Low-cost electrochemical sensing platform for DNA analysis

M.Tech Thesis

Submitted in partial fulfillment of the requirements
for the degree of

Master of Technology
Solid State Devices (EE7)

by

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Thesis Approval

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Declaration

I declare that this written submission represents my ideas in my own words, and where other ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented, fabricated, or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will cause disciplinary action by the Institute or from whom proper permission has not been taken when needed.

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June 29, 2022

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Abstract

The ongoing COVID-19 pandemic has highlighted the need for sensitive and cost-effective diagnostics. The gold standard test to diagnose COVID-19 is real time PCR and although there have been multiple advances in PCR testing, the real time PCR thermocyclers are bulky and expensive. Given that PCR is one of the most sensitive and specific testing methods for diagnosis, it is necessary to find a solution to make it affordable especially for low middle income countries(LMICS). This led to the objective of this project to develop a low-cost, portable, and automated real time PCR device. This project includes WELPCR (portable thermocycler), and WELStat (potentiostat that can be connected to a smartphone for electrochemical measurements). The user has to add PCR master mix to pre-processed samples and place them in the holder on WELPCR. The temperature profile and other parameters can be visualized on the LCD display. An electroless nickel immersion (ENIG) PCB electrode is immersed in the PCR sample tubes to note the electrochemical measurements for real-time quantification.

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Chapter 1

Introduction

The need for accurate, sensitive and low cost diagnostics was sky rocketed during the COVID-19 pandemic. Although there are multiple other rapid technologies for diagnosing infectious diseases such as antigen-antibody based detection, or biochemical assays, they lack the sensitivity and specificity offered by the gold-standard RT-PCR tests. While performing molecular diagnosis it is not possible to detect only a few copies of genomic DNA or RNA. Polymerase chain reaction (PCR) amplifies millions to billion copies of target DNA quickly and accurately which can be studies in greater detail.PCR is a temperature controlled device that cycles between three different temperatures for short period of times. It is essential to maintain these temperatures accurately ensuring a faster transition time between these temperatures to have a faster overall turn around time of the reaction. To develop a PCR device it is essential to consider the need for accurate heating and temperature sensing requirements in such a way that the device is low-cost and not bulky.

There have been multiple recent advances in designing portable real time thermocyclers, for instance a group of researchers at the University of Central Florida, Orlando, USA, have developed a 3D- printed real time PCR device operated using battery for diagnosing infectious diseases [1]. The thermal processing and real time fluorescence based detection is controlled by a closed loop feedback and a microcontroller. Multiple open source PCR devices are available such as the Chai Open qPCR that has been used for water monitoring and detecting malarial parasites from blood [2][3]. Despite the advances and open source PCR designs available on the internet, the existing thermocyclers require exhaustive assembling of the design, have relatively lower ramp rates ($\leq 1^{\circ}C/s$) resulting in longer timescales to complete a reaction of approximately 35-40 cycles.

After the amplification of target DNA the detection and quantification in a

laboratory is usually done using agarose gel electrophoresis. Quantification using agarose gel electrophoresis is end-point detection and is not feasible for on-field or point of care detection. For real-time detection of amplified fragments either optical or electrochemical sensors are used.

In this work, we have tried to make a portable real time PCR device using electrochemical sensing method with minimal need of assembling. The Peltier heater gives a ramp rate of ($\leq 2°C/s$) resulting in a shorter turnaround time. For detection of the amplicons using electrochemical methods such as cyclic voltammetry we have designed a portable potentiostat, 'WELSTAT', that can be connected to a smartphone or laptop. The designed PCR device can be used an indispensable tool for multiple application such as forensics, isolation of genomic nucleic acid and testing its purity, detection and classification of organisms, genomic cloning, genotyping, detection of resistant genes, mutation screening, detection of single nucleotide polymorphism, population morphism, gene expression studies, genomic analysis and sequencing and so on.

1.1 Motivation

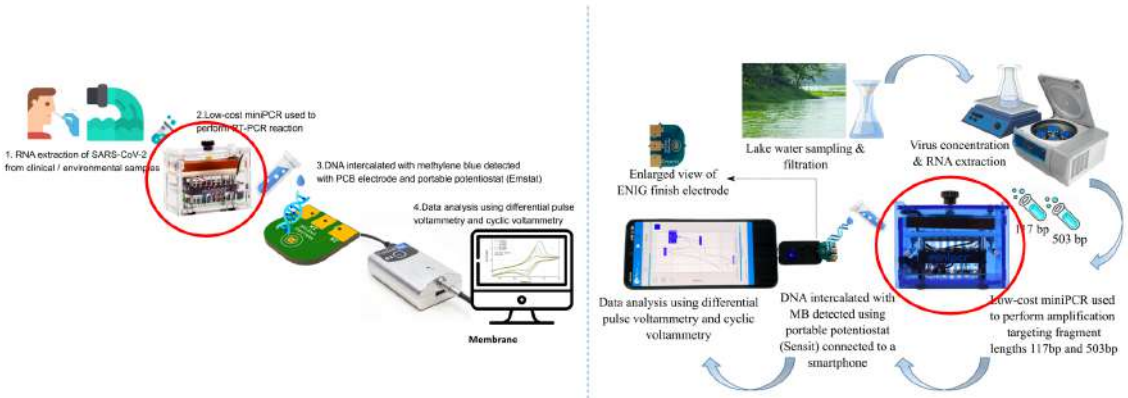


Figure 1.1: Work done in AIMS lab, EE, IITB

The group I am associated with at AIMS lab, EE, IITB have successfully detected SARS-CoV-2 [4] and bacteriophage Phi-6 [5] from wastewater samples using PCB based electrochemical sensing. While testing the samples, amplification was performed using the MiniPCR thermocycler. The device costs around $800-1000+shipping charges (imported from US). We faced several maintenance and

service issues on the PCR device while performing experiments. This led to the motivation of designing a PCR device that can be used for virus (or any pathogen) detection using electrochemical sensing.For electrochemical sensing they have used PalmSens potentiostat Sensit and EMSTAT Blue. Although the potentiostat is portable with a user-friendly software, we decided to develop a low-cost potentiostat that could serve our purpose of end-point and real-time PCR.

1.2 Objectives

- Develop a low-cost, portable, user-friendly PCR device
- Provisions for simultaneous testing of multiple samples (¿ 8 wells)
- Ramp rate (> 1.5°C/s) similar to commercially available PCR device
- Develop a low-cost Potentiostat to perform electrochemical measurements (Cyclic Voltammetry)

The two main aspects of this thesis include a PCR device (WELPCR) and a potentiostat (WELSTAT). The detailed specifications and design of these two devices are discussed in following two chapters.

Chapter 2

WELPCR

Figure 2.1: WELPCR

WELPCR is a PCR (Polymerase Chain Reaction) machine made by taking OpenPCR [6] as a reference. The main improvement made in WELPCR with respect to OpenPCR is that WELPCR is a standalone device and does not require external device like computer or mobile to configure it. Also it us designed for real-time electrochemical measurements.

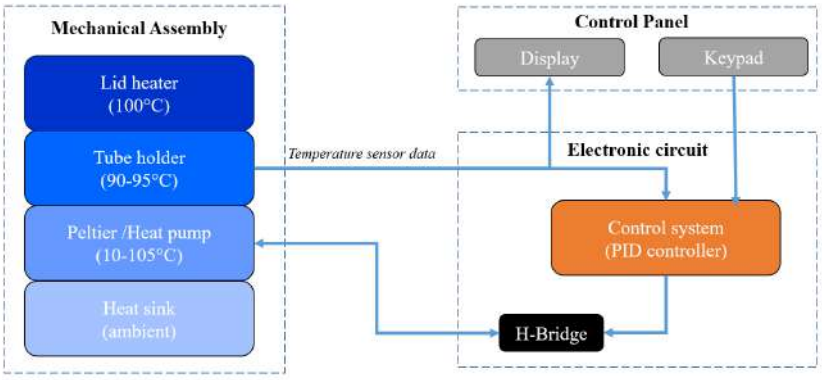


Figure 2.2: WELPCR block diagram

Figure 2.2 Main part in the mechanical assembly is the peltier. Peltier is basically a heat pump. If current flows in forward direction, it draws heat from heat sink and the tube holder is heated and if current flows in reverse direction then heat is drawn from the tube holder and tube holder is cooled down. Peltier requires bidirectional current so we are using H-bridge. To change and maintain the temperature to different set points PID controller is used. For user interface the control panel consists of display and keys.

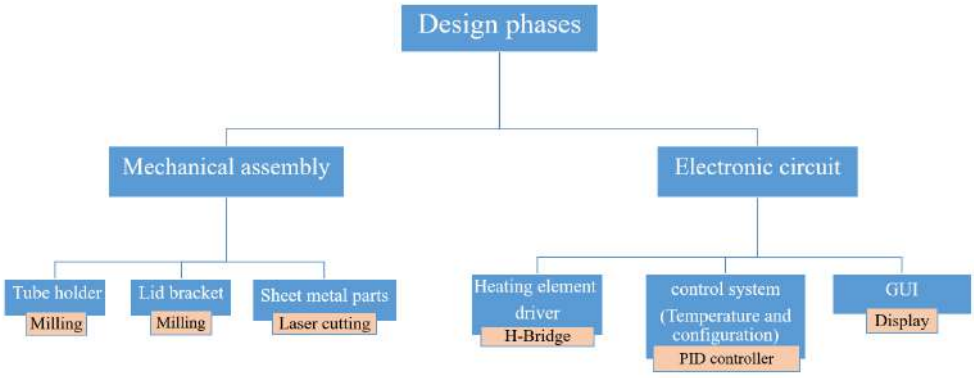


Figure 2.3: Design phases of WELPCR

The whole design of WELPCR was divided into 2 parts. The first one is Me-

chanical Assembly that was finished in Phase-I of this project and the second is Electronic circuit that was completed in this semester. The mechanical assembly includes manufacturing the tube holder, lid bracket and sheet metal parts. Tube holder and lid bracket are 3d parts which are manufactured using milling technique in our Machines and tools lab, Mechanical department, IITB. For 2 dimensional parts were manufacture from aluminium sheet metal. Metal laser cutting facility available at Bhandup was used for sheet metal cutting.
The electronic circuit includes heating element driver, control system for temperature and GUI. As peltier is driven by bi-directional current, H-bridge with driving capacity of 15A is used to drive it. H-Bridge is a is a circuit which allows to pass bi-directional current from the load. Circuit used for H-bridge is given at the top right of the figure B.6. H-bridge has two PMOS and two N-MOS which are IRF9540 and IRF540 respectively.

2.1 The Mechanical Structure of the PCR Machine

(a) (b)

Figure 2.4: (a) WELPCR designed in CAD tool Autodesk fusion 360 (b) Actual WELPCR after manufacturing

WELPCR has lot of 2D and 3D mechanical parts so it was necessary to design the full system in a Computer Aided Design (CAD) tool in which we can design electronic circuit as well as the mechanical parts. Eagle and Fusion 360 are such

CAD tool using which we can design electronic circuit and mechanical parts respectively.Before manufacturing the WELPCR, its model was designed in a CAD tool Autodesk Fusion 360 as shown in figure 2.4. While designing each and every part, care was taken about its Manufacturability, as milling technique has some limitations. Whole assembly is enclosed in a transparent acrylic casing. Acrylic casing is faces are usually fixed with a adhesive. But we need to disassemble the casing many times. So we use the L-bracket with nut-bolt to connect two right angular faces. For maintenance we may need to open one face of the casing. For that, front face is fixed using a hinged on one side. Front of the WELPCR can be opened easily for maintenance purpose.

Figure 2.5 represents the cross-section view of the PCR machine. The CPU cooler is not shown fully.

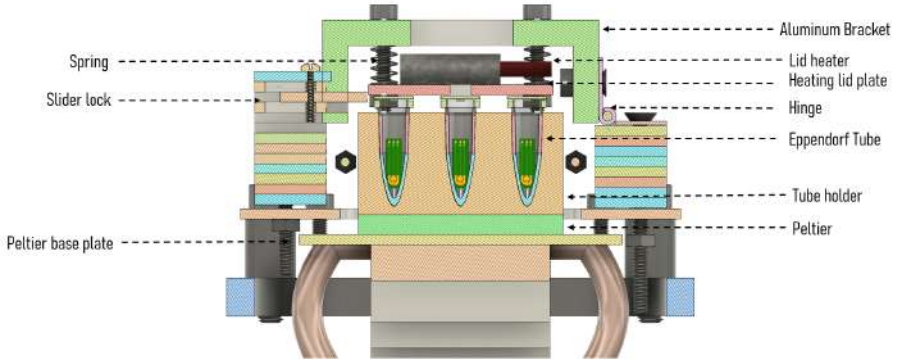


Figure 2.5: Cross section view of PCR

Following is the description for parts in the above image

- **Tube holder**: The tube holder is the main part of this PCR. 3 Eppendorf tubes can be inserted into this block. This block is heated by Peltier.
- **Peltier**: A Peltier is a heat pump that can pump heat from one side to the other side depending on the direction of current flow. We can heat and cool the same side just by inverting the supply connections. So both rising and lowering the temperature can be controlled precisely.
- **Lid heater**: Lid heater is a ceramic 12v 40W ceramic cartridge heater, commonly used in 3d printers.
- **Heating lid plate**: The heating lid plate is heated by a lid heater. This plate is used to heat the lids of tubes to avoid condensation of liquid sample vapour underneath the tube cap.

- **Aluminium Bracket**: is a solid square bracket '[' shaped lid. This is the lid that presses the heating lid plate on the lids of the tube. Tension on the heating lid is maintained due to the spring between the bracket and the heating plate.
- **CPU cooler**: There is no CPU in this PCR machine, but this big heat sink with a cooling fan is a part of the desktop CPU used for cooling the microprocessor. This CPU cooler is used to sink(while decreasing the temperature) and source(while increasing the temperature) the heat.
- **Peltier base plate**: Peltier base plate is used as an interface between the CPU cooler and the Peltier. The shape and area of the Peltier and the CPU cooler are not the same so to collect heat from the whole area of the Peltier and to pass that heat to the CPU cooler, this Peltier base plate is used.
- **eppendorf tube**: These are tubes made for preparing, mixing, centrifuging, storing, and transporting solid and liquid reagents and samples. We use 0.2ml eppendorf tubes.

Manufacturing the the 3D parts

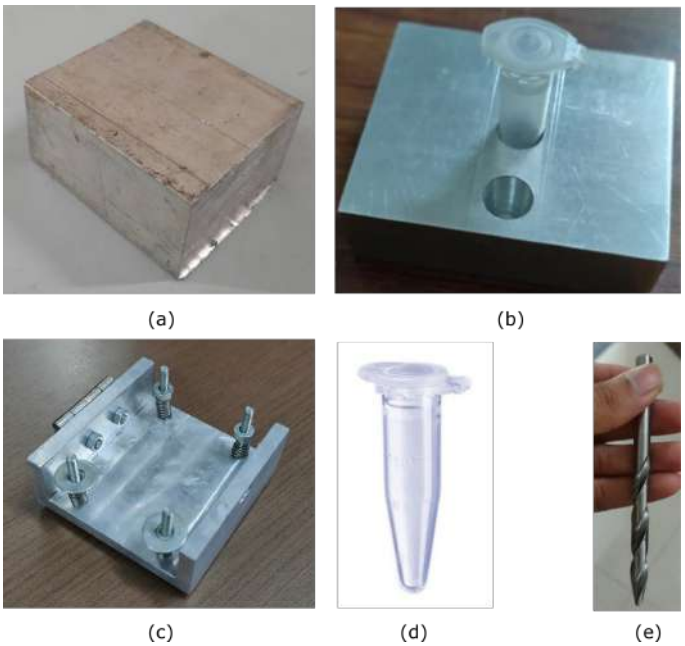


Figure 2.6: (a) Raw Aluminium block (b) Tube holder after manufacturing (c) bracket after manufacturing (d) Eppendorf tube (e) modified drill bit

After designing parts in CAD software fusion 360, we manufactured these parts using milling technique and laser cutting. Milling is machining technique in which we remove the material from workpiece using rotary cutter. 3D parts were manufactured using milling in machine and tools lab, IITB. There are two 3d metal parts in our design, that are tube holder and bracket. Aluminium metal is used for both, as it is light weight and requires less efforts in milling.

Above figure B.1 shows the raw aluminium block (figure B.1(a)), tube holder (figure B.1(b)) and bracket (figure B.1(c)). Actual thermal cycling is performed at the bottom of the tube. So bottom of the tube must in good thermal contact with the tube holder. Eppendort tube shown in figure B.1(d) has a conical shape bottom. The main challenge while manufacturing the tube holder was to make a conical hole for the tube. Because when we make a hole using drill bit it makes a cylindrical hole but we need a conical hole. Outer profile of the drill bit was modified such that it matches with the outer profile of the tube as shown in figure B.1(e)

Manufacturing the the sheet metal parts

There were some 2 dimensional parts in WELPCR design like a heated lid plate, a plate between peltier and heat sink . These parts were manufactured using the metal laser cutting machine.

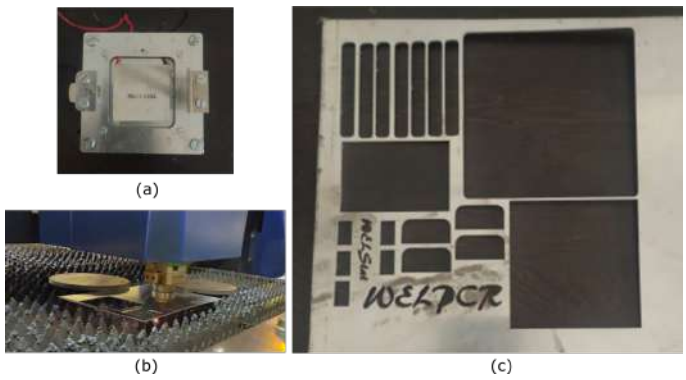


Figure 2.7: (a) 2D parts in WELPCR (b) 2mm aluminium sheet under laser cutting machine (c) Aluminium sheet after laser cutting

2.2 PCR Cycle

PCR or Polymerase Chain Reaction is a temperature controlled device. PCR cycles through different temperatures for different time. The first step is denaturation which requires 90-95°C [7] . It causes the denaturation i.e DNA is broken into double-stranded DNA template as shown in figure 2.8. Second step is annealing which requires 50-60°C. In this step primers is annealed to each of the single-stranded DNA templates. Third step is extension which requires 72°C. In this step,new DNA strand complementary to the DNA template strand is synthesized. Each cycle doubles the number of DNAs .This cycle is repeated for n number of times. so it multiplies the no. of DNA by 2 raised to n. We need to design a system which cycles through these three steps for n number of times.

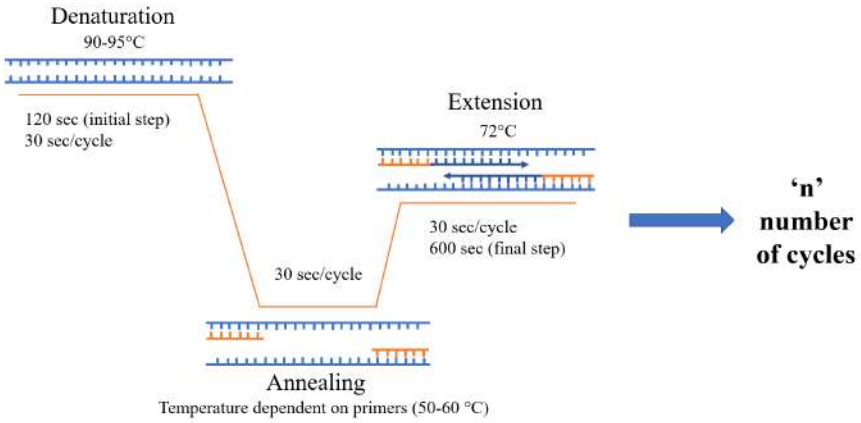


Figure 2.8: PCR temperature profile for single cycle

Figure 2.9 represents the temperature profile for a single cycle of PCR. The blue curve shows the temperature profile of the tube holder, which is directly attached to the Peltier. An orange curve shows the temperature profile of the sample inside the test tube. The sample temperature is always less than the holder temperature by 1-2 degrees because the sensor measuring the temperature of the holder is closer to the heater. Also, there is heat loss at the interface of the tube and the holder. Heat loss across this interface is minimized by using thermal conducting paste.

To measure the temperature, we used a 1 mm 4.7K thermistor. The temperature sensor needs to be as small as possible because the sample is 20ul which fills the test tube just by 2-3 mm, so a big thermistor adds a thermal load and results in heat loss as well as wrong temperature readings because the big thermistor does not dip fully into the sample.

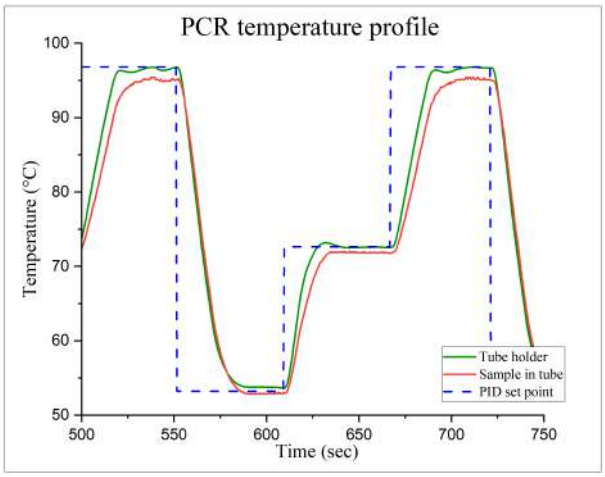


Figure 2.9: Actual temperature profile of WELPCR

2.3 PID controller

In our design, we used Peltier for heating as well as cooling the tube holder. A PID controller is designed to raise the temperature of the tube holder as fast as possible. As PCR is temperature sensitive and primers may be damaged because of overshoot in the temperature, we need a critically damped temperature control system. To achieve this critically damped system, we used the try and error method. Following equation is used for PID

$$\text{PWM duty cycle} = \mathrm{k}_p \times e(t) + k_d \times \frac{\partial e(t)}{\partial t} + k_i \times \int e(t)dt + \beta$$

where :
$k_p = Proportional\ gain$
$k_d = Derivative\ gain$
$k_i = Integral\ gain$
$e(t) = Error\ (difference\ between\ set\ point\ and\ current\ temperature)$
$\beta = bias\ depend\ on\ the\ temperature$

What is β in PID equation?

To maintain the constant temperature peltier require some constant current bias. This required current bias may change from its calculated value due to the surrounding temperature. For example, We want to set tube holder temperature at 72°C. consider at 50% duty cycle tube holder temperature remain constant at 72°C, then β will be equal to 500 for 72°C. For 50% , β is 500 because we put 1000 in timer countdown register in micro-controller. value of β is directly proportion to

the temperature. equation of β is linear for the range of nearly 10°C. so equation of β is different for 90±5°C, 72±5°C and 56±5°C. Following are the equations for different temperature ranges of PCR cycle:

$$\beta = 8.2 \times temperature - 300 \quad for\ 51^oC < temperature < 61^oC$$
$$\beta = 8.5 \times temperature - 292 \quad for\ 67^oC < temperature < 77^oC$$
$$\beta = 9.2 \times temperature - 345 \quad for\ 88^oC < temperature < 98^oC$$

If peltier is driven by only βwithout PID controller, there will some constant error in steady stead as the surrounding temperature changes. for example, if surrounding temperature is lower then PWM duty cycle needs to be higher and if surrounding temperature is higher, PWM duty cycle needs to be lower. Three terms of PID takes care of this change in surrounding temperature. Proportional, integral and derivative terms of the PID get added to or substracted from the βdepending on the sign of error.
Now next task is to tune the PID controller. That is to find the k_p, k_i and k_d to get the critically damped system.

Trial and error method to tune the PID

- Increase the k_p till we get constant oscillations in the steady state.
- Increase the k_d till the oscillation amplitude is within the acceptable range.
- Increase the k_i till you get a steady state error in the acceptable range.

Figure 2.10 shows the temperature vs time plot for different PID constants. We can see from the graph Figure 2.10(a) that for $k_p = 10$ we get the oscillations in the steady state. As k_d and k_i are zero, this is only a proportional controller. To suppress these oscillations, we need to increase the k_d. We directly used $k_d = 70$ but it did not have any effect on the oscillations. So we tried $k_d = 70$ and it was increasing the oscillations. So we decreased the k_d till 35 and we got the curve as shown in the above graph (green, $k_p = 10$, $k_d = 35$). But still, there is a problem with the PD controller. In a steady state, we can see there is a constant drift in the temperature. To suppress this constant drift, we need to add an integral controller. We get the following curves after adding the integral controller.

Figure 2.10(b) shows the curves with the integral (blue) and without the integral (red) controller. For the PID controller (blue), we can see that temperature is constant in the steady state and steady state error is also within the acceptable

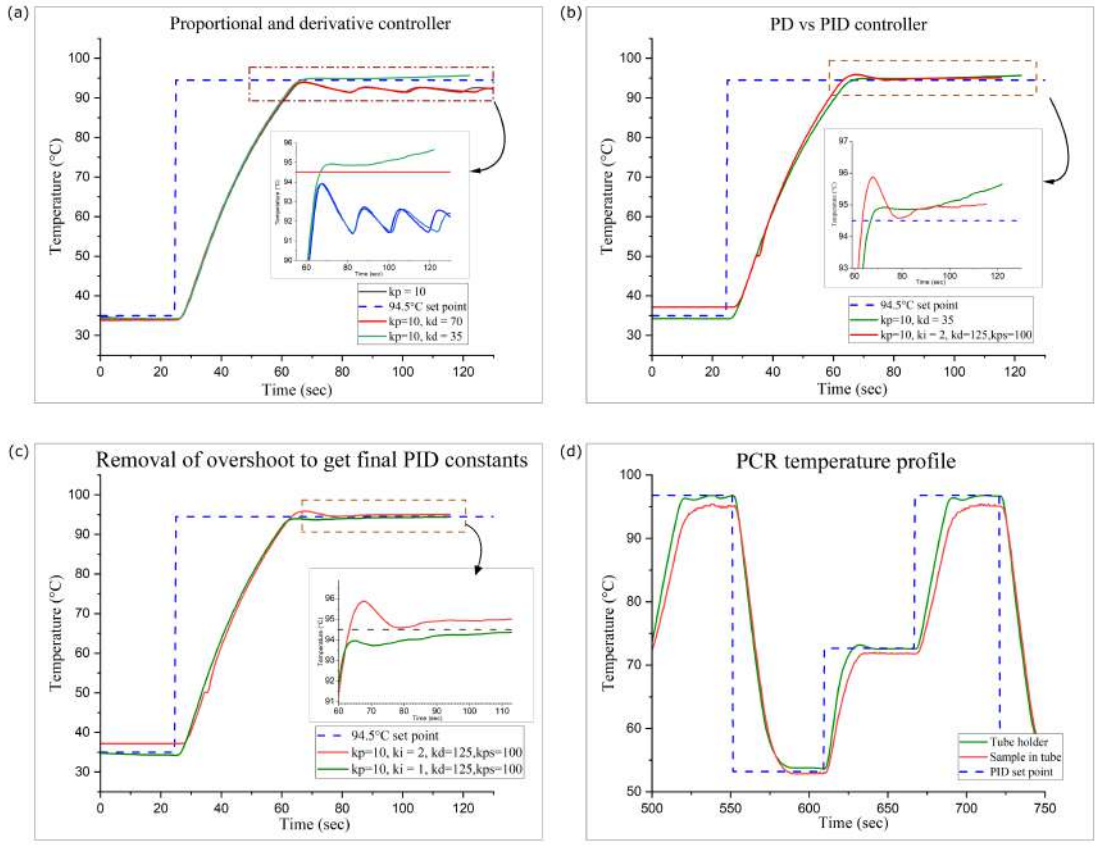


Figure 2.10: (a) Proportional and controller derivative, (b) PD vs PID controller, (c) Removal of overshoot to get final PID controls, (d) PCR temperature profile after PID tuning

range. Still, there is a problem here, and that is the overshoot. This overshoot can damage the chemicals in the test tube, which are temperature sensitive. Overshoot can be decreased by decreasing the integral gain. The following graph shows the effect of decreasing the integral gain.

Figure 2.10(c) shows the response with overshoot with $k_i = 2$ (red) and the critically damped response with $k_i = 1$. We can see there is no overshoot in the critically damped response (blue). Decreasing the k_i solved the problem of overshooting. This is how we got our required PID controller response.

The calculated PID constants are valid only for the temperature around 93±5°C. That is, PID constants change if there is a change in set point. The same method of PID tuning is used to tune the PID controller for the other two set points, which are 55±5°C and 72±5°C. The following plot shows the final PCR cycle.

2.4 Challenges faced during designing

Challenges	Solution	Remark
Poor heat transfer between tube holder and sample	Insulation of tube holder Additional nichrome coil heater along with Peltier	Failed Failed
Condensation on side walls of tube	Tube holder designed for smaller tubes covering entire tube sidewalls	Successful (Solved poor heat transfer problem too)
Slow time response of the temperature sensor	Use of small 1mm thermistor	Successful
Noise in temperature sensor	Connecting full mechanical assembly to ground of the circuit	Successful
Micro-controller out of stock due to chip shortage	De-soldered microcontroller from old board	Successful
Accurate temperature sensor calibrations	Calculated different equations for each temperature sensor	Successful
Time required for self heating of temperature sensor	Use of smaller 1mm sensor	Successful
Size of temperature sensor larger than sample size	Use of smaller 1mm sensor	Successful
SI2302 MOSFET damage exceeding P_{max} of MOSFET	Use of ON/OFF switch instead of PID controller which operates MOSFET in cutoff or linear region	Successful
Damage of power supply due to short circuit	Use of fuse	Successful
Spike in output voltage of supply damaging the controller	Use of Transient Voltage Suppression (TVS) diode	Successful

Table 2.1: List of challenges faced during designing

Poor heat transfer between tube holder and sample

Initially we were using 5ml tube for testing. As side walls of the 5ml tubes are thicker than the 0.2ml tube, it was oberverve that there was difference of 30-40°C

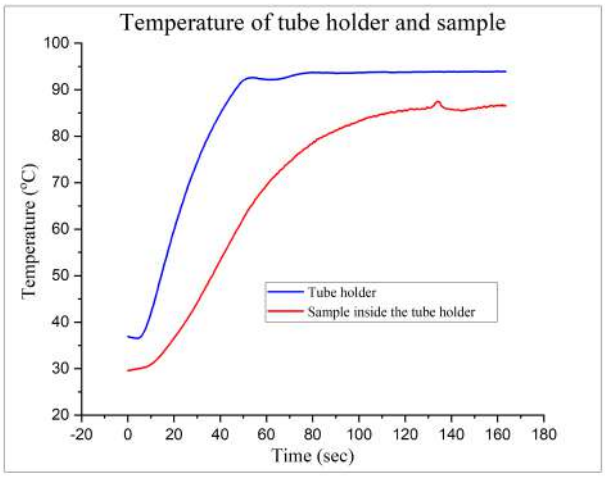


Figure 2.11: Plot showing temperature difference between tube holder and sample

between tube holder and sample temperature as shown in figure 2.11. to solve this problem we first put same thermal paste at the interface of tube holder and tube, but it did not solved the problem.

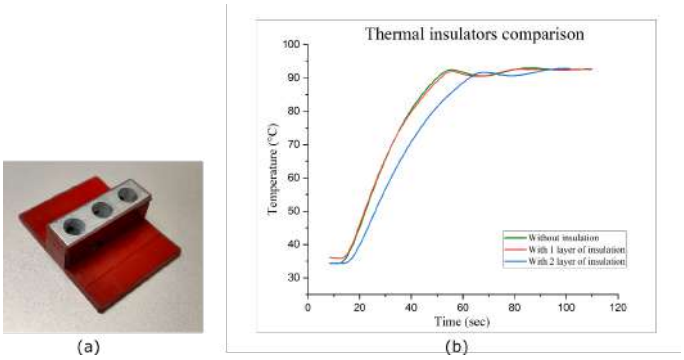


Figure 2.12: (a) Insulation layer (VHB tape) applied on the tube holder (b) Plot showing curves for different no. layers of insulation

We though that there might be heat loss from outer surface of the tube holder which is causing the poor heat transfer. So we applied the insulator on outer surface of the tube holder. For insulation VHB tape was applied on the outer surface as shown in the figure 2.12 (a). But from the figure 2.12 (b), we can observe, as no. of layers of insulation increases, ramp rate decreases. So, though insulation is preventing the heat loss in steady state, it was decreasing the ramp rate in transition state. So this solution failed.

figure 2.12 (b) shows there is huge gap between tube holder and sample temperature. So we decided to raise the temperature of the holder such that we get sample temperature of set point. For example, if we want sample temperature to be 95°C, then increase the temperature to 110-120°C. But the maximum temperature that can be achieved using the peltier is 105°. Also ramp rate is decreases after 80°C so it takes nearly 1.5 minute to reach temperature at 105°C. To solve this problem, we decided to add one more heater. As only assistance for heating was required we decided to use Nichrome coil for heater. Following figure

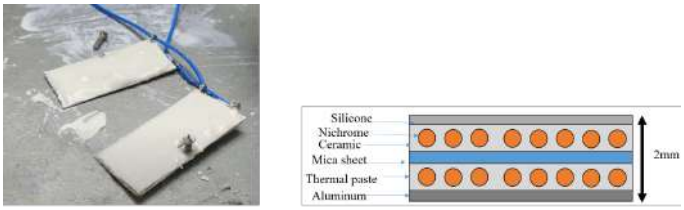


Figure 2.13: (a) Nichrome heater (b) Internal structure of Nichrome heater

Condensation

When vapours of liquid find a surface with lower temperature it get settled on that surface in its liquid form. Initially we used 5ml tubes and designed a tube holder for it which was covering only bottom of the tube. Side walls were not covered with by the holder as shown in the Figure 2.14(a). So though temperature of bottom of the tube was near 95°C, side walls were not reaching to such high temperature. It results in the condensation of the sample on side walls of tubes. This condansation would not have been affected the test if sample quantity was large but sample we put in the tube is just 20µL, which fills just 3mm of tube from bottom. So due to condensation entire 20µL sample leaves the bottom of the tube where actual thermal cycling is performed. The heated lid prevents condensation under the lid of the tube. Sample droplets, which are condensed on the side wall, do not undergo the PCR reaction. As our sample under the test is just 20µL, condensation will affect the PCR process.

To solve this problem, we replaced the 0.5ml test tube with a 0.2ml test tube and redesigned the test tube holder to cover the full sidewall of the test tube, as shown Figure 2.14 (b).

Figure 2.14: (a) Condensation observed in 0.5ml tubes, (b) Tubeholder redesigned for0.2ml tubes to solve the condensation challenge

Time response of the temperature sensor

Selecting the temperature sensor with faster time response was one of the challange in this project. Initially we tried LM35 as it has linear output and it is already calibrated. But problem with LM35 was it was taking nearly 1 minute to attend high temperature 93°C. It might be due to its our packaging which was causing slower heat transfer to the actual sensor inside the package. So we were in search of faster temperature sensor which can have time response such at least 2°C/sec.

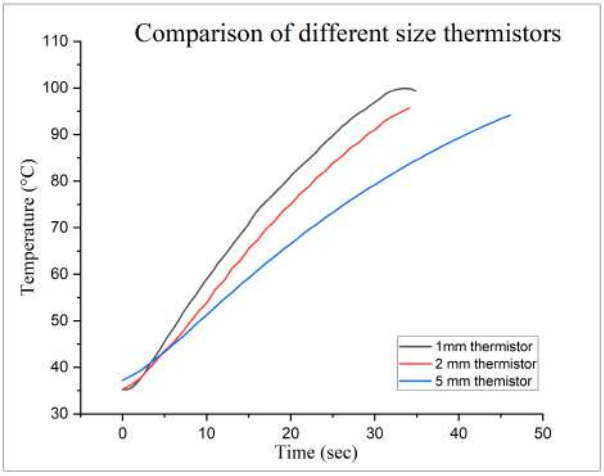


Figure 2.15: Comparison of time response for 1mm, 2mm and 5mm thermistor

After discarding LM35, we decided to test the thermistor for our application. Learned from the previous mistake this time we plotted time response of different size thermistor. We can see from the above figure 2.15,as the size of thermistor decreases, time response of the thermistor improves. We decided to use 1mm thermistor as its time response was better than 2mm and 5mm thermistors. Also we need to measure the temperature of liquid sample inside the tube. Amount of sam-

ple that we use for the PCR is just 20L and it fills just 3mm of the tube from the bottom. so using 2mm or 5mm thermistor add more thermal load in addition to the actual sample. So sensor itself takes time for self heating. Also if sensor is not fully dipped into the sample, it give 3-10°C less reading than the actual. To prevent such problems 1mm themistor was the best choice among other sensor.

The temperature sensor and its calibration

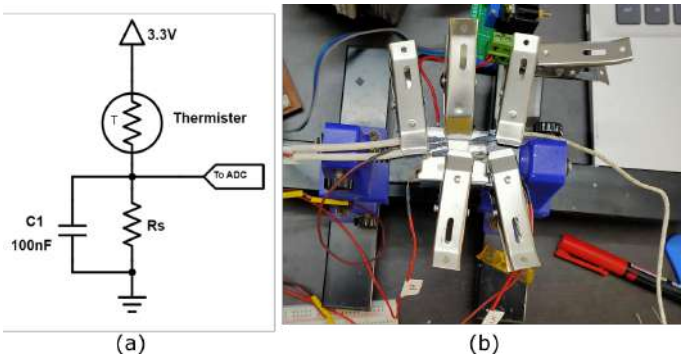


Figure 2.16: (a) Thermistor connected in voltage divider, (b)Set-up for calibrating the thermistor

Figure 2.16 shows the set-up for calibrating the thermistor. Initially, we were using 10k Ohm thermistor. In circuit diagram **??**(a) value of Rs is geometric mean of the resistance values of thermistor for the temperature range and capacitor C1 is 100nF to for noise filtering [8]. For calculating the temperature from the resistance, we used βmodel equation of the thermistor show in the following equation:

$R = R_0(e^{\beta(\frac{1}{T}-\frac{1}{298.15}))})$

βvalue of the thermistor was taken from the datasheet.After some testing, we found that every thermistor has some deviation from its standard β value given in the datasheet. This deviation of the β value results in a deviation of +-3 degrees of temperature, which is very high as PCR is a temperature-sensitive reaction.

To solve this problem, we decided to treat every thermistor differently to get a temperature value from it. In firmware, the thermistor library is updated such that it takes a β value of the thermistor when we initialize the structure for a thermistor. To calculate the β value we made a small heater using a nichrome coil. To control the temperature, the current was controlled using the transistor. We took resistance readings for different temperatures. A temperature reading was

taken using a standard thermocouple thermometer and a resistance reading was taken using a multimeter. The fixture and setup are shown in the Figure 2.17

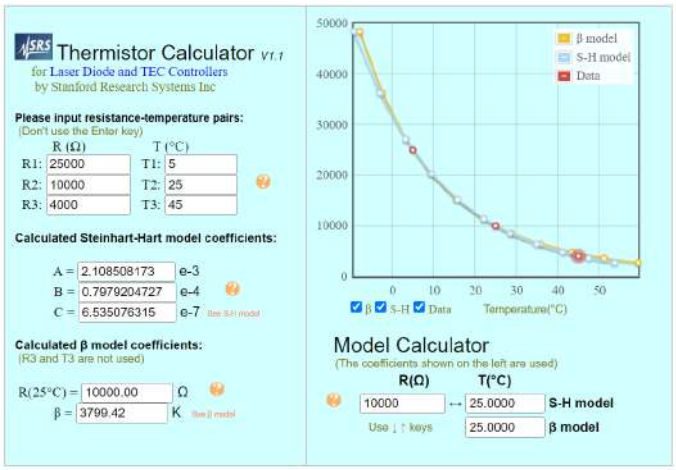


Figure 2.17: 'Thermistor Calculator' by Stanford Research System, Inc

After taking readings of resistance for different temperatures, β value was calculated using a 'Thermistor Calculator' by Stanford Research System, Inc [9] shown in figure 2.17.

2.5 WELPCR Results

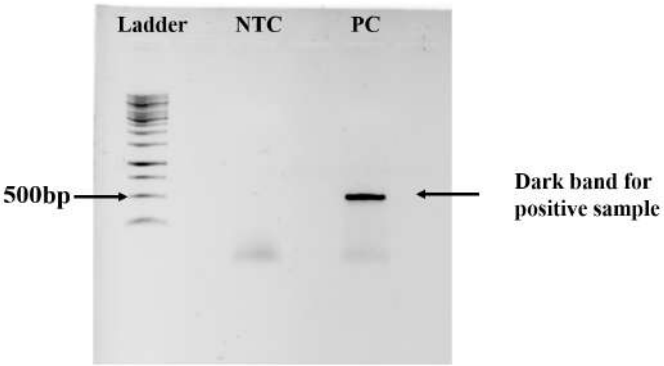


Figure 2.18: Agarose gel showing 503bp fragment of bacteriophage Phi6 after 35 cycles amplified using WELPCR. NTC is the negative sample and PC is the positive sample

After completing the PCR assembly, we needed to check whether it amplifies RNA or not. For that, we ran 35 cycles of PCR for a 503bp fragment of Phi6 bacteriophage. The Phi6 bacteriophage is a commonly used surrogate for SARS-CoV-2,

which is non-infectious and has a lipid envelope similar as the virus responsible for Covid-19. After running PCR for 35 cycles, amplification was verified using agarose gel electrophoresis . As shown in figure 2.18, we can see the dark band for the positive sample (sample with the virus) and no band for the negative sample (sample without virus). The intensity of band is proportional to the amount of the amplified DNA.

2.5.1 Real-time sensing using EMStat Potentiostat

Real-time PCR refers to the detection of amplicons in real-time, i.e, as they are amplifying during the PCR reaction. This saves time and cost of power requirements to run a specific number of cycle depending on the pathogen. We use WELPCR for amplification and dipped in unmodified PCR electrodes in the reaction tubes for real-time sensing using PalmSens EMStat Potentiostat. The DNA intercalating redox dye was added along with PCR master mix reagents before starting the reaction and not at the end of the reaction with an additional one hour of incubtion. As seen in Figure 2.19, there is no change in the peak current for the No-template control (NTC) with increase in number of PCR cycles. Whereas, for the positive control we sea clear decline in the peak current with increase in the number of PCR cycles. It can be clearly seen that the positive control can be distinguished from the negative control at the 12th PCR cycle. This result concludes that WELPCR can be combined with an electrochemical sensor in order to achieve

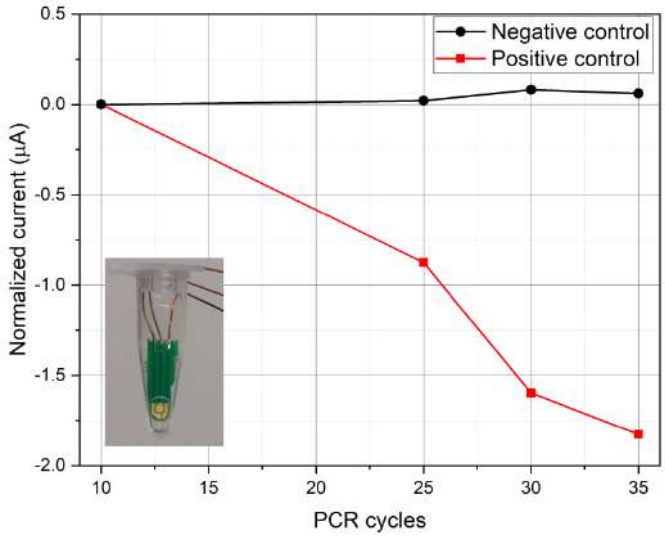


Figure 2.19: Current vs number of cycles plot showing decrease in current with increase in cycles. Inset showing micro-electrode inserted in the tube

2.6 WELPCR Comparison

WELPCR is inspired from the openPCR [6]. We improved lot of features in WELPCR. WELPCR has a higher ramp rate almost double of openPCR. Reverse transcription can be performed using WELPCR, and other devices except MiniPCR.Currently we have manufactured a sample holder for 9 tubes that can be customized later to have more number of wells. User interface (read as given in the boxes). Our device weighs 1.9 kg which can be reduced, that is discussed in future improvements. The Bill of material price of our device is less than 10000. Following table shows comparison of WELPCR with openPCR and MiniPCR

	WELPCR	**OPENPCR**	**MiniPCR mini8**
Max Ramp rate	2 °C/s	1 °C/s	2.4 °C/s
Reverse transcription	Yes	No	No
Wells	9		8
Temperature range	10-105°C	20-105°C	Ambient-99°C
User Interface	Keys+Touch screen Display	Computer Software	Mobile App
Weight	1.9kg		0.45kg
Heated lid	Yes	Yes	Yes
Cost	Rs. 10,000 (BOM)	Rs. 38,500 (BOM)	Rs. 75,000 + shipping from USA and taxes

Table 2.2: Specification of WELPCR and Comparison with OpenPCR and MiniPCR

Chapter 3

WELStat 2.0

In last chapter, we have seen details about the WELPCR. WELPCR is used for DNA amplification i.e making copies of DNA. Sample under the test don't have the amount of DNA that is detectable by the sensor. After amplification using PCR our next task is to detect the DNA. This detection can be done by electrochemical method using WELStat. WELStat is a potentiostat designed in the Wadhawani Electronics Lab (WEL). Now lets see what is a potentiostat?

3.1 What is a Potentiostat?

Potentiostat is a essential tool in electrochemical research used for applying the potentiostat and measuring the current. It can do so while keeping the path where the current flows separate from the path where the potential is measured. Now lets see why do we need to separate the voltage and current measurement loop.
Consider we want to plot a current vs voltage plot for a electrolyte. We take that electrolyte in a beaker and dipped two electrodes. To change the voltage between two electrode a variable voltage supply is connected. To measure the current we have connected the ammeter. After connected all of these components our setup will look like as shown in the case-I of figure 3.1. We are interested in measuring the voltage of one electrode only that is called the working electrode and another electrode is called the counter electrode.

Consider case-I, at t=0, potential drop at both the electrode is same i.e $V_1/2$. Now in case-II, after passing some time, the rate of reaction on both the electrode are different. So potential on both electrode changes with times. So the voltage of working electrode measured by the voltmeter will not be correct as it is measured

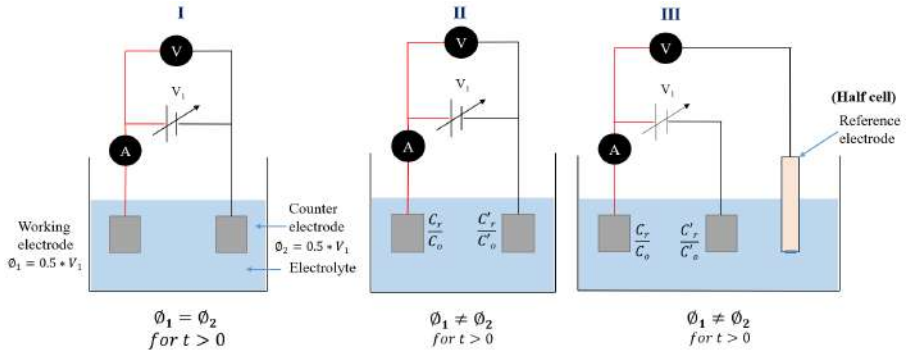


Figure 3.1: Working of the Potentiostat, case-I, case-II, and case-III

with respect to varying reference that is counter electrode. To measure the actual voltage of the working, it must be measured with respect to some constant voltage. So in case-III, we introduced third electrode i.e reference electrode. Reference electrode is a half cell whose potential remain constant irrespective of concentration of the electrolyte or time. In case-III, ground of the voltmeter is connected to the reference. So we are measuring the current passing from loop working electrode - counter electrode - $V_1/2$ and voltage of working electrode is measured with respect to reference electrode. In this way, the loop we are measuring the current is separated from loop in which the voltage is measured.

3.2 WELStat

WELStat 2.0 shown if Figure 3.2 is an improved version of WELStat 1.0. WELStat 1.0 was made with discrete electronic components such as Opamp, ADC, DAC etc. So the previous version required a large PCB which was creating concerns about its portability and power too. In this version i.e. WELStat 2.0, we used Analog Front End (AFE) IC AD5941 IC which has two potentiostats inbuilt and there is no need for any external peripherals except some capacitors and resistors.

3.3 Specifications

Specifications of the WELStat are listed in following table.

(a) (b)

Figure 3.2: (a) WELSTAT designed in Computer Aided Design tool Autodesk Fusion 360, (b) WELSTAT after manufacturing

Parameter	
Excitation voltage range	-1.2 to +2 V
Max current	±3mA
ADC	16 bit
DAC	12bit
No. of Potentiostat	2
No. of channels	1

Table 3.1: Specifications of WELStat

AD5941	EMStat Pico
Price = 700	Price = 45,000
Needs an external micro-controller	Micro-controller + Potentiostat
Not pre-programmed	Pre-programmed
Programing using embedded-C language	human-readable scripting language for ease of programming

Table 3.2: Differences between AD5941 and EMStat Pico

Choosing Analog Front End IC (AFE)

We wanted to make our device as compact as possible. So instead of building potentiostat using discrete components, we decided to use the potentiostat IC that is Analog Front End (AFE) IC. There are multiple potentiostat ICs available. Two of these are AD5941 and EMStat.Both are manufactured by analog devices. Table 3.2 and table 3.1 differences and similarities respectively in AD5941 and EM-

Stat Pico respectively. Both have similar specifications but their price difference is huge. AD5941 costs around Rs.700 and EMStat pico for Rs.45000. Although both EMSTAT Pico and AD5941 have almost similar specifications, the high-cost of EMSTAT Pico is mainly due to the well designed user-friendly software and accurately calibrated potentiostat.so we wanted to try if AD5941 can be used for our application.

AD5941

The AD5941[10] are high precision, low power Analog Front End (AFEs) designed for portable applications that require high precision, electrochemical-based measurement techniques, such as amperometric, voltammetric, or impedance measurements. This AFE has a sequencer that is specifically designed for offloading the AFE operations from external micro-controllers. It can be configured and data can be read from it using SPI protocol.

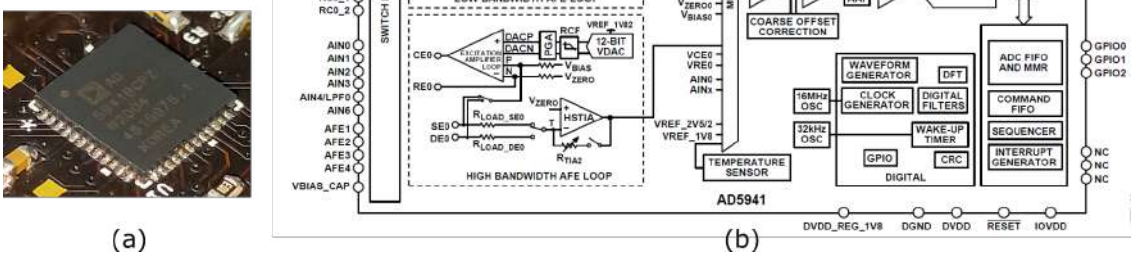


Figure 3.3: (a) AD5941, (b) functional block diagram of AD5941 (taken from its datasheet

Figure 3.3 shows the overall block functional block diagram of AD5941. There are two types of potentiostat in the block diagram, indicated by 'LOW BANDWIDTH AFE LOOP' and 'HIGH BANDWIDTH AFE LOOP'. In most of our applications, we are going to use 'LOW BANDWIDTH AFE LOOP' which is also called Low Power (LP) potentiostat. It can measure the current from 50 pA to 3mA. For excitation, it has a 12-bit Digital to Analog Converter (DAC). For measuring current, the current is converted to voltage using the Trans-Impedance Amplifier (TIA). This output voltage of TIA is measured by Analog to Digital Converter(ADC). This

measured voltage is proportional to the input current to TIA. The proportionality constant is decided by the gain of the TIA which is programmable. This ADC data can be read by the micro-controller instantly or this data can be stored in the inbuilt FIFO and then read once the applied excitation wave is completed.

Testing AD5941

To test whether AFE AD5491 is suitable for our application or not, we tested it using an off the shelf evaluation board as shown in Figure 3.4 (a). After achieving fair results (Figure 3.4(b))on the evaluation board of AD5941, we decided to use it for our application.

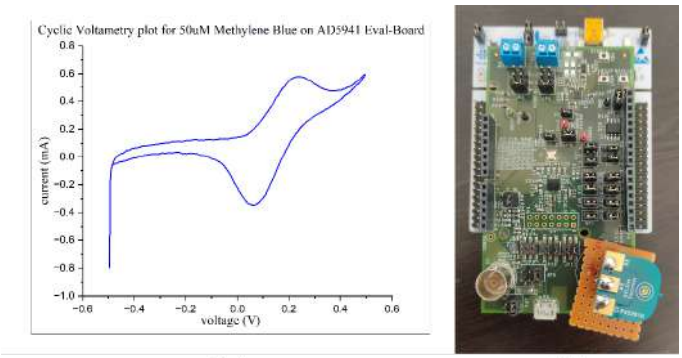


Figure 3.4: (a)Testing AD5941 on evaluation board, (b) Cyclic Voltammetry plot for 50µM Methylene blue on AD5941 Evaluation-board

STM32 Micro-controller

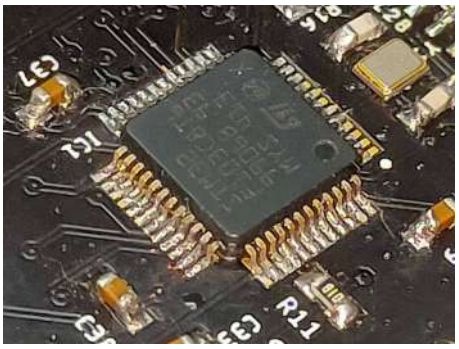


Figure 3.5: STM32F103C8PC IC

As shown in Figure 3.5 STM32F103C8T6 [11] is used as a host micro-controller.

For configuring AFE, requirements from the host micro-controller are: it should have SPI, UART communication protocols and at least 64kb of flash memory.

3.4 First PCB

Figure 3.6: First PCB for testing minimal circuit for AD5941

Figure 3.6 represents a red board mounted on the whiteboard. The whiteboard is a Nucleo board. The red board is designed for the minimal circuit of the AFE IC. So this version of the AFE PCB, i.e. red PCB, has only the AFE IC on it. The micro-controller is on a Nucleo board. This board was designed just to test the minimal circuit requirement for AFE IC. This circuit was tested using a 30 mM ferrocyanide solution and then compared with commercially available potentiostat. Figure... represents the CV plot for that. We got a similar shape but there is some shifting x as well as the y axis. We are still working on finding the reason behind it.

3.5 Second PCB

As seen in the first PCB, there was only AFE IC. While making the second PCB we planned to make a single PCB for AFE as well as micro-controller STM32. So this second circuit is nothing but a combination of the first PCB and a Nucleo board.

As seen in Figure 3.7, the left male USB port is connected to the USB pins of the STM32 micro-controller. The right side female USB port is for the USB electrode. A three-pin header near the male USB port with the jumper is used to select the 5v supply source, i.e whether to take 5V from the male USB port or the UART to USB converted port (golden colour thermal tape). Four pin header in the above PCB is used by the ST-Link programmer to program the micro-controller

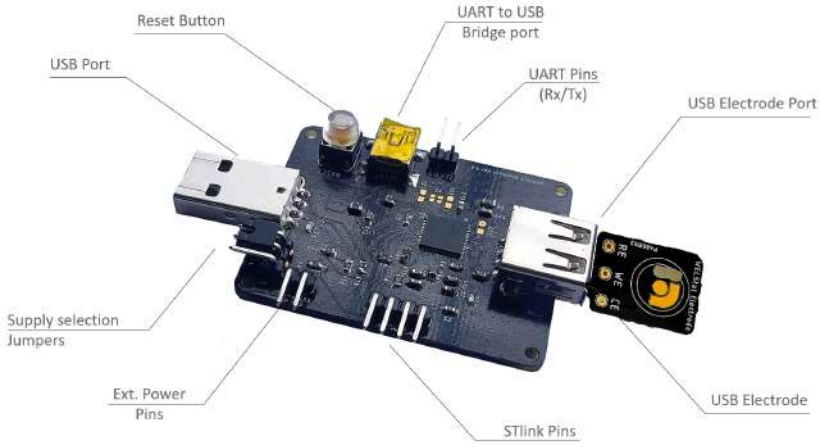


Figure 3.7: Second PCB with both AFE and micro-controller on same board

STM32 which is mounted on the bottom side of the PCB. Figure... represents good cathodic and anodic peaks with respect to commercially available PalmSens Sensit Smart potentiostat.

3.6 WELStat Software

3.6.1 GUI: Overview

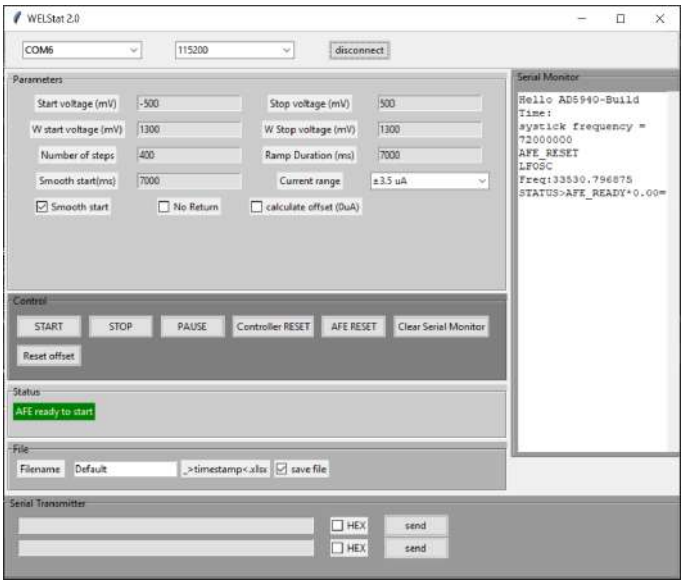


Figure 3.8: WELStat software GUI

WELStat Software figure 3.8 is a program used for configuring WELStat and then plotting the graph of the data collected from the WELStat via UART. This GUI is made using python language.

It stores the data in .xlsx format i.e. excel file. Once it has all the data points, it automatically plots the data. Also, it has the feature to plot multiple graphs in a single plot. Following are some of the shortcuts.

- ctrl+f : select spreadsheet
- ctrl+a : plot graph
- ctrl+x : clear
- ctrl+s : save all graphs in on spreadsheet

3.6.2 DATA String formats

From Software to the controller : datatype>parameter*value=

datatype [10]	**Parameter [15]**	**Default value [10]**
COM (command)	RAMP_START	0
COM (command)	RAMP_STOPNOW	0
COM (command)	RAMP_STOPSYNC	0
COM (command)	RAMP_SHUTDOWN	0
COM (command)	RESET(AFE reset)	0
COM (command)	RESET(AFE reset)	0
COM (command)	CONTROLLERRESET	0
CHG (parameter change)	RampStartVolt	±1300
CHG (parameter change)	RampPeakVolt	±1300
CHG (parameter change)	bRampOneDir	0 or 1
CHG (parameter change)	VzeroStart	1300
CHG (parameter change)	VzeroPeak	1300
CHG (parameter change)	StepNumber	400
CHG (parameter change)	RampDuration	7+
CHG (parameter change)	Smoothstart	1
CHG (parameter change)	LPTIARtiaSel	0-26

Table 3.3: Data string format from software to controller

- COM>RAMPSTART*0=
- COM>RAMPSTOPSYNC*0=
- COM>RAMPSTOPNOW*0=
- COM>RAMPSHUTDOWN*0=
- COM>RESET*0=
- CHG>bRampOneDir*1=
- CHG>RampPeakVolt*300=
- CHG>StepNumber*300=
- CHG>RampStartVolt*-200=
- CHG>RampDuration*10000=]

From controller to Software datatype>parameter*value=

3.7 Challenges faced during designing

Challenges	Solution	Remark
Soldering QFN and QFP package ICs	Self-learnt soldering QFN and QFP package ICs	Successful
Porting the example program given by the manufacturer for STM32 controller	Converted the program given for STM32F411 to STM32F103	Successful
Electromagnetic interference (EMI)	Care taken while PCB designing to minimize electrode track length as small as possible. No signal carrying track should be near the electrode tracks.	Successful
Every time connector needs to be soldered on electrode	USB male connector directly printed on electrode	Successful
Real-time electrochemical sensing of PCR amplicons	Need for further calibration of a potentiostat and make it Noise-proof	Future scope

Problems while testing AD5941 evaluation board

AD5941 was easy for getting started as example codes were given for different applications like amperometry and cyclic voltammetry. But we were not getting noise free results for the current for the order of 1mA and lower. To test the AD5941 board we plotted current vs voltage plot for 1 Mohm resistor. We observed a lot of noise in cyclic voltammetry plots as shown in figure 3.9(d). This noise was due to the Electromagnetic interference (EMI) of the nearby clock or data carrying wires, for example, USB wire connected to the computer shown in figure 3.9(a). Electromagnetic interference or EMI is a unwanted interference or noise due to external sources. So we tested the board by separating the electrode wire from other wires as shown in figure 3.9(b). It reduced the noise to some extent as shown in the figure 3.9(e) but still the plot is noisy. So to avoid long wire, we used a port

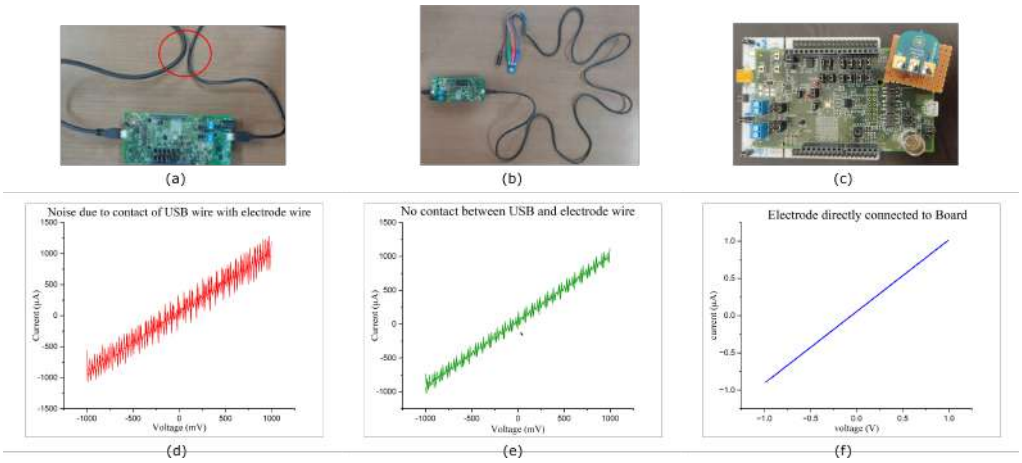


Figure 3.9: (a)USB wire touching the the electrode wire, (b) Using long wire to connect the electrode, (c) connecting electrode on PCB, (d) IV plot for 1Mohm when USB wire was touching the the electrode wire (e) IV plot for 1Mohm when long wire was used to connect the electrode, (f) IV plot for 1Mohm when electrode was directly connected on PCB

given on the board nearer to the AD5941 IC and we did not see any noise that we can see in the Figure 3.9 (f). Learning from this we decided to minimize the distance between electrodes and AD5941 IC while PCB designing.
To make electrode connection more simple and free from the external components like male and female headers, we decided to use a USB type A female port on AFE PCB and the electrode itself will be a USB type A male port. The only thing that needs to be remembered while using a USB male port printed on a PCB is that the thickness of the PCB must be 2mm.

3.8 WELStat Results

Figure 3.10 is the cyclic voltammetry plot plot for different concentration of Methylene blue using WELStat. As we can see in the graph with increase in MB concentration there is an increase in current.

3.8.1 End point PCR testing using WELPCR + WELStat

After verifying that our WELPCR amplifies the sample successfully by agarose gel electrophoresis, we wanted to check whether the amplification could be detected using our potentiostat, WELStat. We did the same procedure as before. That

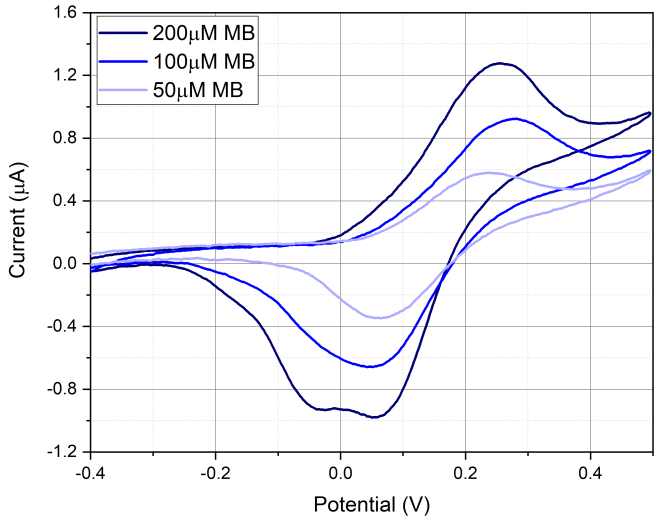


Figure 3.10: Cyclic voltammetry plot for different concentrations of Methylene Blue

is, running 35 PCR cycles for the 503bp fragment of bacteriophage Phi6. After PCR, this time instead of testing amplification using gel electrophoresis, we checked amplification by plotting a cyclic voltammetry plot using the WELStat.

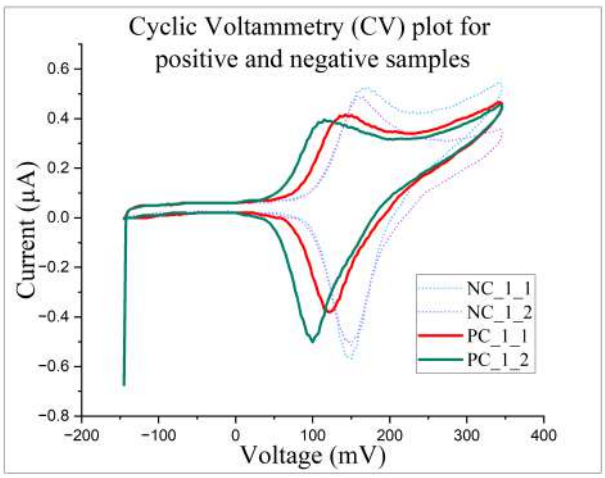


Figure 3.11: Cyclic voltammetry plot for 503bp fragment of Phi6 amplified after 35 cycles using WELPCR and electrochemically detected using WELSTAT. NC is the no-template control, PC is the positive control

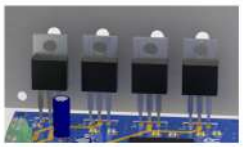
For electrochemical measurements, we need to add methylene blue (MB), which is a redox indicator dye that intercalates DNA. If the sample is positive, amplified DNA or RNA gets intercalated with the MB, resulting in a deacrease as well as

shifting of the peaks in the CV plot.

To check the repeatability of the result, we plotted the CV plot for three different samples of Phi6 bacteriophage whose PCR was done at different times. From the figure 3.11, we can see the decrease and shifting of the peaks. The decrease in peak current indicates amplification of the target DNA or RNA.

3.9 Future improvements

- **Using H-bridge IC:** Current WELPCR is over-designed so size of the WELPCR can be reduced. The MOSFETs used in the current device require a big heat sink thereby occupying more space. These MOSFET, gate driver and heat sink can be replaced by VNH2SP30, which is an H-bridge motor driver IC to reduce the overall size of the device.



H-bridge using discrete MOSFET

VNH2SP30: H-bridge IC

Figure 3.12: H-brige using 4 discrete MOSFET and VNH2SP30 IC

- **Use of smaller heat-sink for peltier:** Current WELPCR use a big heat sink for peltier. After some testing it was found that such a big heat-sink is not requires for the peltier. Smaller heat-sink with size 50% smaller than the current heat-sink.

- **Protection circuit:** While testing the WELPCR, peltier was damaged due to the overcurrent. So we can have a high current protection circuit before the peltier port.

- **Smaller version of WELPCR:** Current WELPCR size is big just because of the big 15A power supply. Such high current rating power supply is required just to get the high temperature ramp rate. So we can have a smaller version of WELPCR which will have slower ramp rate so it will require smaller power supply. This PCR will be optimized for the size instead of the ramp rate

- **Real-time testing using the WELStat:** In real time electrochemical sensing the is lot of noise due to PWM which drives the peltier. So we need to reduce noise in WELSTAT to perform the Real-time electrochemical PCR.

Appendix A

WELPCR User Manual

A.0.1 User Interface

Figure A.1: (a)PCR parameter window, (b) PCR parameter monitor window

As shown in Figure A.1 WELPCR has a 2.4" touch screen 240x320 display. The display has two windows. The first window is the 'PCR Parameter Window," and the second window is the 'PCR Parameter Monitor Window'. In the PCR parameter window, we can see the current parameters of the single PCR cycle, which are temperature and time for denaturation, annealing, and extension. These parameters can be selected and changed by the navigation keys. The second window, the 'PCR Parameter Monitor Window', is for monitoring the live parameters.

PCR parameter window:

1. The total number of cycles
2. The number of cycles completed
3. Extension temperature
4. Extension time for the last cycle

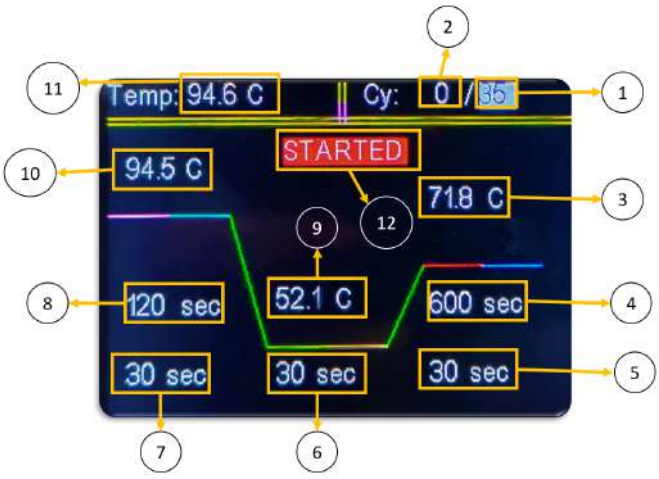


Figure A.2: Details about the PCR parameter window

5. Extension time of a single cycle
6. Annealing time
7. denaturation time
8. Initial denaturation temperature
9. Annealing temperature
10. Denaturation temperature
11. the current temperature of the tube holder
12. 'STARTED' or 'STOPPED' indicates the current state of PCR.

PCR parameter monitor window:

1. The number of cycles completed
2. The total number of cycles
3. The current set point of the PID controller
4. Percentage progress of the current PCR test
5. Time remaining for the current PCR test.
6. Temperature measured by the temperature sensor probe

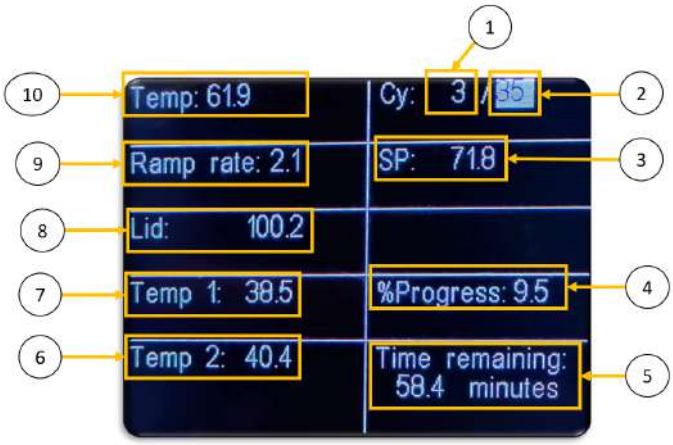


Figure A.3: Details about the PCR parameter monitor window

7. Temperature measured by the temperature sensor probe 2.
8. Temperature of the heated lid
9. Current Ramp rate
10. Current tube holder temperature

PCR front panel

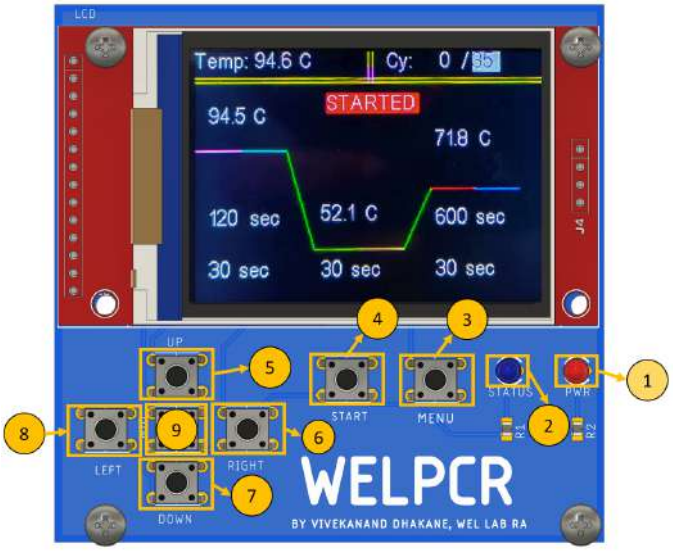


Figure A.4: Front panel of WELPCR

1. Power LED

2. Status LED
3. MENU: To change the window
4. START: Long press to start or stop the test.
5. UP: Shift the cursor up.
6. RIGHT: Shift the cursor right.
7. Down: Shift the cursor down.
8. LEFT: Shift the cursor left.
9. MID: Select the parameters.

Procedure

On PCR Parameter Window, navigate using the navigation keys. Select the parameter to be changed using the MID-button and adjust it using the navigation key. UP and DOWN keys change the parameter by 0.1 if it is temperature and change it by 1 if it is time. LEFT and RIGHT buttons change the parameters by 10.

Once parameters are set, long-press the START button to start the test. The status on the screen will be changed to STARTED Change the window using the MENU button to see real-time parameters like remaining time and percentage progress.

Once the test is completed, the status on the screen will be changed to STOPPED.

Important points to remember while testing

If we want to plot the temp of the sample, the temperature sensor must be fully dipped in the sample. While taking real time reading using WELStat or any other potentiostat, the ground of the measuring instrument must be connected to the ground of the PCR or body of the PCR (as the PCR body is connected to the ground of the PCR).

Appendix B

Design Files

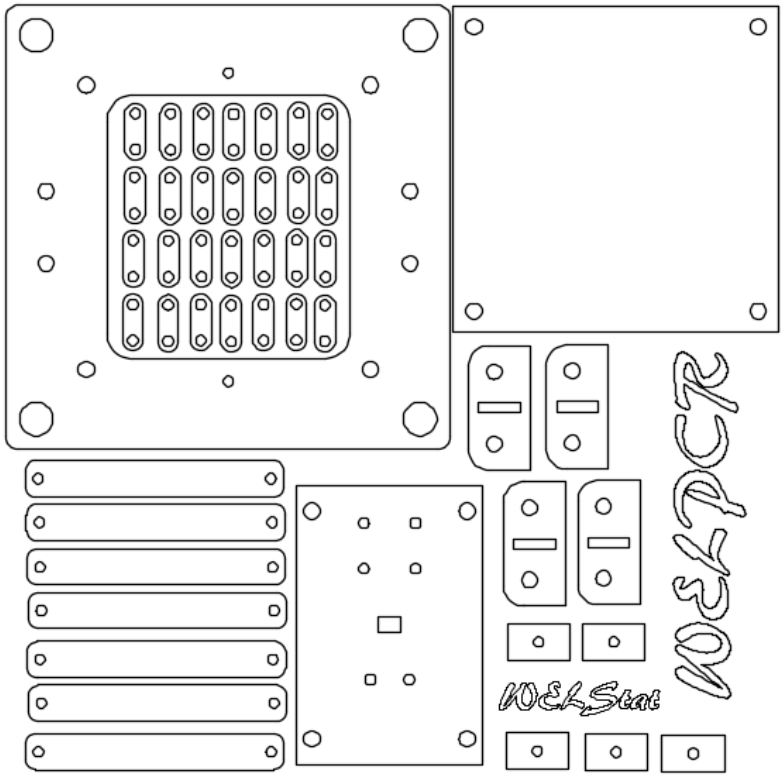


Figure B.1: Drawing for laser cutting

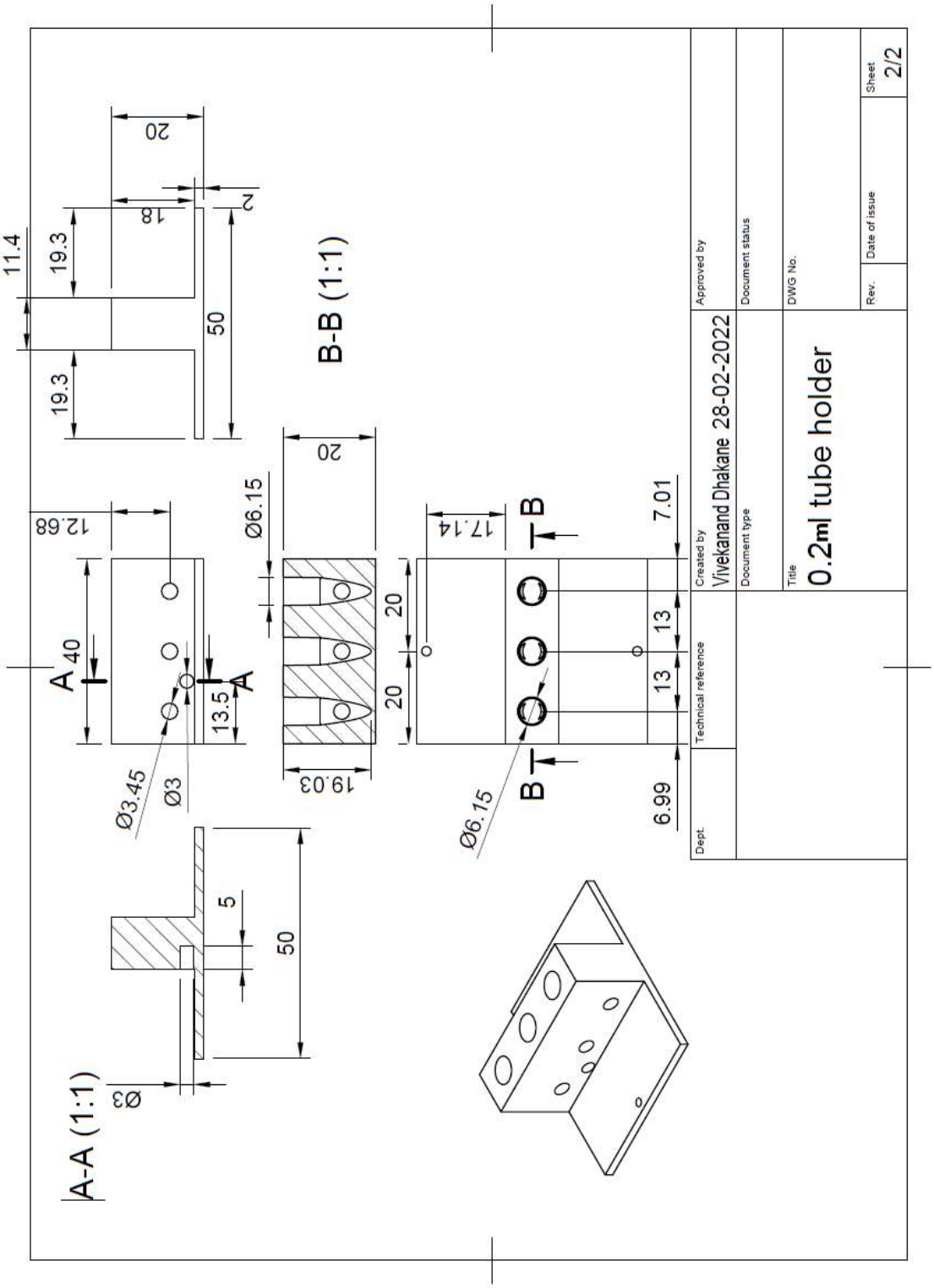


Figure B.2: Tube holder drawing

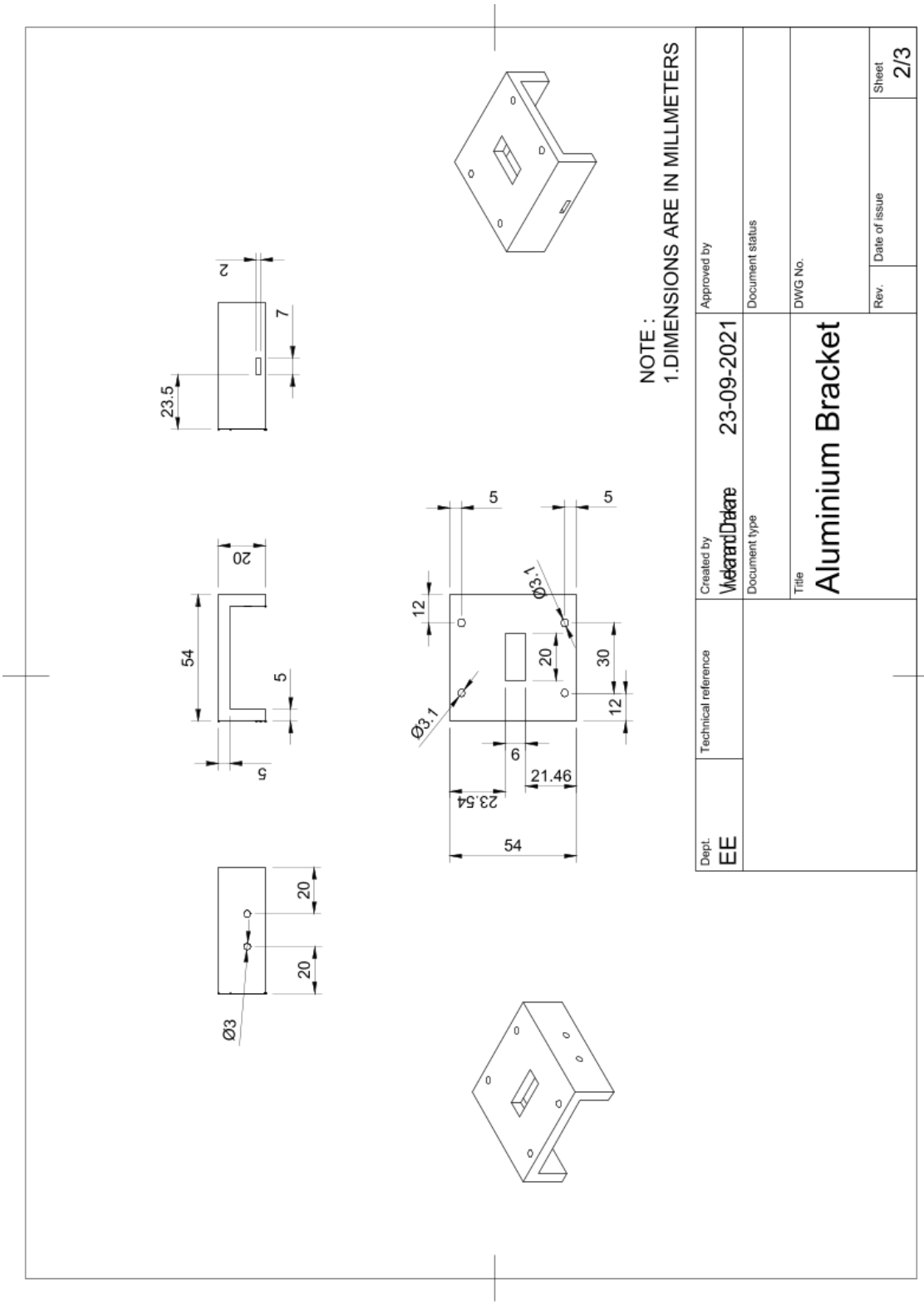


Figure B.3: Aluminium Bracket drawing

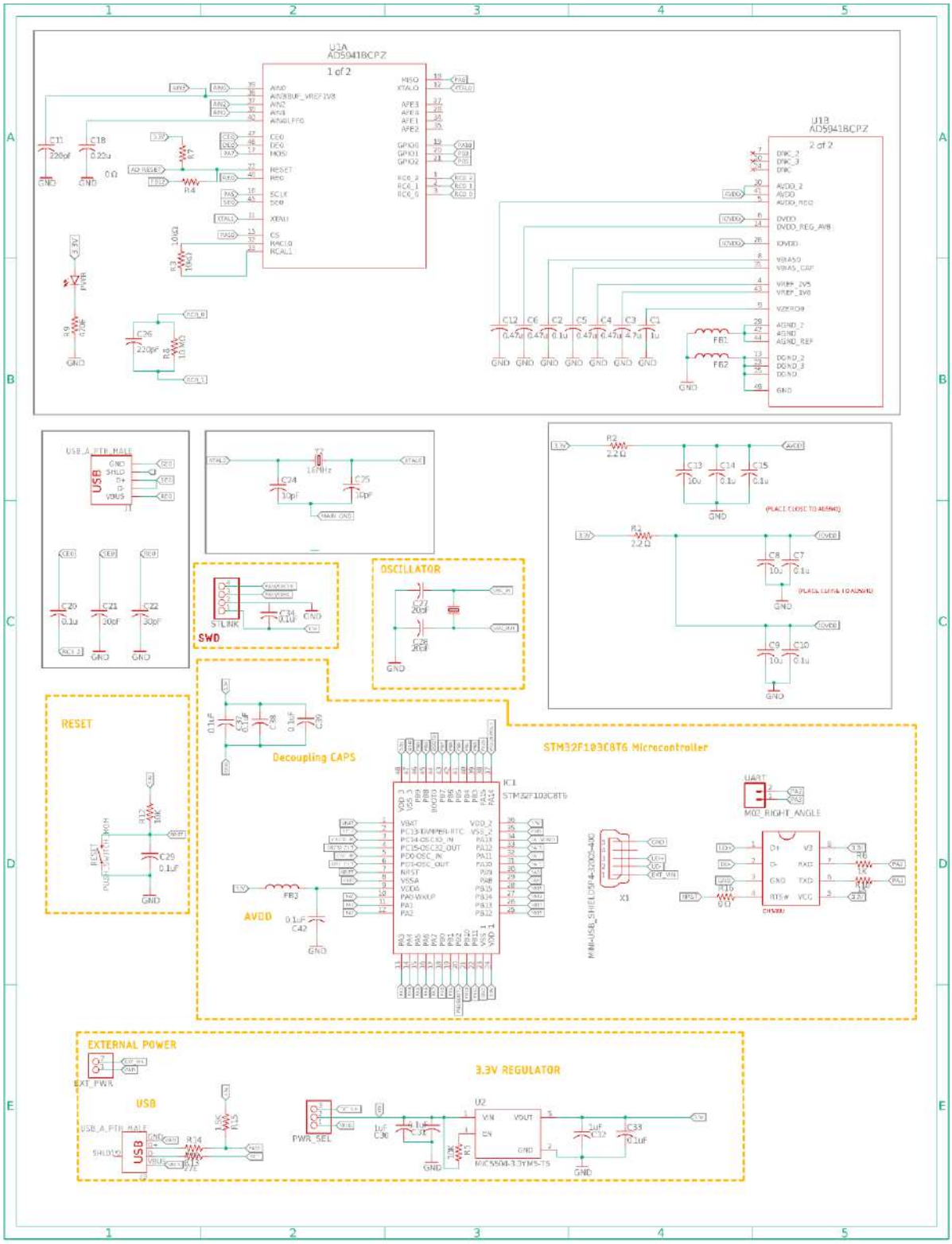


Figure B.4: WELStat schematic

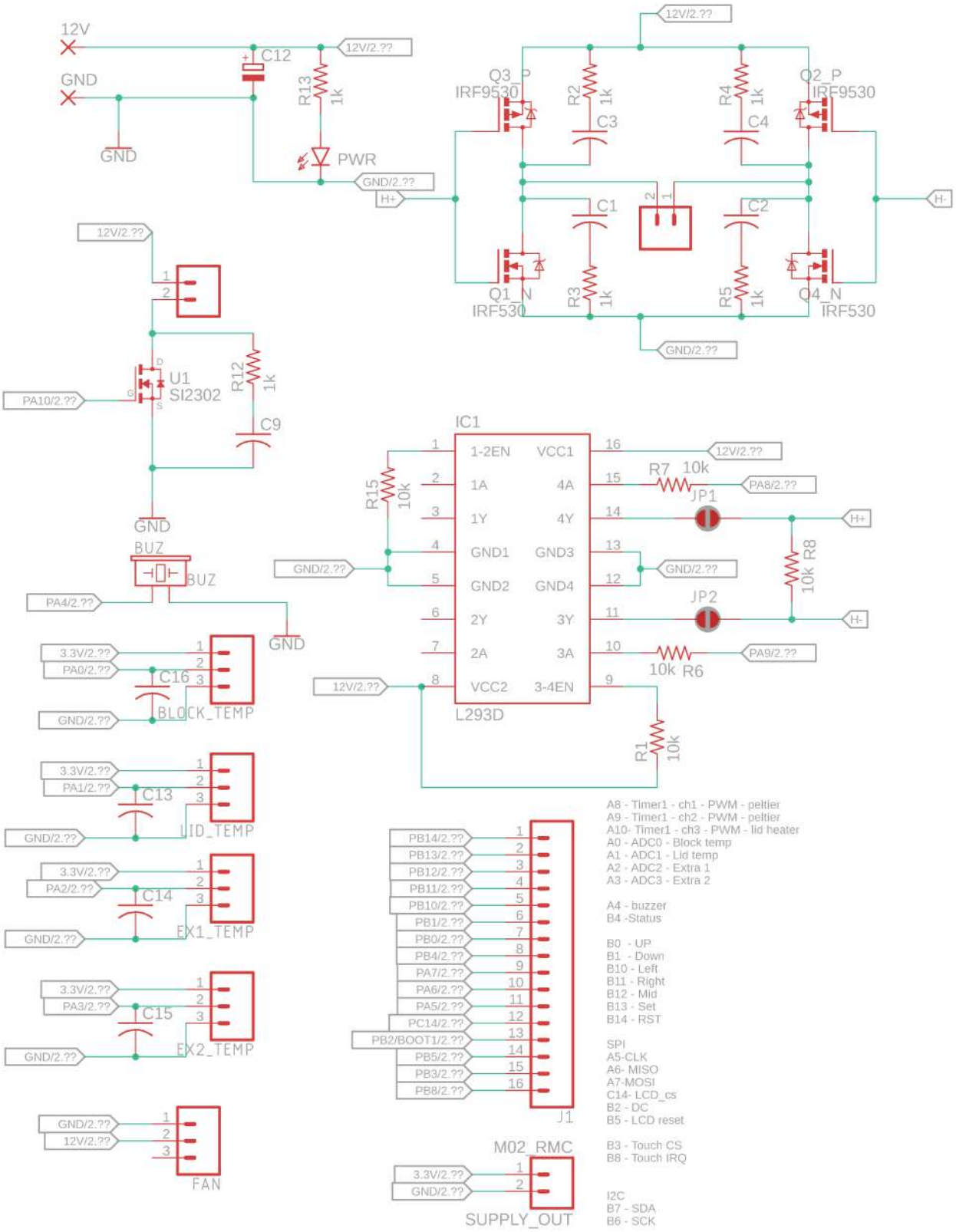


Figure B.5: PCR motherboard schematic sheet 1/2

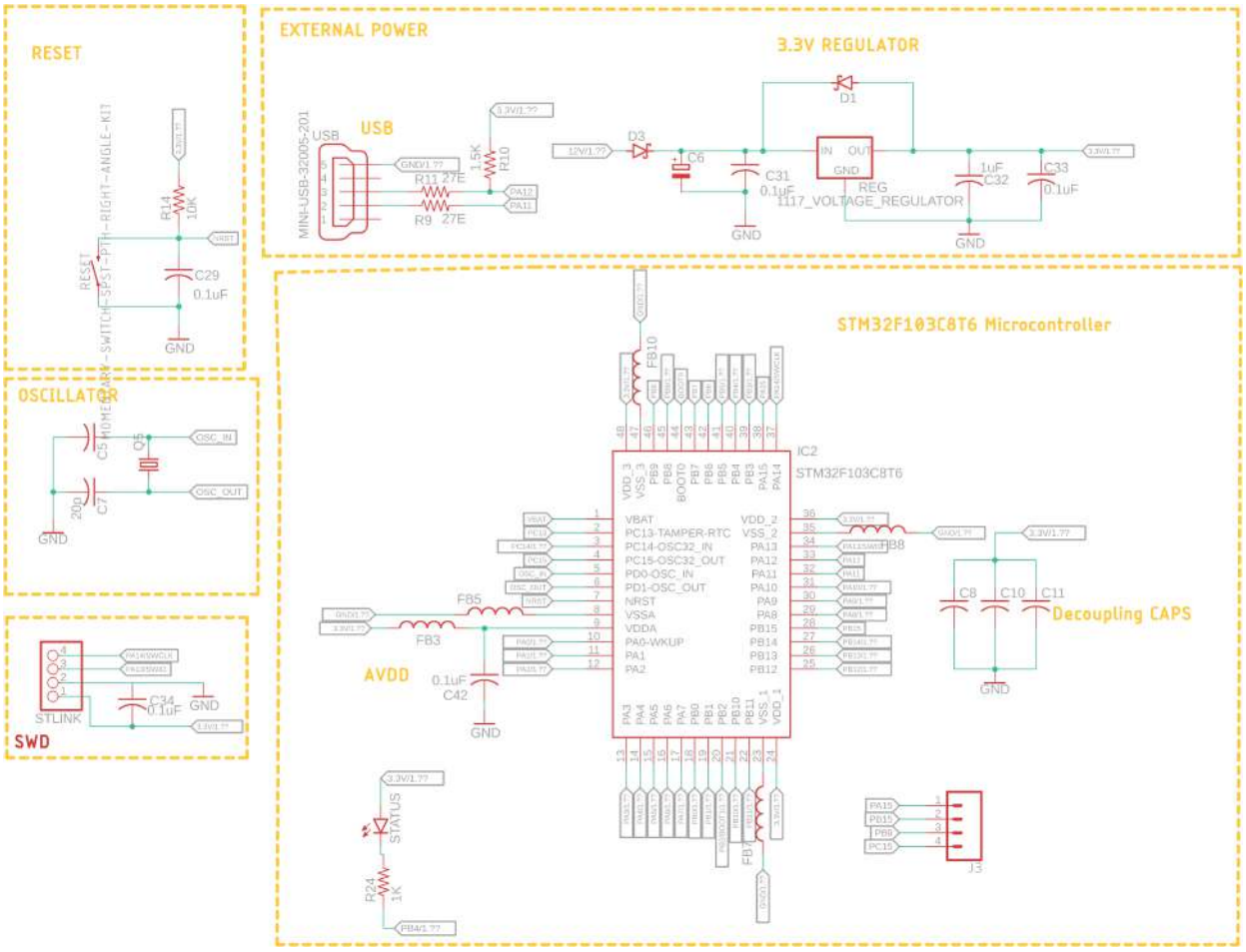


Figure B.6: PCR motherboard schematic sheet 2/2

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