IOSR Journal of Electrical and Electronics Engineering (IOSR-JEEE)
e-ISSN: 2278-1676,p-ISSN: 2320-3331, Volume 14, Issue 3 Ser. III (May. – June. 2019), PP 45-53
www.iosrjournals.org

Design and Development of Embedded System Based PCR Thermal Cycler

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Abstract: *In this paper we present the design and development of embedded system based Polymerase Chain Reaction (PCR) Thermal Cycler. The development steps of PCR thermal cycler are explained. Important design features of thermal cycler and their implementation are discussed. The results of the developed PCR thermal cycler are compared with those of benchmark instruments.*

Keywords: *PCR, Peltier, Hardware, Software, Thermal Cycling.*

Date of Submission: 01-07-2019 Date of acceptance: 16-07-2019

I. Introduction

Polymerase Chain Reaction (PCR), is a method of in vitro enzymatic synthesis and amplification of specific DNA fragments. PCR was invented by Dr. Kary Mullis in the 1980s. Mullis was awarded Nobel Prize in Chemistry in 1993 for this invention [1]. With the development and breakthrough of science and technology, PCR technology has been widely used in many fields, such as microbial detection, veterinary medicine, aquaculture and so on. PCR has various applications, including molecular diagnostics of human and animal diseases, forensics [5], food technology [6] and environmental studies [7, 8]. This technique has strong sensitivity, accuracy and specificity, And it can be detected quickly, so its application field is extending continuously [2, 3]. PCR technology is based on the known DNA sequence, to be amplified with the synthetic DNA two chain end complementary two oligonucleotide primers, in vitro to be detected DNA sequences (template) were amplified in enzymatic action. During amplification, the sample is cycled between denaturation, annealing and extension temperature. The typical ranges of denaturation and annealing are, respectively, 90-95°C and 50-65°C. The extension happens around 72°C. The whole technical process of PCR by several cycles, one cycle consists of 3 steps: The first step is the continuous DNA template degeneration under high temperature conditions, namely the template DNA at 93~94 ℃ under the condition of denaturation chain; the second step is that annealing synthetic oligonucleotide primer and template DNA chain. By the end of the cooling to 55 ℃annealing; the third step is to extend, which exist at the same time in 4 kinds of d NTP substrate, with the help of Taq DNA polymerase, primers chain along the 5'-3' direction and new chain of complementary template. After this cycle, a new chain is synthesized that can be continued as a DNA template and thus recycled. During the cycle, the amount of amplified products increased exponentially, and the single copy gene is 25~40 times [4], and DNA could amplify l00 million times. The steps of the PCR reaction are simple, but the specific operations are complex, such as the determination of the annealing temperature, the length of the extension and the number of cycles. Experimental steps of the PCR procedure are sample preparation, DNA amplification and product detection. The PCR protocol is as shown in Table 1.

A PCR thermal cycler is a device temperature-monitoring and controlling of the temperature of an aluminum block in order to deliver the temperature required by each chain reaction step of PCR protocol. Thermal block in the Fig.1 is to conduct three steps of PCR, denaturation, annealing and extension. DNA reaction mixture contains the target DNA, primers, nucleotides and DNA polymerase. Heated lid at the top of the mixture is to suppress condensation in the tube. Temperature of the heated lid remains the same and is kept above the denaturation temperature. PCR thermal cycler is also called qualitative PCR. Its components are of macroscopic size. Normally, it uses Peltier cell thermal cycling. Other parts include power system and temperature control system. Micro litres of DNA mixture of is needed. Several samples can be processed or analyzed simultaneously. DNA amplification up to millions is possible in hours time scale.

Table 1: Thermal Cycling Protocol Example.

Step No.	Temperature (°C)	Duration (sec)
1	95	300
2	94	45
3	56	30
4	72	60
5	GOTO	35
6	72	600
7	20	∞

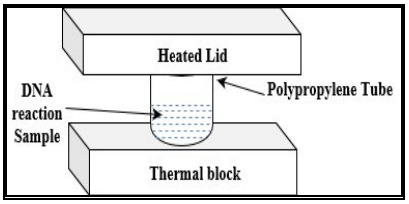

Fig.1: Schematic of Typical function of PCR thermal cycler

In the following section, the design issues, hardware implementation details, and the software implementation issues with the usage of the multiple units are presented. The implementation results indicated that the presented system had the same performance with the conventional thermal cycler.

II. Hardware Design

In well-based PCR, where the reaction is performed in a chamber that is cyclically heated and cooled, control instrumentation is needed to dynamically cycle the temperature of PCR samples. Also, the thermal mass associated with the heater and PCR well helps the achievable thermal uniformity. The functional blocks in the system of a conventional thermal cycler is shown in Fig. 2 The PCR protocol is edited using the operation panel and LCD, and the completed protocol is saved in the EEPROM memory embedded in the PCR thermal cycler. Since several users use one cycler in general, it can save multiple protocols and enable users to select one is required.

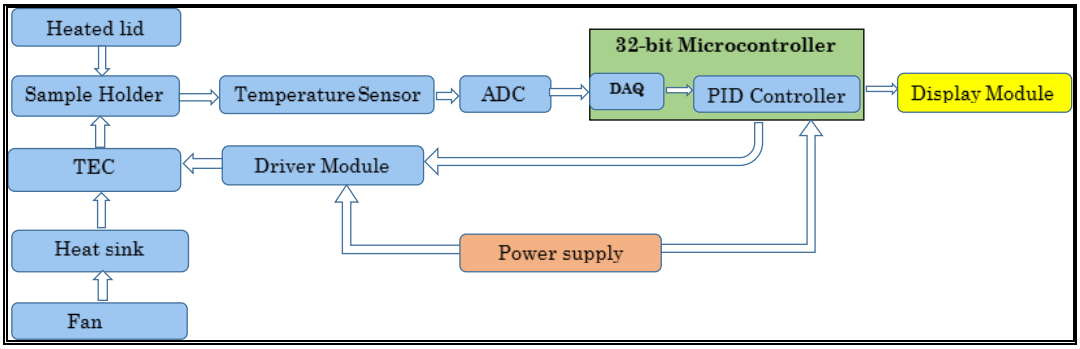

Fig. 2: Functional Block diagram of embedded system based PCR Thermal Cycler

The system consists of three main parts: 1) the lid heater for heating the top lids of the tubes, 2) the heat block module including the 96 well block, 6 Peltier modules and 3) the heat sink. The Heating metal block, having 96 micro vials fitted on a 10 mm thick aluminum holder and in tight contact with standardized 0.2µL PCR tubes. The wells in the block are exactly the same shape and size as the PCR tubes allowing for maximum heat exchange as shown in fig. 3. DNA mixture is placed in the PCR block to perform DNA amplification. The block is built with aluminum alloy (1060) material. Aluminum alloy is selected because it has high thermal conductivity and low heat capacity. The Thermal mass of the metal plate and required number of peltier modules for 96 well block are shown in Table 3. The designed 96 well plate dimensions of the developed system are shown in the Table 2. Most of the PCR block in commercial thermal cycler is using aluminum material. The 96 well plate is among the most common in industry. This plate density is used in general assays, cell-based assays, and immunoassays. The 384 well plate is second to the 96 well plate in popularity, and use in industry today. These micro plates are ideal for high throughput assays, as they require small working volumes making these plates much more cost efficient to process. Peltier (Thermoelectric Cooler), which is also inexpensive and easily available, is selected as the device for temperature control of the 96 well aluminum block

where the reagent tubes are installed. The 96 well block is placed on the cool side of the thermoelectric module while the other side of TEC is installed with Heat sink and fans for heat transfer. The top side of the Peltier absorbs heat in the cooling state lowering the heat block temperature, and the bottom side ventilates the heat absorbed in order to lower the temperature of the top side. Sufficient heat must be released from the bottom side of the Peltier in order to prevent the heat from re-entry to the top side. Therefore, the heat of the Peltier is released by using the heat sink module. A heat sink with a fan having dimensions of 120x120x38 mm running at the speed of 3600 rpm is employed to additionally to improve the efficiency of the system. Thermal contact enhancement between peltier, 96 well block and heat sink can be achieved by using either thermal graphite pads EYG-A121802DM or silicone free gap Filler TGS-Z-nS.

Six interchangeable NTC thermistors with the standard accuracy are applied to measure the temperature of 96 well heat block. Two thermistors are used to measure the temperature of the heat sink and heated lid. Heat sink temperature is not displayed and is only utilized to monitor the system failure. The temperatures of the lid and the 96 well heat block are shown to users to control and monitor the PCR temperature conditions for the biochemical process. It used NTC thermistors (50Kohm) as a temperature feedback system to measure and maintain the temperature of 96 well block. A proportional-integral (PI) controller algorithm is used to control the thermal electric modules and set the temperatures of PCR block. The PI constants are tuned using Ziegler-Nichols closed loop method. The output from PI controller is applied as PWM to control dual full-bridge drivers. The full-bridge temperature controller controls the current supply to the thermoelectric modules and the resistive heated lid of the thermal cycler. A Data acquisition system with a 50 kHz, 24-bit resolution Analog to Digital Converter and 8:1 multiplexer used to read the temperature of 96-well block and gives the data to PI controller. A 24-bit $\sum\Delta$ ADC which greatly improves the precision. An 8:1 Multiplexer is used in series with ADC instead of using multichannel ADC. In order to calculate temperature from thermistor resistance, used the Steinhart-Hart equation, which is a model for semiconductor resistance.

$$\frac{1}{T} = A + B\ln(R) + C[\ln(R)]^3$$

Table 2: Designed 96-well plate dimensions.

Sample Holder									
Material	**Density**	**Type**	**Peltier**	**Dimensions (mm)**				**Volume**	**Mass**
	(g/cc)		**Dimensions (mm)**	**H**	**L**	**W**	**Bottom Thickness**	**(cc)**	**(g) (=d*V)**
Aluminum	2.705	Type-I	36x36	10	110	74	3.5	32.2252	87.169166
		Type-II	40x40		120	80		37.5452	101.559766
							1	33.7452	91.280766
CarbAl-G	1.75						3.5	37.5452	65.7041

Table 3: Different material Properties and mass of PCR 96 well plate

Material Properties											PCR plate Volume	Output: Variation in Mass	
Alloy and quality		**Tensile strength**	**Yield strength**	**Stretch (%)**	**Thermal Conductivity (W/m°C)**	**Electric Resistivity**	**Thermal diffusivity**	**Conductance**	**Specific heat capacity J/(kg*K)**	**Density (g/cc)**		**Mass of the PCR Plate (g)**	**Remarks**
		(N/mm2) Mpa	**(N/mm2) Mpa**			**(μΩ•cm)**	**(cm2/sec)**	**(IACS%)**			**(cc)**	**m= d*V**	
Conventional Aluminum materials	A1100-O	90	35	35	222	-	-	59	904	2.71	37.545	101.75	Low thermal conductivity.
	A1100-H16	165	138	6	222	-	-	59	904	2.71		101.75	
	A1100-H18	165	150	5	218	-	-	57	900	2.71		101.75	
	A5052-O	195	90	25	137	-	-	35	880	2.7		101.37	
	A5052-H32	230	195	12	137	-	-	35	880	2.7		101.37	Low thermal conductivity
	Al1050-H18	160	150	43	230	-	-	58	900	2.705		101.56	1. High Thermal 2. Low mass - Low cost
	Al1060	-	-	-	204	2.8	0.84	-	900	2.7		101.37	3. Easily Available
Other metals	Aluminum Silicon	-	-	-	200	20.7	0.86	-	786	2.96		111.13	Low thermal conductivity
	CarbAl™-G	-	-	-	350	400	2.9	-	690	1.75		65.7	1. High Thermal 2. Low mass
	Copper	233	69	45	390	1.7	1.1	100	394	8.9		334.15	High thermal conductivity High Thermal mass
	Mild steel S15C	420	255	30	52	-	-	12	460	7.9		296.61	High density Low thermal conductivity
	Magnesium (Casting)	265	95	10	70	-	-	12	1050	1.8		67.58	
	Silver	207	124	40	429	-	-	105	245	10.49		393.85	High cost, High density
	Gold	124	-	45	318	-	-	70	129	19.32		725.37	High cost, High density
	steel	790	380	55	15	-		3 to 15	500	7.85		294.73	High density, Low thermal conductivity

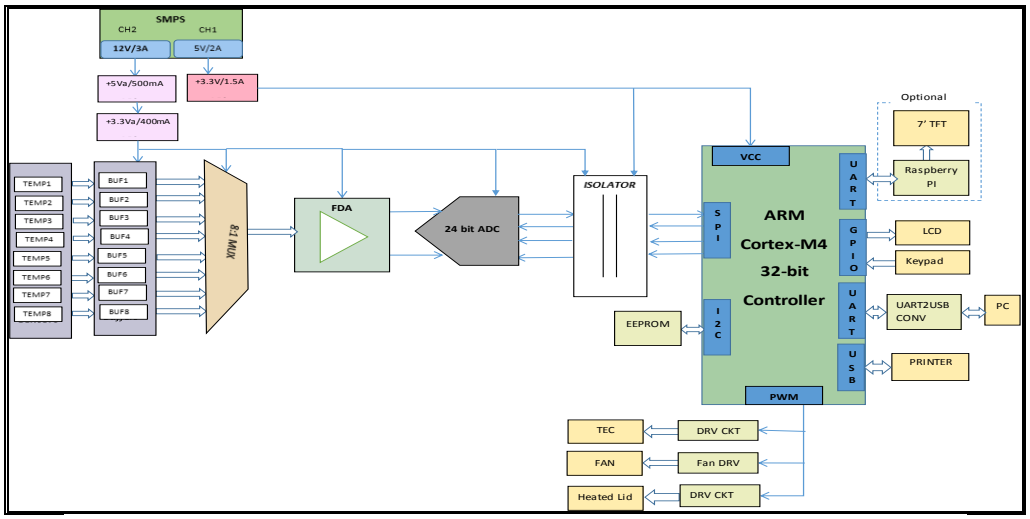

Fig. 3: Schematic diagram of designed embedded system based PCR thermal Cycler

The embedded system-based PCR thermal cycler is designed and implemented. The PCR protocol includes, PCR temperature steps, dew times, number of cycles for a particular application are defined using Graphical User Interface. Based on the protocol, Thermoelctric modules are used to heat or cool 96 well sample holder. TEC modules are driven by a driver module which is controlled using pulse width modulation feature of microcontroller. Holes are drilled into the middle of the 96 well aluminum block for the main thermistors. The thermistors 50kΩ wires must be shielded to reduce noise and so achieve a more accurate temperature measurement. The thermistors are connected to a constant current source and in parallel to a fixed resistor. The resistance of which must be precisely known to accurately measure the temperature so the resistor used has a tolerance of ±0.1% enabling the temperature to be accurately measured. Sensor output is fed to signal conditioner and a buffer to a filter, which eliminates the noise of low and high pass frequencies and allow specific bandwidth of frequency. The analog section houses the ∑Δ 24 – bit analog to digital converter (ADC) chip, 2.048V precision reference chip. The signal is fed to a multiplexer to read multiple temperature signals with a single channel 24-bit ADC. The signal is driven to a low offset opamp as buffer to avoid loading effect. To eliminate noise due to single ended configuration the signal is fed to a fully differential amplifier (FDA). FDA generates fully differential signal based to the common mode signal and fed to ADC. ADC converts the analog signal into equivalent 24-bit digital data with 50kSPS sample rate of conversion. The converted digital signal is isolated from microcontroller using a SPI (Serial peripheral interface) isolator to eliminate noise. ADC is interfaced to microcontroller through SPI protocol. The received temperature data from DAQ (data acquisition system) is fed to a PID (Proportional Integral Derivative) control logic in software to find the error based on set point (SP) and process value (PV) temperature. PID logic calculates the error equivalent PWM with 40 kHz based on Flex Timer module in Microcontroller. The PWM is fed to TEC driver to drive sufficient TEC current for heating or cooling the 96 well sample block. Fan for thermal block is controlled based the mode of the sample (heating/ cooling). The final processed data is displayed on LCD. The system also contains a real-time clock chip and coin cell battery backup for data-logging. Heated Lid prevents the samples from evaporating. The temperature of hot lid is constantly maintaining at 105^0C, as the PCR tubes are sealed, some of the sample evaporates but the air in the tube quickly become saturated with water vapour, preventing any further evaporation which would disrupt the PCR reaction. Spring system in lid allows the height of the heated lid to be adjusted so that it is in contact with all PCR tubes.

Fig. 3: Picture of designed 96 well sample holder

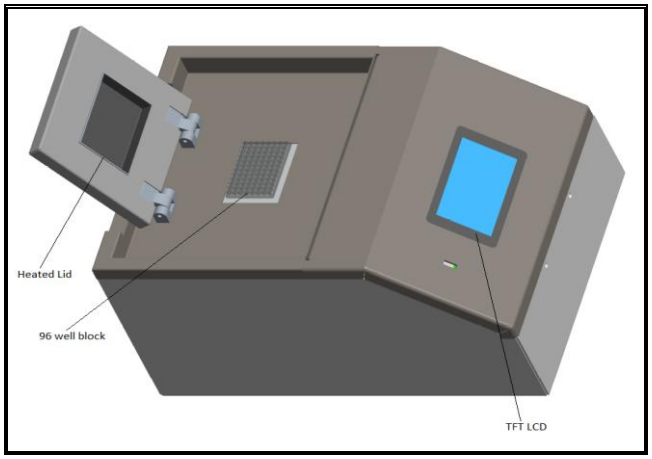

Fig. 4: 3D model of designed embedded system based PCR thermal Cycler

Both the peltiers and lid heaters require a very high current and the heaters are controlled by switching this current on and off very quickly, this is an effective method of controlling the heaters but it causes a lot of problems. Switching the current on and off causes a rapidly changing magnetic field which induces small currents in any wires or pieces of metal inside the machine. This is a massive problem as it caused the sensitive analog to digital converter (ADC) to crash or incorrectly measure the temperature. To solve this, changed several different ADC chips, some of which were better at coping with the noisy environment, to find the most suitable. Also added a lot of electromagnetic shielding between the heater and the boards and around all the heater power lines and thermistor wires, this greatly reduced the amount of electromagnetic noise. Furthermore, adjusted the frequency of the PWM (the rate at which the heater is turned on and off) to limit the induction.

III. Software Design

Implementing the protocol of PCR, including user interactions, can be difficult compared to the basic functions such as protocol handling or temperature control implemented in an embedded system. The developed software has more effective system architecture. The system of the designed PCR thermal cycler has input or output devices LCD and Keypad. A UART interface is used to enable the system PC communication. Software is developed using Embedded C and Free RTOS system calls. The Flow chart of the PCR Protocol Execution is shown in Fig.5.

The software is developed with support of FreeRTOS APIs using MCUXpresso cross compiler for DNA amplification with PCR protocol. The main role of the software in the present study is to govern the following activities using ARM 32-bit microcontroller. a) To acquire the block temperature data from temperature sensors placed in 96 well block, b) To make the data acquisition system and convert the analog to digital data. C) To get the set point temperatures, dew times and number of cycles of the protocol from the key pad or EEPROM. d) To compute the temperatures, ramp rates and display on LCD. e) To make the different functional units of system work in a systematic and sequential manner.

The software program developed in the present study is divided into 6 tasks. a) Keypad / TFT GUI task to enter and display PCR parameters. b) Data acquisition task to read 96 well block and lid temperature. c) PID drive task to calculate PWM based on derived PI constants and update PWM to PWM drive task. d) PWM drive task, e) TEC driver error monitoring task to monitor and display the error of the TEC driver modules.

One of the biggest problems encountered when writing the software is controlling the temperature, particularly holding the block at a stable temperature without fluctuations and reaching this stable temperature quickly. Initially adjusting the heater proportionally to the error (difference between the actual temperature and the set point) would be sufficient but there were many issues with this, could not get the temperature to stabilize without massively over or undershooting and small fluctuations could throw the controller off. To solve this problem implemented a PI (proportional-integral) control loop, this adjusts the temperature according to the equation shown below. The heater is set to full until the temperature gets close to the target, then the PI control is switched on, this reduces overshoot and allows a more stable temperature to be reached very quickly.

$$\mathrm{MV}(t) = K_p e + K_d \frac{de}{dt} + K_i \int_0^t e(t)\,dt$$

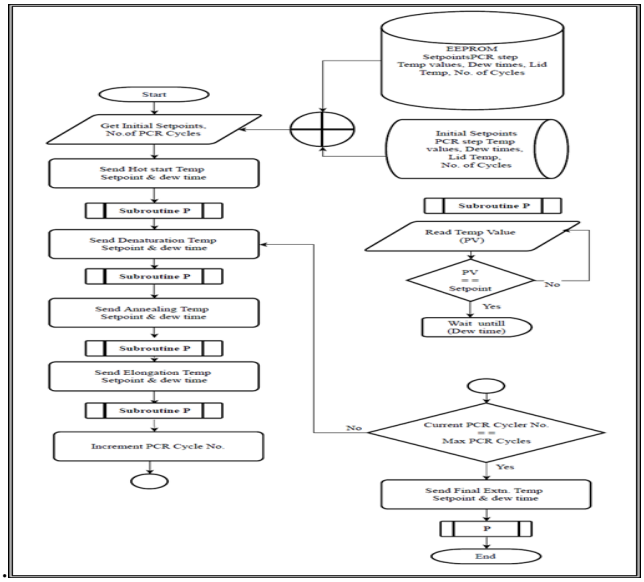

Fig. 5: Flow chart of PCR protocol execution

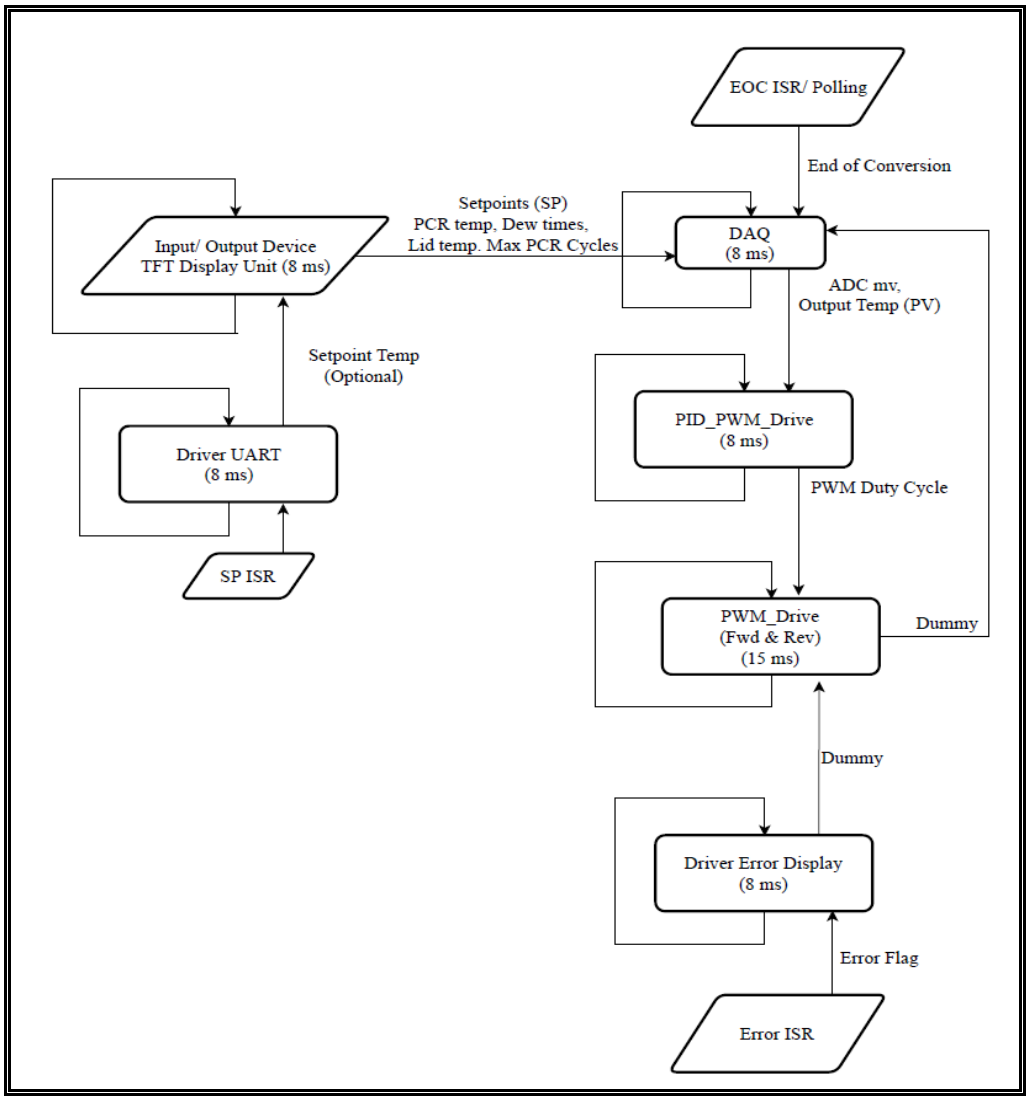

Fig.6: Block diagram of Software flow of designed system

IV. Results and Discussions

A thermal cycler with 96 well block and 6 peltier heaters is successfully developed and tested. The PCR system is initially tested the performance for heating and cooling cycles. The developed PCR system is then experimented for the full PCR thermal cycling for 30 cycles and the temperature response of the first two cycles are shown in Fig.7. In each cycle, DNAs were thermo cycled at 94^0 C (denaturation) for 30 seconds, 55^0 C (annealing) for 30 seconds and 72^0 C (extension) for 60 seconds, respectively. The total cycle time including heating and cooling time is approximately 400 seconds which is as fast as commercial PCR device. The result shows that the PI controller can maintain and control a constant temperature.

Thermistor (Semitec) are used as temperature probes. The thermistors are implemented in 200uL PCR tubes. The lead wires of a sensor are arranged to pass the tube wall through a fine hole provided, which helped air-tight sealing against expanding pressure of boiling water. Seams around the guide were sealed with epoxy glue, and satisfactory sealing is confirmed if no significant loss of test solution is detected after leaving the test solution in the closed temperature probes for more than 15 min at 95°C. A typical temperature probe is shown in Fig 8.

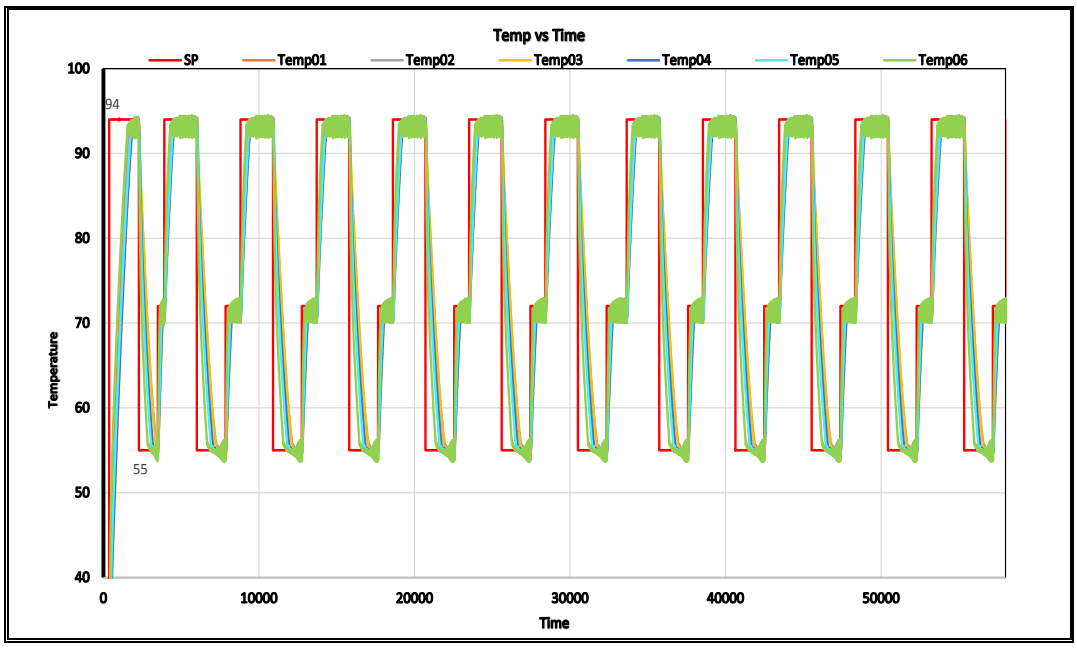

Fig. 7: Thermal Cycling profile with 6 Thermistors inside of 96 well block.

Fig. 8: A picture of a temperature measurement probe.

The temperature change speed of the heat block is compared with benchtop thermal cycler (Applied Biosystems, GENEAMP 2700 and Eppendorf Master Cycler). Table 4 shows the results of the temperature change speed comparison for the three PCR thermal cyclers. The temperature change speed of the designed system is similar to the benchtop instruments and the heating speed of the developed system is faster among the three cyclers.

Table 4: Comparison of Temperature changing speed of Sample holder block (°C/s)

	94 to 55	**55 to 72**	**72 to 94**
Designed PCR Thermal Cycler	-1.4	2.3	1.8
Eppendorf Master cycler	-1.2	1.2	1.1
GENEAMP 2700	-2.2	1.3	1

Genomic DNA is extracted from the cultures Microbial Type Culture Collection and Gene Bank (MTCC) MTCC:424 and from of the selected bacterial isolates using QIAGEN DNA isolation kit (Qiagen), suspended in 100 µl of elution buffer (10 mM/L Tris-HCl, pH 8.5) and quantified by measuring OD at 260 nm. PCR amplification is performed using a 50 µL reaction mixture containing 100 ng of template DNA, 20 µmol of 16S rRNA primers, 200 µM of dNTPs, 1.5 mM of MgCl2, 1U of Taq DNA polymerase (MBI Fermentas) and 10 µL of 10x Taq polymerase buffer. The sequences of 16S rRNA primers used were as follows.
27f: (5'-AGAGTTTGATCCTGGCTCAG-3')
1522r: (5′-AAGGAGGTGATCCANCCRCA-3')

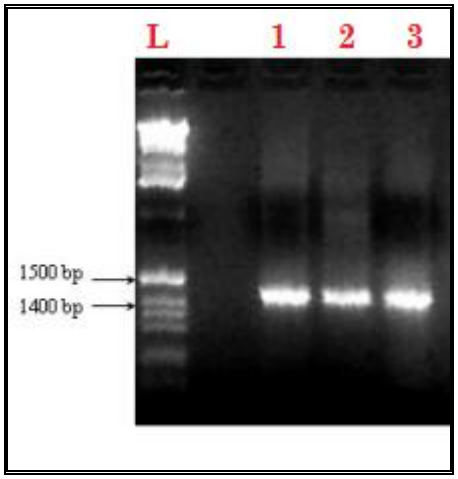

Fig. 9: Agarose Gel run for amplified DNA. L- ladder, DNA amplified by 1- Bench top instrument, 2,3 – developed PCR Thermal Cycler

Amplification is carried out with an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min using a thermocycler (Master Cycler Personal; Eppendorf) and the developed PCR thermal cycler. PCR products were analyzed on 1% agarose gel for 16S rRNA amplicons in 1x TBE buffer at 100 V as shown in Fig.9.

V. Conclusion

A 96 well PCR thermal cycler, using 6 Peltier modules of high performance, is designed, fabricated and characterized. The temperature control of the designed system is evaluated and the results showed fast temperature change speed and reliable uniformity. The performance of the amplified PCR product is also compared between the developed system and the conventional one, and the results showed no difference. PCR reaction as fast as benchtop machines, has been achieved and further improvement in thermal protocol optimization is still ongoing.

Acknowledgements

Authors are thankful to M/s ELICO Limited, Hyderabad, INDIA for financial aid and providing infrastructure facilities.

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IOSR Journal of Electrical and Electronics Engineering (IOSR-JEEE) is UGC approved Journal with Sl. No. 4198, Journal no. 45125.

V.Sailaja. " Design and Development of Embedded System Based PCR Thermal Cycler." IOSR Journal of Electrical and Electronics Engineering (IOSR-JEEE) 14.3 (2019): 45-53.