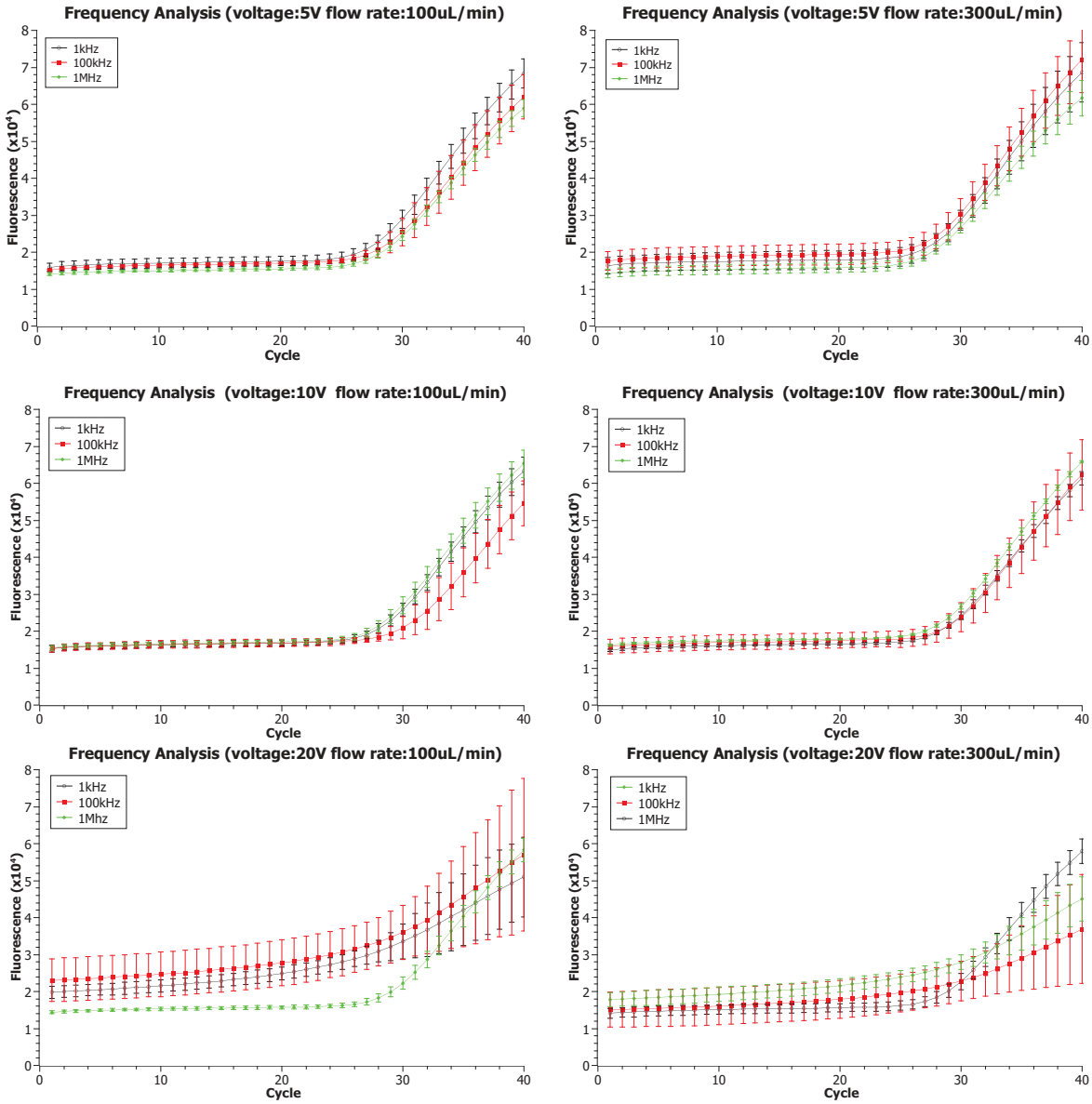


(a) Flow Rate: 100uL/min

(b) Flow Rate: 300uL/min

Source: Autor.

Figure 23 – Frequency Analsys.

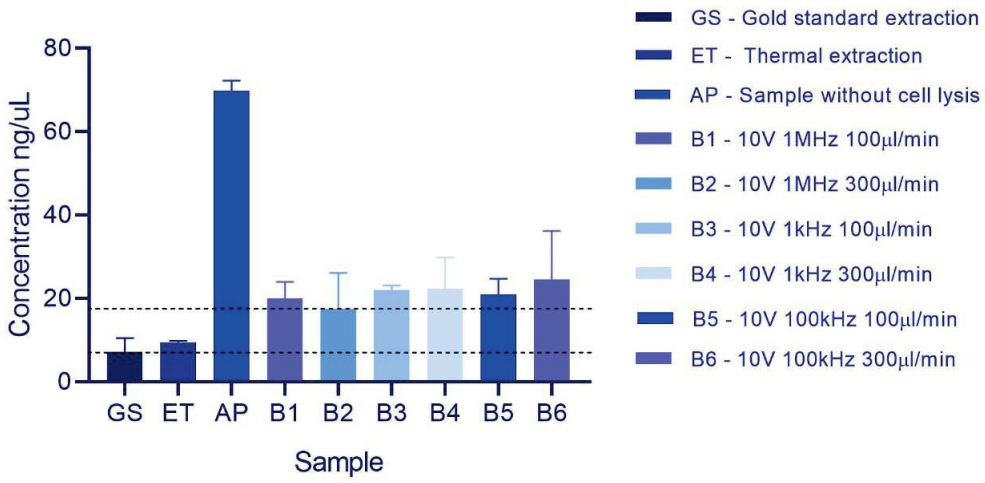


(a) Flow Rate: 100uL/min

(b) Flow Rate: 300uL/min

Source: Autor.

Figure 24 – Spectrophomter results from best group of samples.



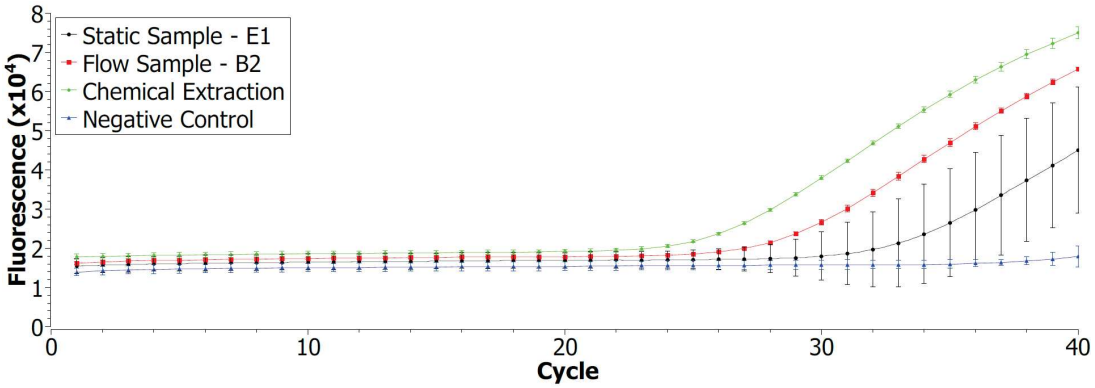
Source: Autor.

even in the absence of sample purification steps. The variation of the flow of sample in the channel was not relevant in the PCR amplification. The results from the PCR analysis were the same for both flow rate: 100 µL/min and 300 µL/min. With exception in the devices analyzed at the frequency of 100 kHz with a voltage of 20V that shown higher amplification at lower flow. However, this result got a high deviation standard, therefore it was not considered for the analysis of the flow rate.

As for the potential applied to the electrodes, the voltage of 5V and 10V got better results compared to higher voltage, as they presented greater amplification (fluorescence generated) along with fewer thermocycling cycles. The highest amplification obtained had as parameters: voltage of 10V, frequency of 1 MHz and flow rate of 300 µL/min. The graphs show in figures 22 and 23 the comparison of voltages and frequencies respectively.

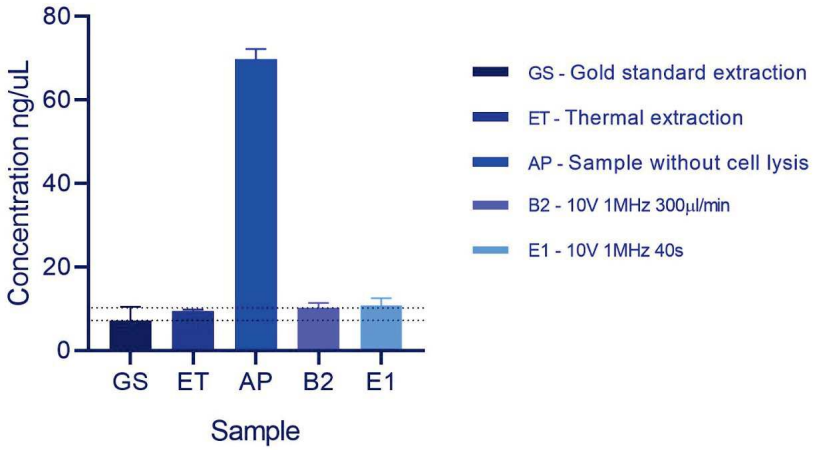
Based on the spectrophotometry chart, it is possible to infer that the samples with higher purity, which means higher RNA concentration in relation to protein concentration are the ones from gold standard extraction and thermal extraction. This is justified since in these methodologies are used washing steps or thermal shocks to respectively obtain protein washing or degradation. Even so, in all tested parameters there was lower genetic material/protein ratio than in the pure sample. Moreover, the set of parameters with better performances were: B1, B2 and B5. In relation to pure sample there was a significant difference from 70 ng/µL RNA/protein concentration to less than 20 ng/µL in the samples lysed by the electrical method subject of this study. According to the results, the flow rate effect was not significant in the parameters investigated. For this reason, a last set of tests was added in order to evaluate the device with static sample meaning no flow in the channel (E1). The purpose of this test was to evaluate the need to use a syringe pump. To accomplish this test, a volume of 10 µL was inserted in the microchannel, afterwards an excitation was applied for 40 seconds with the parameters of voltage

Figure 25 – Polymerase Chain Reaction Analysis.



Source: Autor.

Figure 26 – Concentration analysis through spectrophotometry.

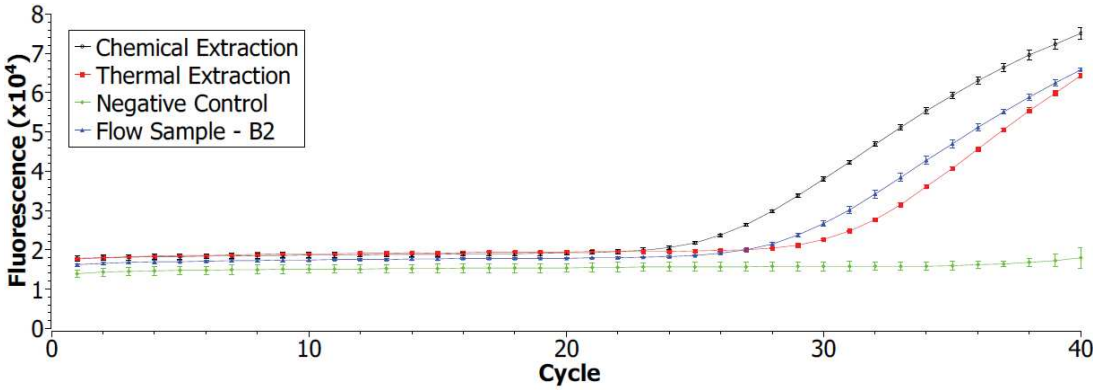


Source: Autor.

and frequency of group B2 that showed better performance in the PCR and spectrophotometry analysis based on the evaluation of previous experiments. After 30 seconds the signal generator was turned off, then a volume of 5 µL was removed from the device for proof-of-concept analyses. This experiment was also carried out in triplicate. In addition to static test, the experiment that obtained the best result in the previous analyzes with the sample flowing along the channel was repeated.

In the proof of concept using PCR and spectrophotometer with the sample lysed in a static condition was evidenced an RNA/protein concentration of the same order of magnitude than the one with flowing sample, however in PCR assay the triplicate of static samples showed lower amplification compared to flowing samples, which indicates less genetic material availble on the sample. In addition, there was a high variation on PCR assay in the static samples unlike the results from flowing conditions.

Figure 27 – Comparison with standard techniques.



Source: Autor.

The results obtained are consistent with recently published works, such as the study by Smyrlaki et al. (2020), which showed the detection of Sars-CoV 2 with a nasopharyngeal sample and oropharynx without purification of genetic material. In this work, a lysis step was carried out in the thermocycler for 5 minutes at 95 °C excluding purification steps. From the results achieved in this thesis and based on published studies, it is possible to assume that purification steps are dispensable for qPCR tests. Also,the results presented endorse faster methodologies, especially in a pandemic scenario where cutting steps in sample analysis can led to faster diagnosis.

5 CONCLUSION

The study developed described the process of cell lysis and cell extraction. Throughout the work, techniques used in the laboratory context were described and proposed a new cell lysis microdevice that employs an electric field to break the membrane cell. Based on the literature review and the proposed methodology, it can be stated that the objectives of this work were achieved. Regarding the manufacture and assembly of the device, the technique used to manufacture the platinum lift off electrodes proved to be adequate. form, the microchannels that were fabricated using the soft lithography technique. the results of the proof of concept demonstrate that the device, manufactured using two techniques of microfabrication, was able to lyse cells.

Based on the proof-of-concept tests, it was possible to prove that the device presented performance that can be compared to the gold standard method of chemical extraction and by column, and showed better performance than the widely used thermal method in clinical analysis laboratories. In addition, in this work, purification was dispensed with. of the biological sample in the extraction process, since the performance of the sample without purification was able to present a sufficient amount of genetic material to carry out the PCR technique. The elimination of this step resulted in a significant reduction of the time to 1 minute of sample preparation.

Throughout the work, parameters relevant to the operation of the device such as voltage, frequency and flow. In the latter, no differences were observed. significant in the final result at the flow rates tested. The parameter that most influenced the result was the voltage that at the highest applied potential showed the lowest amplification of the genetic material compared to minor stresses. By analogy with what happens in other cell types such as in the work of Geng et al. (2012), this happens due to the degradation of the genetic material due to the high electric field. Regarding the frequency of the applied signal, third parameter evaluated, also had a significant impact on the result.

The samples tested at the highest frequency had a higher amplification compared to 1 kHz and 100 kHz. This behavior is justified by the variation in amplitude of the field due to the frequency applied, which at lower frequencies the sample is less time subject to the maximum field value which reduces the lysis efficiency. All parameters tested were able to lyse cells, despite showing differences in amplification levels and fluorescence level enabling the assessment of the best set of parameters tested based on shortest cycle amplification and highest fluorescence level.

The best set of parameters in this study (B2), when compared to the technique of cell lysis and gold standard extraction (chemical method with amplification starting in cycle 25) demonstrated a slightly lower level of amplification starting at cycle 28. The rapid extraction kit using the thermal method had a lower fluorescence level starting to amplify from cycle 29, the

device in that same set of parameters showed a higher amplification level starting to amplify in cycle 28. This performance it was shown to be satisfactory considering that no washing steps were used after cell lysis.

Based on the results obtained, the developed device presented performance superior to the thermal method and slightly inferior to the gold standard extraction method. Is still It is important to emphasize that the sample preparation time with the proposed device is shorter in compared to other methods, which reduces the time needed for analysis (1 minutes versus two hours).

The developed device has advantages over standard extraction methods such as reducing the probability of contamination due to the integration of necessary steps for the extraction process, reducing human error as a result restriction of sample handling, significantly speeding up the extraction process, these are characteristics that enhance the use of the device developed in this work.

5.1 Future perspectives

This study showed a proof of concept of a microfluidic device capable of perform cell lysis faster compared to current methods. The proposed device in this work, its main advantage is the significant reduction in process time, a fact that enhances the market insertion of the developed device. for continuity development studies are still needed. In this work it was proven functioning of electrical lysis using a type of biological sample nasopharyngeal secretion and oropharynx. Other types of samples can also be tested, such as blood, urine and saliva. Ideal voltage and frequency parameters may require adaptations accordingly with the type of sample.

With regard to making the proposed device into a product, it is necessary to expand the number of samples tested to validate the efficiency of the proposed device, planned step in the second part of this research. In this sense, tests on other genes of research beyond the RNAseP gene. In the same vein, the methods used for manufacturing of the device in the present studies did not consider the cost factor. Other methodologies of fabrication should be explored as screen printing for the fabrication of machined electrodes and channels with laser instead of the soft lithography process which despite having great flexibility in the manufacture of microchannels, it involves several steps, which makes the cost of this process high.

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ANEXO 1

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO FASE 1

Você está sendo convidado(a) a participar do projeto de pesquisa "DESENVOLVIMENTO DE DISPOSITIVO MICROFLUÍDICO PARA LISE CELULAR" do aluno de mestrado SAMUEL TAVARES DA SILVA MARASCHIN, sob responsabilidade do professor Prof. Dr. Paulo Ricardo da Silva Pereira (UNISINOS). Esta pesquisa tem por objetivo verificar a eficácia de um novo método de lise celular. Esse novo teste, além de ser rápido e de baixo custo, pode ser realizado fora do laboratório. A pesquisa utilizará suas amostras de secreção nasofaringe e orofaringe coletadas e armazenadas no banco de amostras do Hemocord. Os testes experimentais serão dívidos em duas etapas, na primeira etapa será incluída uma amostra de paciente que aceitar livremente participar da pesquisa, essa amostra será utilizada para testar os parâmetros de funcionalidade do dispositivo. Caso os resultados forem satisfatórios na primeira fase, serão incluídas vinte amostras na segunda fase, para provar o conceito de funcionamento da tecnologia desenvolvida. Todos os pacientes que serão incluídos no estudo assinarão o Termo de Consentimento Livre Esclarecido em duas vias, ficando uma com o pesquisador e outra com o participante, sua participação no estudo é voluntária e não acarretará em qualquer forma de pagamento. Você não necessitará passar por mais de um procedimento, pois o material já colhido de secreção nasofaringe e orofaringe será suficiente para realizar o estudo. Esta pesquisa não apresenta risco a sua saúde, uma vez que utilizaremos sua amostra já coletada, também garantiremos o sigilo de sua identificação em todas as etapas do projeto. Sua participação é isenta de remuneração ou ônus. Todos os resultados obtidos serão confidenciais e ficarão sob a tutela e total responsabilidade dos pesquisadores deste projeto, podendo a qualquer momento ser consultados e/ou eliminados da pesquisa caso você desista de sua participação como voluntário(a). Você tem a liberdade de abandonar a pesquisa em qualquer fase dela, sem que isto leve à penalização alguma ou qualquer prejuízo posterior a você ou a sua família. Os pesquisadores envolvidos no Projeto garantem a você o direito a qualquer pergunta e/ou esclarecimentos mais específicos dos procedimentos realizados e/ou interpretação dos resultados obtidos nos exames. Os dados obtidos serão utilizados somente para este estudo e serão armazenados por um período de 5 anos após o qual serão eliminados por meio de picotagem. Em caso de eventuais dúvidas sobre a pesquisa, você terá plena liberdade de entrar em contato com o pesquisador responsável, buscando maiores esclarecimento pelo telefone: (51) 3591-1122 ramais 3720 ou 3181 ou pelos seguintes correios eletrônicos: samuelmaraschin@hotmail.com ou prpereira@unisinos.br.

São Leopoldo,____ de _____ de _____.

_____ _____

Nome do(a) Voluntário(a) Assinatura

_____ _____

Samuel Tavares da Silva Maraschin Paulo Ricardo da Silva Pereira

ANEXO 2

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO FASE 2

Você está sendo convidado(a) a participar do projeto de pesquisa "DESENVOLVIMENTO DE DISPOSITIVO MICROFLUÍDICO PARA LISE CELULAR" do aluno de mestrado SAMUEL TAVARES DA SILVA MARASCHIN, sob responsabilidade do professor Prof. Dr. Paulo Ricardo da Silva Pereira (UNISINOS). Esta pesquisa tem por objetivo verificar a eficácia de um novo método de lise celular. Esse novo teste, além de ser rápido e de baixo custo, pode ser realizado fora do laboratório. A pesquisa utilizará suas amostras de secreção nasofaringe e orofaringe coletadas e armazenadas no banco de amostras do Hemocord. Os testes experimentais serão dívidos em duas etapas, na primeira etapa será incluída uma amostra de paciente que aceitar livremente participar da pesquisa, essa amostra será utilizada para testar os parâmetros de funcionalidade do dispositivo. Caso os resultados forem satisfatórios na primeira fase, serão incluídas vinte amostras na segunda fase, para provar o conceito de funcionamento da tecnologia desenvolvida. Todos os pacientes que serão incluídos no estudo assinarão o Termo de Consentimento Livre Esclarecido em duas vias, ficando uma com o pesquisador e outra com o participante, sua participação no estudo é voluntária e não acarretará em qualquer forma de pagamento. Você não necessitará passar por mais de um procedimento, pois o material já colhido de secreção nasofaringe e orofaringe será suficiente para realizar o estudo. Esta pesquisa não apresenta risco a sua saúde, uma vez que utilizaremos sua amostra já coletada, também garantiremos o sigilo de sua identificação em todas as etapas do projeto. Sua participação é isenta de remuneração ou ônus. Todos os resultados obtidos serão confidenciais e ficarão sob a tutela e total responsabilidade dos pesquisadores deste projeto, podendo a qualquer momento ser consultados e/ou eliminados da pesquisa caso você desista de sua participação como voluntário(a). Você tem a liberdade de abandonar a pesquisa em qualquer fase dela, sem que isto leve à penalização alguma ou qualquer prejuízo posterior a você ou a sua família. Os pesquisadores envolvidos no Projeto garantem a você o direito a qualquer pergunta e/ou esclarecimentos mais específicos dos procedimentos realizados e/ou interpretação dos resultados obtidos nos exames. Os dados obtidos serão utilizados somente para este estudo e serão armazenados por um período de 5 anos após o qual serão eliminados por meio de picotagem. Em caso de eventuais dúvidas sobre a pesquisa, você terá plena liberdade de entrar em contato com o pesquisador responsável, buscando maiores esclarecimento pelo telefone: (51) 3591-1122 ramais 3720 ou 3181 ou pelos seguintes correios eletrônicos: samuelmaraschin@hotmail.com ou prpereira@unisinos.br.

São Leopoldo,____ de _____ de _____.

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Nome do(a) Voluntário(a) Assinatura

_____ _____

Samuel Tavares da Silva Maraschin Paulo Ricardo da Silva Pereira