University of Alberta

Desiccated and Preserved Polyacrylamide based Nucleic Acid Diagnostic Systems

by

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ABSTRACT

The In-gel polymerase chain reaction (PCR) technology developed by our group at the University of Alberta makes use of PCR reagents entrapped within nano-liter polyacrylamide gels. To adapt this technology for use at the point of care, methods were developed to preserve gel-based PCR reagents by desiccation within the microchips. The gel-based PCR reagents were desiccated using a vacuum desiccator with trehalose as the lyoprotectant. Long-term stability studies were conducted with these formulations at different temperature conditions. Our results indicate that the desiccated gel-based PCR reagents can retain most of their activity upon rehydration after ten weeks. The shelf life stability of these desiccated gel-based PCR reagents can be maintained for much longer durations with further improvements to the formulations, chip geometries and packaging strategies. With the inclusion of the desiccated gel-based PCR reagents within the chips, the in gel PCR technology has a great potential to be used at point of care.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic acid
FGF	Fibroblast Growth Factor
LDH	Lactate dehydrogenase
LOC	Lab on a Chip
MATCI	Miniature Analytical Thermal Cycling Instrument
MCA	Melt Curve Analysis
MDG	Microfluidics Development Group
NAAT	Nucleic Acid Amplification Test
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
PFK	Phosphofructokinase
qPCR	Quantitative Polymerase Chain Reaction
RDT	Rapid Diagnostic Test
RH	Relative Humidity
SEM	Scanning Electron Microscopy
TEMED	N,N,N',N'-tetramethyleneethylenediamine
TNF	Tumor Necrosis Factor
tPA	Tissue Plasminogen Activator
TTK	Tricorder Tool Kit

Chapter 1: Introduction

1.1 Background

The development of fast, efficient and cheap diagnostic technologies has become an absolute necessity in the present world, especially in low resource settings. The diagnostic tests that are run at present are time consuming and expensive. The accuracy of results from these tests are dependent on several factors such as maintaining the viability of the specimen, constant supplies of reagents and electricity, well equipped laboratories and adequately trained technologists [3]. In many cases, the samples are sent to a central laboratory for analysis, thereby causing a further time delay in the availability of the results. Moreover, this approach may also lead to errors such as loss or mislabeling of samples. A new approach is required to address the needs of the developing countries especially in regions such as sub-Saharan Africa, which have poorly resourced health-care facilities and minimum supporting clinical laboratory infrastructure. Small, fast and easy-to-operate devices to be used at point of care are needed to reduce costs and inefficiencies associated testing in central laboratories[4-6].

A comparison of the laboratory-based method of blood testing and a point of care system are presented in table 1.1.

Laboratory based system	***Point-of-care system***
Test is ordered	Test is ordered
Test request is processed	Blood sample is drawn
Blood sample is drawn	Sample is analysed
Sample is transported to the lab	Results are reviewed
Sample is labelled and stored	Clinician acts on results
Sample is centrifuged	
Serum sample is sorted to Analysers	
Sample is analysed	
Results are reviewed and reported	
Clinician acts on results	

Table 1.1 Comparison of laboratory-based method of blood testing and an ideal point-of-care system [7]

It is therefore critical that diagnostic technologies be developed with "sample in answer out" capabilities that do not require skilled labor and are suitable for field operations.

There are several approaches that have been pursued so far in the development of point of care testing. One of the most common techniques to diagnose malaria and TB infections in the developing world is microscopy. It can be used to identify and count the number of malarial parasites in strained blood smears. However, this process is time consuming and requires trained personnel to carry out the sample preparation and analysis procedures [8].

Rapid diagnostic tests (RDTs) that are based on the detection of pathogen-specific antigens or antibodies are the most common tests that are currently in use in low resource settings. Immunochromatographic strip tests/lateral flow tests that detect pathogen-specific antibodies provide immediate results in the form of a visible band. This system is comprised of mobile and immobile phases. The mobile phase consists of monoclonal antibodies prepared against an antigen target and bound to either gold particles or a liposome containing selenium dye. The immobile phase consists of a second monoclonal antibody, which is applied to a strip of nitrocellulose. The mobile phase captures the parasite antigen and the antibody-antigen complex migrates along the nitrocellulose strip. This complex is then captured by the monoclonal antibody in the immobile phase thereby producing a visible colored line [9-11]. The agglutination test is another example, where immunoassays are used for the detection of microbial infections. An example of a lateral flow test is presented in Figure 1.1. However, these tests are not quantitative and cannot be made sufficiently sensitive to detect all biomarkers. Cheesbrough [12, 13] has provided a detailed description of different types of immunochromatographic tests and other RDTs specific for different pathogens in his review.

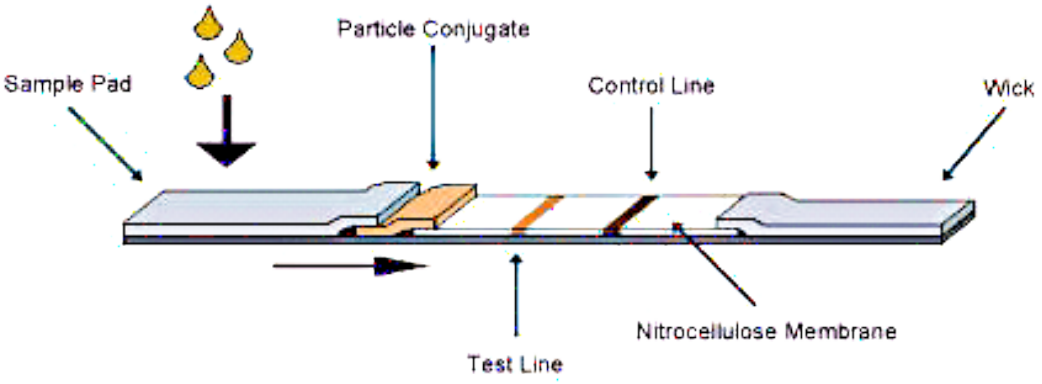

Figure 1.1: Format of a Lateral Flow Assay. (Source: www.prismabiotech.org)

Flow-cytometry is another approach that is being pursued in the development of point of care systems. Flow-cytometry is one of the best methods for type specific cell counting. In flow-cytometry cells are suspended in a stream of fluid and passed through an electronic detection system. A schematic representation of flow cytometric system is presented in Figure 1.2. Conventional flow cytometry (Figure 1.2) involves staining of cells with a fluorochrome –tagged antibody followed by washing and re-suspension in buffer. These stained cells are then introduced to the flow cytometer, which adjusts fluid flow such that individual cells pass through a flow cell and lasers excite any bound fluorochromes to emit light. The cells tagged with the excited fluorochrome, which indicates that they have bound antibody, are then counted to provide a measure of their frequency. This basic approach is modified for proposed point of care systems. To avoid a

need for prolonged manipulations for staining of cells with fluorescence tagged antibodies, cells are suspended in a stream of fluid containing fluorochrome antibodies, which bind to the cells. This stream is then passed through an electronic detection system with a laser, which scatters light that is measured by photo detectors. Depending on the type of fluorescent dye used, the signals generated by the detectors must be compensated electronically and computationally. EasyCD4 assay (Figure 1.3)(Guava Technologies, Hayward, California, USA)[14] is a system based on this principle which makes use of a capillary based microfluorometric assay to count the number of CD4+ lymphocytes present in the sample. Another example of such a system is the CyFlow^{green} (Partec, Muenster, Germany)[15, 16], which is a portable volumetric flow cytometer that has been proven to be effective in low resource settings.

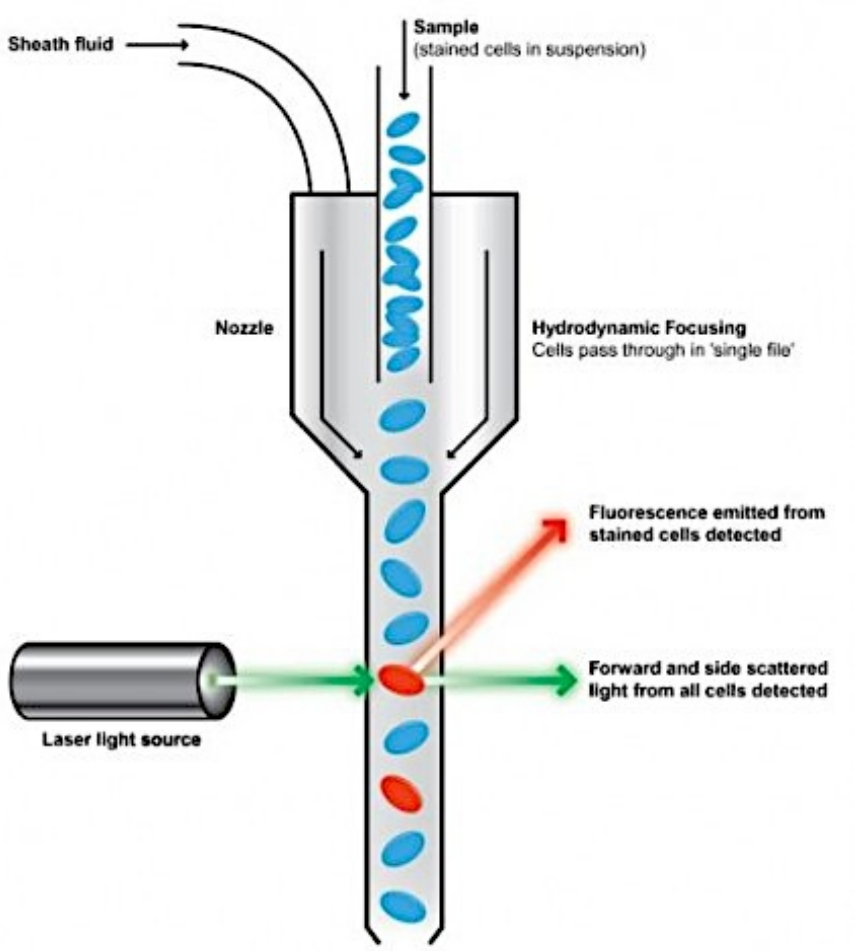

Figure 1.2: Schematic diagram of a conventional flow cytometer. (Source: www.sonyinsider.com)

Figure 1.3: Guava EasyCyte Plus (Source: www.guavatechnologies.com)

All the systems that have been described so far are either expensive and require trained personnel or in most cases they are target specific and cannot be used to detect multiple biomarkers. Although conventional flow cytometry has extensive multiple marker detection capabilities, the instruments are expensive, requiring a controlled environment, a skilled technologist to run and maintain them, and costly service contracts. This is not feasible for resource-deprived settings. A system that is fast, efficient and requires minimal technical expertise is required to address the needs of resource-deprived settings. The most promising approach to resolve these issues and provide a system that can test for multiple pathogens is nucleic acid amplification testing which is also been viewed as the diagnostic approach of the future [17-19].

Nucleic acid amplification tests (NAATs) have excellent specificity and sensitivity. The most commonly used NAAT is the polymerase chain reaction (PCR).

1.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a nucleic acid amplification method used to generate millions of copies of DNA from a few copies. This technique developed by Kary Mullis in 1983, is based on the ability of the enzyme DNA polymerase to synthesize a new strand of DNA complementary to the template.

1.2.1 Components involved in PCR

The main components of PCR include: the DNA template that contains the target sequence to be amplified, forward and reverse Primers, which are short DNA fragments that are complementary to the sense (DNA sequence same as the RNA sequence that is translatable into a protein) and antisense (complimentary sequence to the sense strand) strands of the segment of interest, Taq polymerase, which is a thermo-stable enzyme that was originally isolated from the bacterium *Thermus aquaticus* by Thomas D. Brock in in 1965 and is capable of enzymatically assembling a new strand of DNA complementary to the template by using nucleotides (dNTPs) which are the building blocks of DNA, and PCR buffer.

1.2.2 Reaction Mechanism

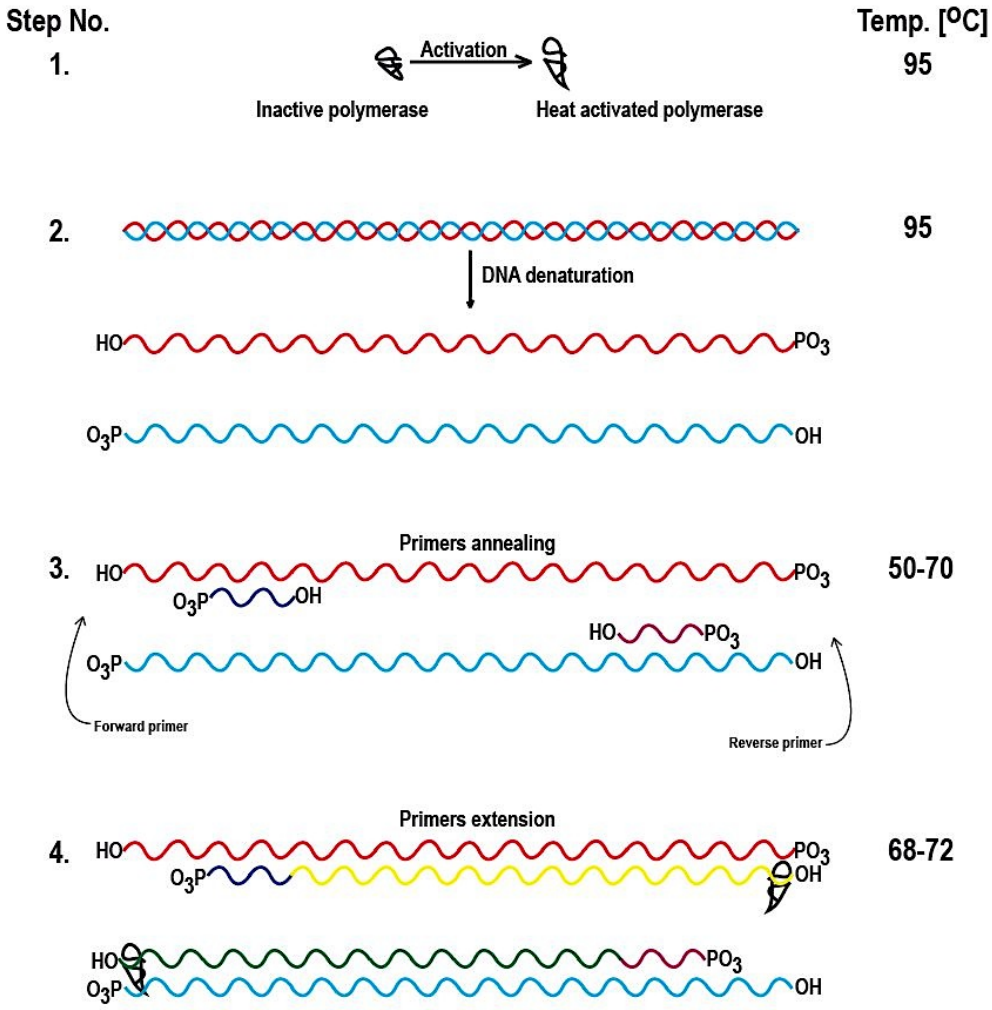

Figure 1.4: Schematic of a typical Polymerase Chain Reaction. (Source: bio-ggs.blogspot.com)

This reaction relies on temperature cycling and has multiple steps. The first step is the *denaturation step* where the temperature is increased above 90°C to denature/melt the DNA by producing single stranded DNA. The next step is the *annealing step* in which, the temperature is lowered to 50-65 °C depending on the primer set. The primers anneal complementary to the segment of interest of the DNA strand and the enzyme binds to the primer-DNA complex. The third step is the *extension/elongation step* wherein, the temperature is increased to 70-75°C depending on the enzyme. *Taq* polymerase has maximum activity at 72°C. At this step, the DNA polymerase generates a new strand of DNA by adding dNTPs complementary to the target region. The double stranded DNA generated during this step serves as the template for the next cycle and this process is repeated until the required amplification is achieved or until the reaction components are exhausted [20, 21]. Theoretically PCR can generate billions of copies of DNA from a few copies. It is highly sensitive as it can amplify very minute amounts of DNA thereby allowing subsequent testing of the target of interest and is also highly specific due to the complementarity of the base-pairing interactions. Conventional PCR normally involves reaction volumes of 10-200 µl and is carried out in small thin-walled reaction tubes in a thermocycler (Figure 1.5a). Most commercial thermocyclers have a block that holds the reaction tubes and can be heated or cooled just by reversing the electric current (Peltier effect [22]). The amplified product is then analyzed using gel electrophoresis (Figure 1.5b) [23] to determine whether the target of the

right size has been amplified. The analysis methods may vary depending on the type of PCR performed.

However, conventional PCR is not quantitative and the results obtained during analysis may be insufficient in the diagnostic setting.

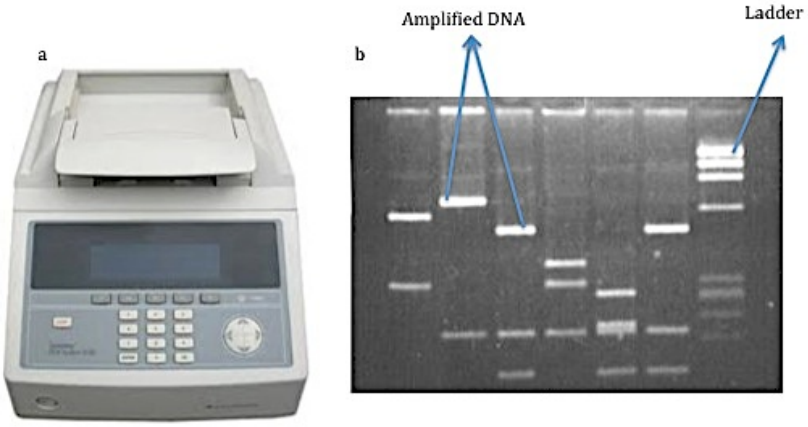

Figure 1.5: (a) Conventional Thermocycler (Applied Biosystems). (b) Gel Electrophoresis results. The DNA ladder is a molecular weight size marker that can be used to determine the size of the product. (Source: www.mun.ca)

1.2.3 Quantitative Real-Time PCR

Quantitative Real-Time PCR (qPCR) is another variant of PCR, which is used to quantitatively measure the PCR product in real time, effectively measuring the amount of amplified product generated at each cycle of PCR. Quantitative PCR makes use of fluorescent dyes such as SYBR Green, LC Green or

fluorophore containing DNA probes, to measure the quantity of the amplified product in real time. These dyes bind to double stranded DNA and fluoresce when bound. Hence, as the amount of DNA increases, the fluorescence intensity increases thereby allowing us to quantify the amount of DNA in real time. Real time PCR has several advantages over conventional PCR in terms of the number of steps required in the amplification and analysis of the sample and consequently helps in reducing the costs in terms of labor and materials. Real time PCR also helps in avoiding the contamination that may be introduced between the amplification and analysis steps in conventional PCR, as it requires to user interference between these steps.

Real time PCR machines such as the Lightcycler (Roche Diagnostics Inc.) [24] (Figure 1.6) combine amplification and detection of the specific product by fluorescence, thereby speeding up the process [18]. The Lightcycler determines the threshold values where the fluorescence of PCR rises above the background signal [25, 26], thereby identifying the cycle number at which the DNA copy numbers begin to increase exponentially. Because the amount of PCR product is measured during stages of the amplification process when reagents are not yet limiting, the amount of product is directly proportional to the number of templates in the original sample. This is unlike conventional PCR which involves measurement of products at the end of the thermocycling, when the reation has leveled off due to reagent depletion and thus does not adequately measure the number of original templates added to

the reaction. For real-time PCR, the cycle number vs the fluorescence intensity is plotted by the software, allowing quantitation of the amount of product. However the DNA bindng dyes bind to any double stranded DNA and thus do not confirm that the correct product has been amplified.

To confirm product identity, melt curve analysis (MCA) is performed. The dye binding is lost when double stranded DNA melts. The melting temperature (temperature at which the denaturation/melting of DNA occurs) of a given DNA product depends on its size and its GC content, making this a suitably accurate method to confirm that the correct product has been amplified. Melt curves are obtained by measuring the decrease in fluorescence intensity due to the denaturation of DNA. The temperature at which there is an exponential decrease in fluorescence is referred to as the melt temperature (Tm) of the product. The melt temperatures are product specific and can be used to identify if the correct product is amplified. The temperature versus the negative first derivative of the melt curves plotted by the Lightcycler software represents the melt peaks. Narrow peaks represent a properly amplified product while broad peaks suggest the presence of large or unwanted amplification products. The predicted peak for a given product can then be compared to the actual peak obtained in the reaction.

Figure 1.6: Lightcycler 2.0 (Roche Diagnostics

(Source: www.perkinelmer.com)

Quantitative PCR instruments that are commercially available are expensive (≈ $10,000) and require skilled professionals to handle the systems. In order to make this technology accessible at point of care, the entire system must be miniaturized and automated. Miniaturization of the system will help in reducing costs by reducing the consumption of reagents, decreasing analysis times, increasing efficiency and also enabling automation [27]. Several attempts have been made to adapt qPCR for low-cost, point of care testing in low-resource settings. One of the most common approaches that is widely taken to develop such technologies is the Lab-on-a-Chip approach.

1.3 Lab on a Chip

A lab-on-a-chip (LOC) is a miniaturized device that integrates one or several laboratory functions such as sample preparation, nucleic acid amplification and detection on a single chip. The sample volumes involved in LOCs are extremely small in the order of nanoliters. There are several advantages to this approach. Low sample volumes ensure less waste and lower reagent costs. The response and analysis times are faster due to fast heating, higher surface to volume ratios and small heat capacities. Most of the LOCs are compact and have multi-functionality. The small size of the LOCs helps in lowering fabrication costs. The reaction unit of LOCs can also be made disposable.

Various research groups have taken this approach in building diagnostic tools that can perform sample preparation, sample delivery, gene amplification and product detection. Liu et al. [28] developed a disposable PDMS rotary microfluidic device integrated with pneumatically controlled on-chip valves and pumps to run PCR and the sample volume required for this setup is just 12 nL. Grodzinski et al. [29] have integrated sample preparation, cell capture, cell pre-concentration and purification, cell lysis, PCR, DNA hybridization, and electrochemical detection to analyze DNA from pathogenic bacteria on a self-contained device in plastic [29]. Sun et al. developed a transparent micro-channel chip with an integrated heater for continuous-flow PCR by using quartz glass as the channel substrate and

indium-tin oxide films as thermal sources [30]. Yoon et al. fabricated Si-based micro-machined PCR chips with an integrated platinum thin-film micro-heater and a temperature sensor allowing for precise temperature control and rapid thermal cycling [31]. Rodriguez et al. [32] have achieved PCR cycling times of 16s/cycle by integrating silicon micro-fabricated PCR chambers with aluminum heaters and temperature sensors onto a glass cover. Chou and co-workers designed and fabricated the first miniaturized cyclic PCR device in low temperature ceramics [33]. Obeid et al. developed a microfabricated, reusable glass chip for the functional integration of reverse transcription and PCR in a continuous-flow mode that allowed for the selection of the number of amplification cycles [34]. Jabasini et al carried out multiplex PCR on a 12-channel microchip electrophoresis system and were able to detect 72 markers simultaneously in approximately 100 s [35]. Cepheid developed a miniature analytical thermal cycling instrument (MATCI) that consists of silicon-micromachined reaction chambers with integrated heaters, optical windows, and diode-based fluorescence detection[36]. Kaigala et al. [2] developed a 3 layered glass-polydimethylsiloxane (PDMS)-glass chips with an integrated platinum heater which has the capability to perform both PCR and capillary electrophoresis on peltier cycle based heating module call the Tri-corder Tool Kit (TTK) (Figure 1.7). There are several other technologies with on-chip PCR amplification and detection capabilities that have been developed [32-35].

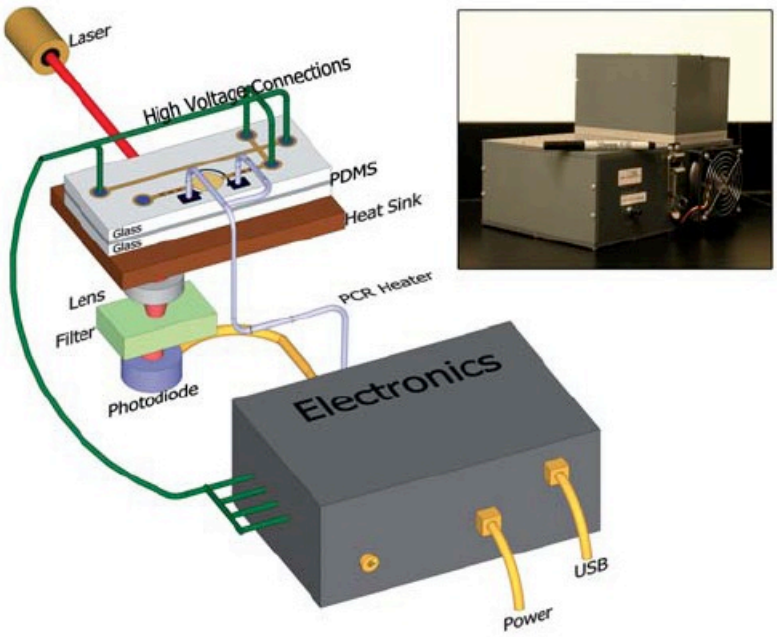

Figure 1.7: Schematic of the Tricorder Tool Kit [2]. Picture is taken from the reference.

Most of these technologies require complex mechanics such as the use of silicon rubber-based or PDMS based valving and pumping in order to facilitate the movement of samples. Although these approaches are potentially powerful, challenges may arise due to pressure seal and/or evaporation, pressure buffering, chemical interference through surface interactions, and evaporation/contamination via the porous, gas permeable membranes used in pumps and valves [1].

Another approach adapted by certain groups involves the amplification of nucleic acids in an immobilized solid medium. The concept of "PCR in a solid media" was first introduced in 1997 by Chetverin [37]. This method differs from the conventional methods of nucleic acid amplification, in the sense that it is performed in an immobilized solid medium instead of a liquid medium. Their invention is based on the hypothesis that nucleic acid molecules can be, like microorganisms, grown as colonies in an immobilized medium. The advantages of using a solid medium over liquid media are:

- The entrapment of reactants and products within a solid matrix can ensure the prevention of heat transfer by convection.
- The templates and reaction products can be restricted to limited zones at fixed locations of the reaction volume.
- Several samples can be tested simultaneously.
- The possibilities of contamination are lowered to a great extent because the reaction units are physically separated.

This approach was also pursued by the Atrazhev et al. [1] in the development of their in-gel technology for gene amplification. This technology is the main focus of my thesis.

1.4 In-gel technology for Gene Amplification

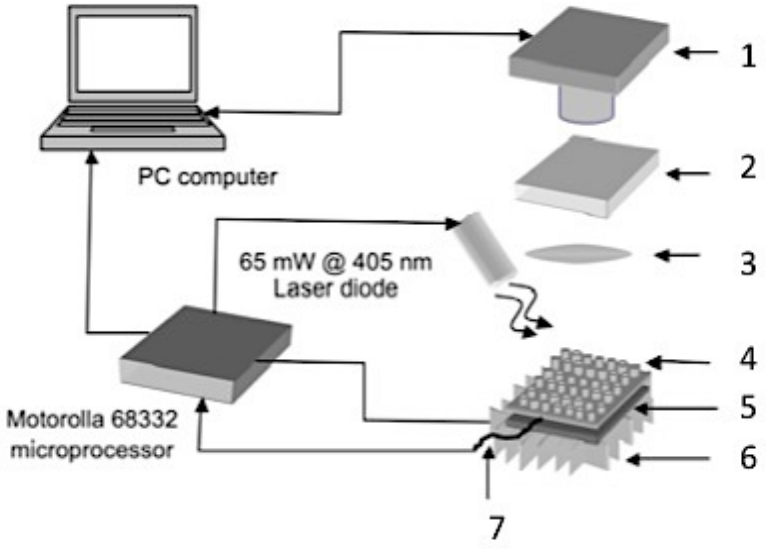

Figure 1.8: Infrastructure needed to operated the In-gel Gene amplification system [1]. 1. Picture taken from the reference. 1. Meade Deep Sky Imager. 2. Chroma HQ510/50m optical filter. 3. Newport 25.4mm efl lens. 4. Hydrogel array 5. Marlow XL T2398-01L Peltier. 6. Heat sink. 7. Temperature sensor.

This hydrogel technology makes use of gel posts, which are an array of nanoliter size polyacrylamide gel chambers, which contain the polymerase chain reaction (PCR) reagents and serve as reaction vessels for PCR amplification. Polyacrylamide gel and PCR reagents are photopolymerized in a mold to create an array of semisolid posts that serve as reaction vessels for parallel PCR amplification of an externally added template.

The key features of this system are that it consists of an array of polyacrylamide gel posts within a mold. These posts contain all the reagents required for performing a successful PCR (enzyme, primers, buffer, dye, dNTPs). The template DNA can be added before the reaction or during the preparation of the gel. The gel post assembly is then placed on the peltier heating system shown in Figure 1.8. The gel posts are overlaid with mineral oil in order to avoid drying up of these posts during PCR. PCR and Melt Curve Analysis (MCA)[38] are then performed on this system.

1.4.1 Current fabrication of the in-gel amplification system

The PCR reagents namely Taq Polymerase, primers, dNTPs, buffer, and gel reagents namely acrylamide, azobis, TEMED (initiators for Acrylamide polymerization), LC Green Plus (fluorescent dye for detection) are mixed, degassed and pipetted into the wells of the mold (an array of wells with a diameter of 1mm). The mold is then covered with a cover glass and introduced into the photopolymerization chamber. The template DNA can be added prior to photopolymerization or after photopolymerization. After the addition of the template DNA, the mold is overlaid with oil in order to avoid drying of the gels during PCR. This assembly is now introduced onto the peltier heating system shown in Fig 1.8. PCR and MCA are subsequently performed.

However, for an ideal “sample in result out” system, it is necessary for the chip to be readily usable without any manual steps. The reagents entrapped

within the gel must be inactive and intact during the period of storage and must have their activity restored when required. It is also required that the reagents be stable despite any variations in the surrounding temperature and humidity conditions. It is not possible to maintain the stability of the reagents in a liquid state at room temperature. The enzyme Taq polymerase is active at room temperature and will lead to non-specific reactions, which may adversely affect our PCR.

In order to ensure that the PCR reagents within the gel posts which include the primers, dNTPs and the Taq polymerase, stay intact and inactive during long term storage, it is necessary for them to be in a dry state. The activity of these reagents should be restored upon rehydration.

1.5 Methods for the preservation of reagents

1.5.1. Lyophilization/Freeze-Drying

Lyophilization is one of most common methods used to dehydrate and preserve any perishable material. The principle involves freezing the material below its eutectic temperature (the temperature at which the solid and liquid phases are in a thermal equilibrium) and then sublimating the frozen water present in the material by lowering the pressure and sufficiently increasing the temperature. The process mainly consists of three steps:

1. Freezing: During this step, the material that needs to be dehydrated is cooled below its eutectic temperature (temperature at which both the solid phase and liquid phase of the material are in a chemical equilibrium) and frozen. The freezing temperatures generally range from -40°C to -80°C.

2. Primary drying: During this step, the chamber pressure is lowered to the order of a few millibars and the temperature is increased slightly above the eutectic point, thereby allowing the liquid phase to sublimate. This step occurs at a very slow rate and the time required can range from a couple of hours to a few days depending on the volume and type of material. About 90% of water can be removed during this stage.

3. Secondary drying: During this step, the temperature is further increased stepwise above the eutectic temperature to remove the strongly bound water molecules. The duration of this step may vary depending on the residual

water content that is needed to achieve stable storage [39].

Figure 1.9: Virtis 2.0 Freeze-Dryer (Source: www.spscientific.com)

Lyophilization is an effective method to achieve an optimal dry state for the reagents in terms of residual water levels. The advantages with this method are that the reagents remain in a dry state in the absence of a moisture source. In most cases, the dried reagents can be stored at the room temperature. The reagents also have a longer shelf life as they remain inactive in a dry state. This method works perfectly for reagents in liquid form. However, this method requires freezing which is known to cause major structural changes when applied to gels [40]. There is a major variation in the polymer network resulting in an increased pore size of the gels. These variations may adversely alter the structure of the enzyme rendering it inactive. These structural changes will also affect the diffusion rates of primers and other reagents while performing PCR, which have already been

optimized for the gel post system. These changes arising due to the structural changes in the gel may have negative affects on our gel PCR system.

1.5.2 Spray Drying

Spray drying is a preservation technique generally used to preserve food products, which makes use of a hot gas to rapidly dry and produce a dry powder. A spray takes a liquid stream and separates the solute or suspension as a solid and the solvent into a vapor. The solid is usually collected in a drum or cyclone. The liquid input stream is sprayed through a nozzle into a hot vapor stream and vaporized. Solids form as moisture quickly leaves the droplets. A nozzle is usually used to make the droplets as small as possible, maximizing heat transfer and the rate of water vaporization. Although spray dryers can dry a product very quickly compared to other methods of drying, they are suited only for liquid systems and cannot applied to gels. Moreover, exposure of PCR reagents to hot gases may be detrimental to the stability of the reagents [41, 42].

Figure 1.10: Industry scale spray-dryer (Source: www.thespraydryer.com)

1.5.3 Vacuum Desiccation

Desiccation is the state of extreme dryness, or the process of extreme drying. This state of dryness can be achieved in an open air where the moisture levels are low or can be achieved in a vacuum desiccator. A vacuum desiccator is an enclosed chamber with absolutely no air present. The temperatures in the vacuum desiccator can be varied according to our requirements.

Figure 1.11: Vacuum Oven (Model 282A, Fisher Scientific).

(Source: www.fisherscientific.com)

There have been several reports on the desiccation of gels in air [37, 40]. Preliminary experiments on desiccating polyacrylamide gels in air have also been performed by the Microfluidics Development Group (MDG, University of Alberta) and have been successful. This treatment removes 99% of water from gels. These air-dried gels, when observed under a microscope do not show any alterations in their structure. However detailed analyses have to be done to confirm these observations. With the optimal combination of stabilizers, we may also be able to preserve the activity of the enzyme during desiccation. However, air-drying takes longer and is not a fully controlled drying procedure in the sense that there are many variables in open air. The moisture levels and the temperature conditions are not constant in open air. Moreover, the enzyme is active at room temperature when there is sufficient moisture around it. This may lead to many non-specific reactions during PCR such as oxidation in the presence of oxygen. Exposing the gel with entrapped PCR reagents to open air for such long durations may also lead to contamination. A vacuum desiccator can offer a controlled environment for desiccating the gels. The temperatures do not touch the negative values and hence prevents the freezing of the gels. The process is very fast and does not require exposing the gels to an open environment for long durations.

There have been a few reports on preserving PCR reagents. *Lyophilized reagent for PCR* [43] invention provides a lyophilized PCR formulation which includes a stabilizing and a sedimenting agent. This invention presents the

preservation of PCR reagents at the macro scale. They make use of trehalose and some poly-ols like sorbitol as stabilizers during lyophilization. The methods followed here are mainly designed for liquid media and may not be suitable for solid gels. The preservation of the structure of the gels also becomes an important factor in case of solid gels. However, some aspects of this invention may be useful in determining the best combination of stabilizers for this thesis. Wolff et al. [44]have used gelification techniques developed by Biotools (Biotools, B&M labs, Madrid, Spain) to preserve reagents on chip. However, their methods are also targeted towards liquid media and also their lyophilized product must be stored between -20^{o}C and 4^{o}C. The combination or the concentration of the lyoprotectants used by them may not be optimal for the preservation of PCR reagents at room temperature. The residual moisture levels may also be high and not optimal for storage at ambient temperatures. Such a system may not be applicable to be used in the field as the temperature and humidity conditions vary from region to region and constantly maintaining the temperature of the reagents at -20^{o}C becomes a problem. Chetverin et al [45] have also performed some preliminary experiments on drying PCR reagents entrapped within a gel. They have demonstrated the proof of concept that the PCR reagents can be added onto a dried polyacrylamide gel, which is followed by the diffusion of the reagents into the gel. However, their techniques have not been targeted towards the preservation of the gels containing the entrapped PCR reagents and they are not standardized and optimized for different applications.

Detailed studies on the shelf life of these reagents have not been performed. The key aspect in the preservation of reagents by drying is the development of a correlation between the residual moisture levels, the concentrations and combinations of lyoprotectants and the stability of the reagents. This aspect has not been explored in great detail in the context of preservation of PCR reagents entrapped within a gel.

1.5.3.1 Parameters involved in desiccation

There are several factors that affect the stability of dried protein formulations during storage such as storage temperature, glass transition temperature, residual water content, combination and concentration of lyoprotectants.

Storage Temperature

Storage temperature is one of the most important factors that effects dried protein formulations. Storage of protein formulations at high temperatures leads to an increased mobility of protein molecules, thereby facilitating protein – protein interactions [39].

Glass Transition temperature (Tg)

*T*g of a polymer is defined as the transition temperature between the rubbery (or liquid-like) and glassy (solid-like) states. Glass transition temperature (*T*g) of protein formulations is considered to be one of the major determinants of protein stability [46].

Lyoprotectants

A lyoprotectant is a substance, which is used to protect biological molecules from damage during drying.

Residual water content

Residual water content is the water that remains in the sample after drying.

A detailed description and effects of these parameters on the desiccation process will be discussed in further chapters.

1.6 Thesis Overview

To carry out complex blood tests for detection of pathogens requires skilled labor and well-equipped laboratories. However, it is difficult to have such facilities in low resource settings. We need to develop instruments that can perform field operations without involving skilled labor and in a cost effective manner. The instrument should not require any human intervention. The instrument should be able to perform sample preparation, DNA amplification and analysis in an entirely automated fashion. So far microfluidic chips, which are cost-efficient, have been successfully developed for various medical applications. However, there are complications and issues concerned with these systems, which might include, for example, reagent flow system, cross contamination and sieving matrix. Most of these chips also require the user to prepare the mixtures required to perform PCR,

and need complex strategies for valving and pumping with complex instrumentation. This process requires experienced and well-trained labor, which may not be available in low resource settings.

The in-gel PCR technology presented here does not require any complex mechanics and reduces the cost of the instrumentation. The In-gel PCR system has already been proven to effectively perform PCR and MCA analysis and has been successfully tested by our group to detect malaria, BK virus and other pathogens. Development of methods for preservation of the gel posts containing the PCR reagents at ambient temperatures helps us in designing a ready to use system. This system does not require the user to prepare any mixtures. The in-gel PCR technology coupled with long term storage of the PCR amplification module can prove to be a very effective and sensitive "sample in answer out system", that can be potentially used at the point of care or near point of care.

Hypothesis

That the gel-based PCR formulations can be desiccated and preserved for more than ten weeks at ambient temperatures with a compatible combination of lyoprotectants and residual water content.

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Chapter 2: Evaluation of Trehalose and Sucrose as Lyoprotectants for the Desiccation of Gel PCR Systems

2.1 Introduction

A lyoprotectant is a substance, which is used to protect biological molecules from damage during drying. Lyoprotectants must be able to form hydrogen bonds with the protein that needs to be stabilized. They are generally poly-ols or polysachharides, which have a hydroxyl group like ethylene glycol, trehalose and sucrose. Proteins/enzymes have intramolecular interactions with the surrounding groups and intermolecular interactions with solvent in a solution [1]. Both these interactions affect the stability of the protein and have to be taken into account during dehydration. Lyoprotectants stabilize the protein during dehydration. There are two hypotheses that have been proposed to explain the mechanism of protein stabilization by lyoprotectants. The first hypothesis is the "Glass Dynamics hypothesis" [2], which states that the stabilizers form a rigid matrix around the proteins thereby coupling the motion of the proteins to the motion of the matrix. This greatly reduces the mobility of the protein and hence minimizes molecular interactions thereby maintaining the stability of the protein during and after drying. The second hypothesis is the "Water replacement hypothesis" [3], which states that the lyoprotectants also function by forming hydrogen bonds with biological molecules as water molecules are displaced. Hydrogen bonding in aqueous solutions is important for proper protein function. Thus, as the lyoprotectant replaces the water molecules, the proteins retain their native physiological

structure and function, although they are no longer immersed in an aqueous environment.

In the context of preserving protein formulations, the most common lyoprotectants that have been used in the past are trehalose, sucrose, sorbitol and mannitol. After a thorough review of the literature pertaining lyoprotectants, trehalose and sucrose were chosen to act as stabilizers to preserve the native structure of the enzyme Taq polymerase. Crowe et al [4] have shown that the protein stabilization is influenced not only by the sugar moiety but also by the orientation of the subunit. They have also observed that the addition of trehalose and sucrose to the protein formulations has prevented a conformational change in the protein as opposed to the protein formulations without these stabilizers [5, 6]. Acker et al [7] have demonstrated that membrane integrity (MI) values were high for cells loaded with internal trehalose and low for cells with no internal trehalose, after drying. The addition of sucrose completely inhibited the aggregation of acidic fibroblast growth factor (aFGF) [8] indicating that sugars help in reducing protein aggregation. The recovery of PFK activity during freeze-thawing increased with the increase in the concentration of trehalose in the formulation [9]. Carpenter et al [4] have reported that the optimal stabilization of proteins during drying is better achieved with low molecular weight saccharides like trehalose and sucrose. This may be due to the fact that the long chain lengths of larger saccharides may interfere with

intermolecular hydrogen bonding between the sugars and proteins [10].

A minimum concentration of lyoprotectants is necessary to confer stability to the proteins during drying. Trehalose and glucose at a concentration of 5 – 100 mM, were both ineffective in protecting LDH to a significant level during lyophilization [11]. However, a higher concentration of sugars does not always ensure an increase in protein stability. The freeze-drying recovery of PFK activity increased to 65% with an increase in trehalose concentration upto 150 $mgml^{-1}$ but a further increase in trehalose concentration gradually reduced the protein recovery [9, 12]. The stabilizing effects of sugars also depend on their physical properties and the composition and concentration of the formulations [10]. Trehalose also helps in lowering the melting temperature of the template DNA thereby making it easier to attain the single stranded state, which favors the polymerase chain reaction [4, 9, 12]. Trehalose prevents the denaturation of the enzyme Taq polymerase during drying by forming hydrogen bonds with the enzyme and is also responsible for thermal stabilization of the enzyme after drying. Trehalose also protects the PCR formulation by forming a stable glassy matrix around the constituents of the formulation thereby restricting the mobility of the enzyme and preventing crystallization [13, 14].

Hence, we hypothesized that a combination of both trehalose and sucrose will result in the most stable formulation for carrying of the desiccation or PCR reagents in a gel matrix.

2.2 Objective

To determine a compatible combination and concentration of lyoprotectants required for the preservation of the gel PCR reagents. For this chapter, the efficacy of lyoprotectants in preserving PCR activity of desiccated gel-enclosed reagents was tested using PCR amplification of BK virus templates, followed by melting curve analysis to confirm product identity.

2.3 Materials and Methods:

2.3.1 Quantitative PCR on Light cycler

In order to study the influence of Trehalose on PCR, quantitative PCR was performed on a Lightcycler (V2.0 Roche Diagnostics) with varying concentrations of trehalose to detect BK Virus (BKV). BK Virus is a human polyoma virus that can be accurately detected even when the sample volumes are minute [15, 16].

A formulation of 212.8 µl PCR mix was prepared with 11.2 µl Taq Polymerase (20 units/ µl) (Taq polymerase was prepared in our laboratory and verified to have PCR activity as well as acceptable accuracy, defined as a low introduced mutation rate), 11.2 µl each of 10 µM BKV forward and reverse primers (to produce 100 bp product), 11.2 µl 10mM dNTP, 11.2 µl 1% BSA (Sigma), 56 µl 5 X PCR buffer (333 mM tris-sulfate, pH 8.6, 83 mM $(NH4)_2SO_4$ (Sigma) and 40% sucrose (Sigma, Lot#BCBB7807)), 11.2 µl 50mM $MgCl_2$, 56 µl 10x LC Green Plus (Idaho Technology Inc., Salt Lake City, Utah). Seven centrifuge tubes were taken and to each of these tubes, 30.4 µl of this

formulation, 1.5 M trehalose (Cargill Foods Inc., Cat No. 2K141) and water were added to make 80 ul PCR formulations. The final concentration of trehalose ranged from 0 – 0.6 M between tubes 1 through 7 with an increment of 0.1M. Three LightCycler (V2.0 Roche Diagnostics) capillaries were prepared for each of these formulations, two of which contained 19.5 µl of the formulation and 0.5 µl of template DNA (BKV (10^8 copies/ µl)). The third capillary served as the negative control, which contained 19.5 µl of PCR formulation and 0.5 µl water. These capillaries were then introduced into the Lightcycler to perform PCR with the following parameters: predenaturation at 94 ^oC for 120 s, denaturation at 94 ^oC for 10 s, Anneal at 58 ^oC for 20 s, extension at 72 ^oC for 20 s for 35 cycles. The final elongation step was performed at 72 ^oC for 120 s. Melting curve analysis within the temperature range of 55 ^oC - 95 ^oC , was also performed on these samples. Seven replicates of this experiment were performed. The same procedures were followed to study the influence of sucrose (concentration range 0 – 0.6M) on PCR.

Primer Sequence:

Forward Primer: BKV -F 100bp 5'-TTC CTT TTT GCT AAG TGA CC-3'

Reverse Primer: BKV- Rani 5'-TCA AAC ACC CTA ACC TCT TCT ACC TG-3'

DNA Samples: DNA samples (purified from urine samples of patients infected with BKV) were obtained from the Provincial Laboratories of Public Health in Edmonton, Alberta, the consent for which has been obtained by our group.

The lightcycler software generates Cp (cycle number at which fluorescence rises above threshold value) values by applying curve fitting and determining the threshold values where the fluorescence of PCR rises above the background signal [17], thereby indicating the cycle number (represented by Cp value) at which the DNA copy numbers can be seen to increase exponentially. The cycle number vs the fluorescence intensity plotted by the software during real-time PCR for different samples represent the PCR curves and the melt curves were obtained by measuring the decrease in fluorescence intensity due to the denaturation of DNA when heated from 55 ^{o}C - 95 ^{o}C .

The results were analyzed based on the Cp values generated by the Lightcycler software (version 4.1) during PCR, and melt temperatures and melt peak heights during MCA. Assuming that the data obtained from the experiments follows the Gaussian distribution, Grubb's Test [18, 19], which computes the largest absolute deviation from the sample mean in units of the sample standard deviation, was performed to detect and remove any outliers (unusual observations which are distant from the rest of the data) in the data. The mean Cp values, melt peak heights and melt temperatures and their standard deviations were then computed. One-way ANOVA [20] was then

performed on the computed means to test if the mean values obtained with varying concentrations of sugars are equal with Tukey's Post hoc [20] analysis.

2.3.2 In-gel PCR with trehalose and sucrose

2.3.2.1 Gel PCR mix preparation

Fifty-nine µl PCR mix was prepared with 44 µl PCR reagents and 15 µl gel reagents. The PCR reagents were: 3 µl Taq Polymerase (20 units/ µl), 2 µl each of 10 µM BKV forward and reverse primers to produce 100 bp product, 2 µl 10mM dNTP, 2 µl 1% BSA, 20 µl 5 X PCR buffer, 4 µl 50mM $MgCl_2$, 10 µl 10xLC Green Plus. The gel reagents were: 10 µl of 40% acrylamide (Sigma, cat no. A9099) + 4% bis-acrylamide aqueous solution (N,N-methylene bis-acrylamide, BioRad, Hercules, CA, cat no. BA05-1610201), 2 µl of 3% azobis (Wako, Richmond, cat no. VA-086) and 1 µl of 10% TEMED (N,N,N',N'-tetramethyleneethylenediamine, Sigma, cat no. T7024). This mix was then equally divided into two centrifuge tubes. 10 µl 1.5M trehalose, 2.5 µl 1M sucrose, 7.5 µl water was added to one tube and 20 µl water was added to the other tube, which served as the control (no sugars). The formulations were then de-gassed under vacuum for about thirty seconds. The final concentrations of trehalose and sucrose in the formulation were 0.3 M and 0.1 M respectively.

2.3.2.2 Gel Casting

The formulations were loaded onto 8x6 molds (6x4 array for the positive controls and 2x4 array for the negative controls (Figure 2.1). Each mold (14 mm x 18 mm) is made by permanently bonding two 1.1 mm thick glass slides. One of the glass slides has a 8x6 array of wells drilled into it. Although the experiment was designed to accommodate negative controls, it was observed that avoiding contamination is very difficult with the present architecture of the molds. A 22x22mm cover slip was slid on top of the mold. The mold was then introduced into the gel polymerization chamber where it was exposed to the 405 nm laser for 25 min to photopolymerize the acrylamide mix.

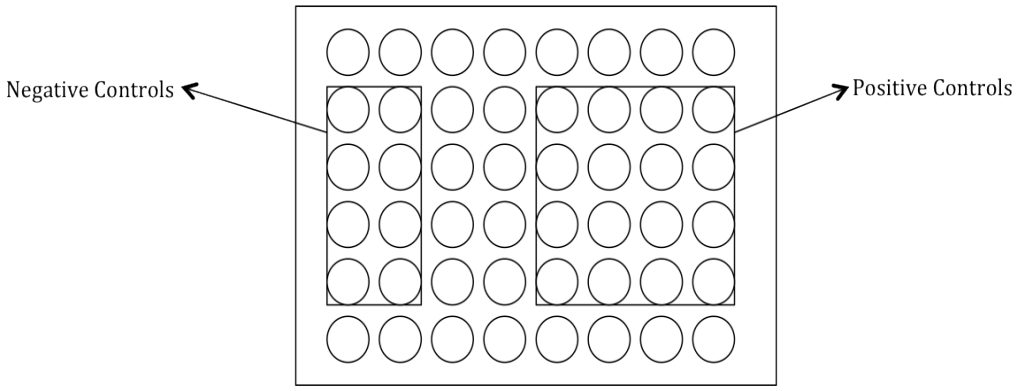


Figure 2.1: Schematic of a 6x8 mold.

After polymerization the cover slip was removed and a 10 µl mix containing 9.5 µl water and 0.5 µl BKV DNA was added on top of the gels. The mold was then left on the bench for approximately 5 min to allow diffusion of the DNA into the gels. Another cover slip was then slid on top of the mold to remove the extra liquid. The mold was then inverted and immersed in mineral oil

(Sigma, cat no. M5904), in an anodized aluminium (23x23mm) pan, which was then placed on the peltier element of the Viriloc instrument (figure 1.3). PCR was then performed with these gels, with the following parameters: predenaturation at 94 ^{o}C for 120 s, denaturation at 94 ^{o}C for 10 s, anneal at 58 ^{o}C for 20 s, extension at 72 ^{o}C for 20 s for 35 cycles. The final elongation step was performed at 72 ^{o}C for 120 s. MCA within the temperature range of 55 ^{o}C - 95 ^{o}C , was also performed on these samples. The Cp values obtained during PCR and peak heights obtained during MCA were considered for analysis.

2.3.2.3 Mold Cleaning and Decontamination

The molds were soaked for one hour in a disinfectant solution (Conflikt, Decon Labs, Inc.) in order to decontaminate. They were then washed with soap solution (Soft Soap, Colgate-Palmolive Inc.) and soaked in a 1 M KOH solution for 20 minutes to remove any left over gel particles. The molds were again washed with soap solution, cleaned with deionized water and blown dry with air to allow subsequent gel casting.

2.3.3 In-gel PCR with trehalose

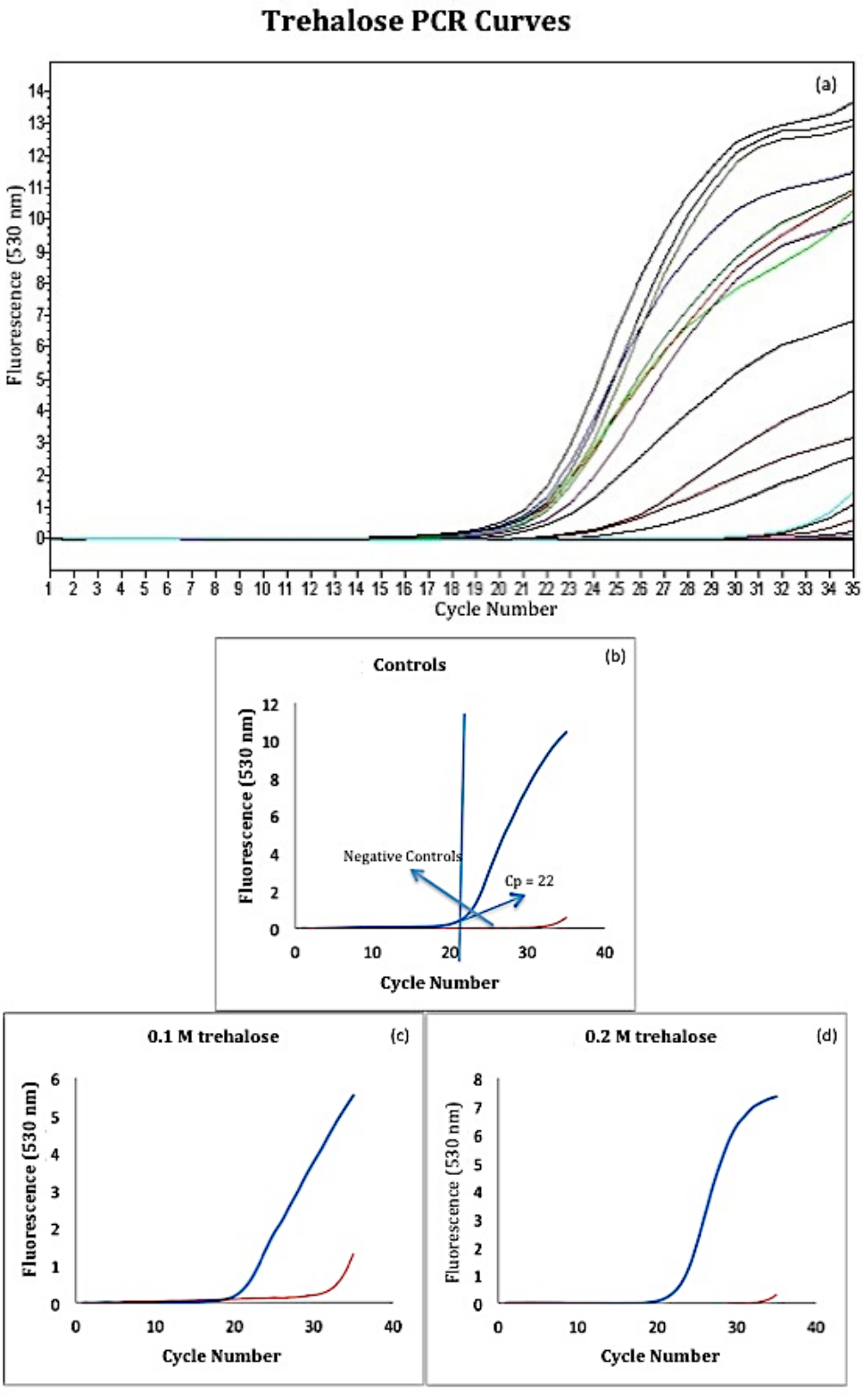
Fifty-nine µl gel PCR mix was prepared following the protocols mentioned in section 2.3.2.1. This mix was then equally divided into two centrifuge tubes. 10 µl 1.5M trehalose, 10 µl water was added to one tube and 20 µl water was added to the other tube, which served as the positive control (no trehalose).

The formulations were then de-gassed under vacuum. The final concentration of trehalose in the formulation was 0.3 M. PCR and MCA were performed with these formulations following the same protocol discussed in section 2.3.2.2.

2.4 Results and Discussion:

2.4.1 Light Cycler Real Time PCR Analysis

The cycle number vs the fluorescence intensity (530 nm) plotted by the software during real-time PCR for different samples pertaining to trehalose and sucrose for a single experiment is shown in Figure 2.2 and 2.3. As shown in the figures, there is an exponential rise in the DNA copy number after cycle 22 (Cp = 22) (Figure 2.2b and 2.3b) for the positive control (sample with no trehalose or sucrose). This rise occurs between cycle numbers 20 (Cp=20) and 22 (Cp=22) for the rest of the samples which contain increasing concentrations of trehalose (Figure 2.2 c-h) and sucrose (Figure 2.3 c-h). The figures also show that there is a small rise in fluorescence intensity after cycle 30 for the negative controls (Figure 2.2 b-h and Figure 2.3 b-h) indicating that there is no noticeable amplification in these samples.


Trehalose PCR Curves
(a)
Fluorescence (530 nm)
Cycle Number
(b)
Controls
Fluorescence (530 nm)
Negative Controls
Cp = 22
Cycle Number
(c)
0.1 M trehalose
Fluorescence (530 nm)
Cycle Number
(d)
0.2 M trehalose
Fluorescence (530 nm)
Cycle Number

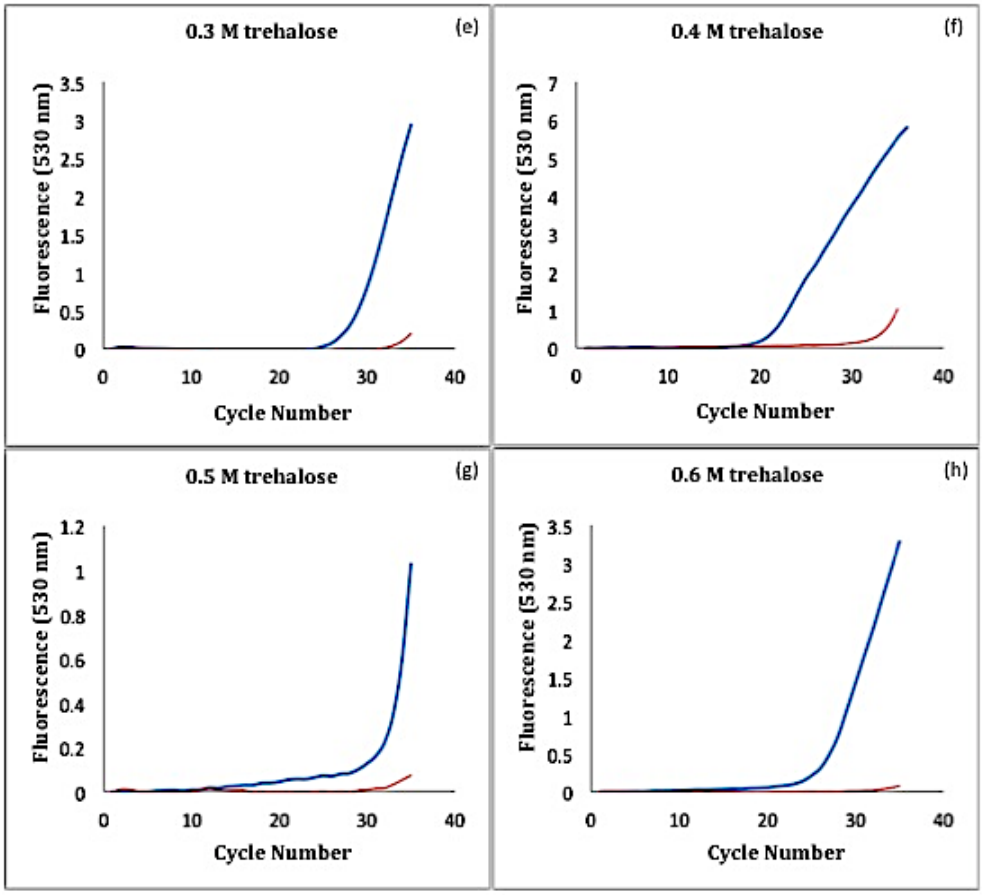
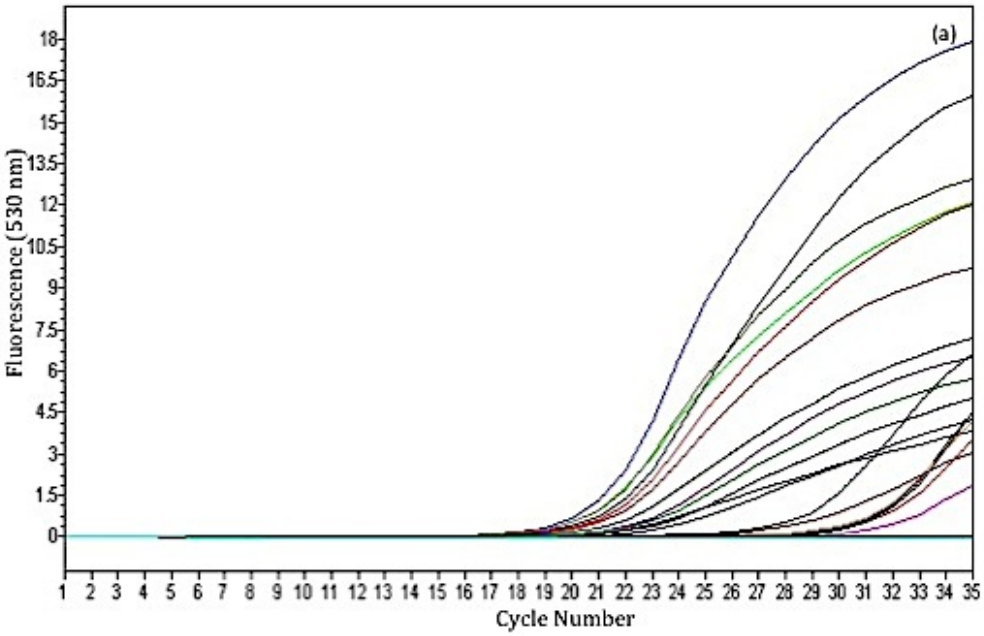
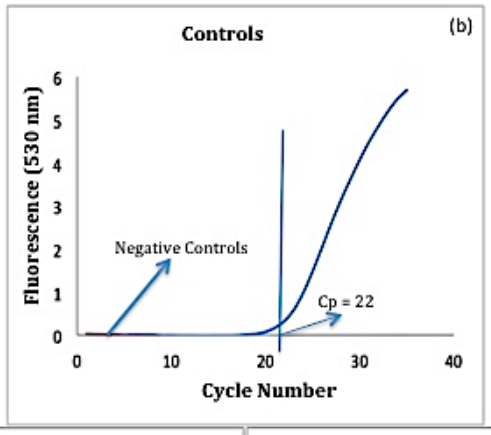
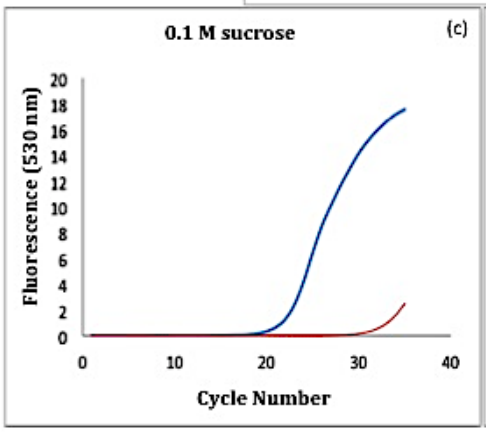


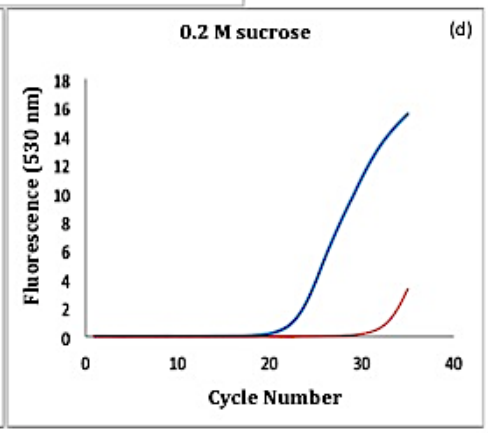
Figure 2.2: Lightcycler PCR curves with increasing concentrations of trehalose. (a) PCR curves corresponding to varying trehalose concentrations. (b) PCR curve representing the control sample (no trehalose) (Cp=22), the lower curve represents the negative controls (no template) (Cp>30). (c) PCR curves representing the sample with 0.1 M trehalose (Cp=20). (d) PCR curves representing the sample with 0.2 M trehalose (Cp=21). (e) PCR curves representing the sample with 0.3 M trehalose (Cp=25). (f) PCR curves representing the sample with 0.4 M trehalose (Cp=21). (g) PCR curves representing the sample with 0.5 M trehalose (Cp=29). (h) PCR curves representing the sample with 0.6 M trehalose (Cp=24).

Sucrose PCR Curves









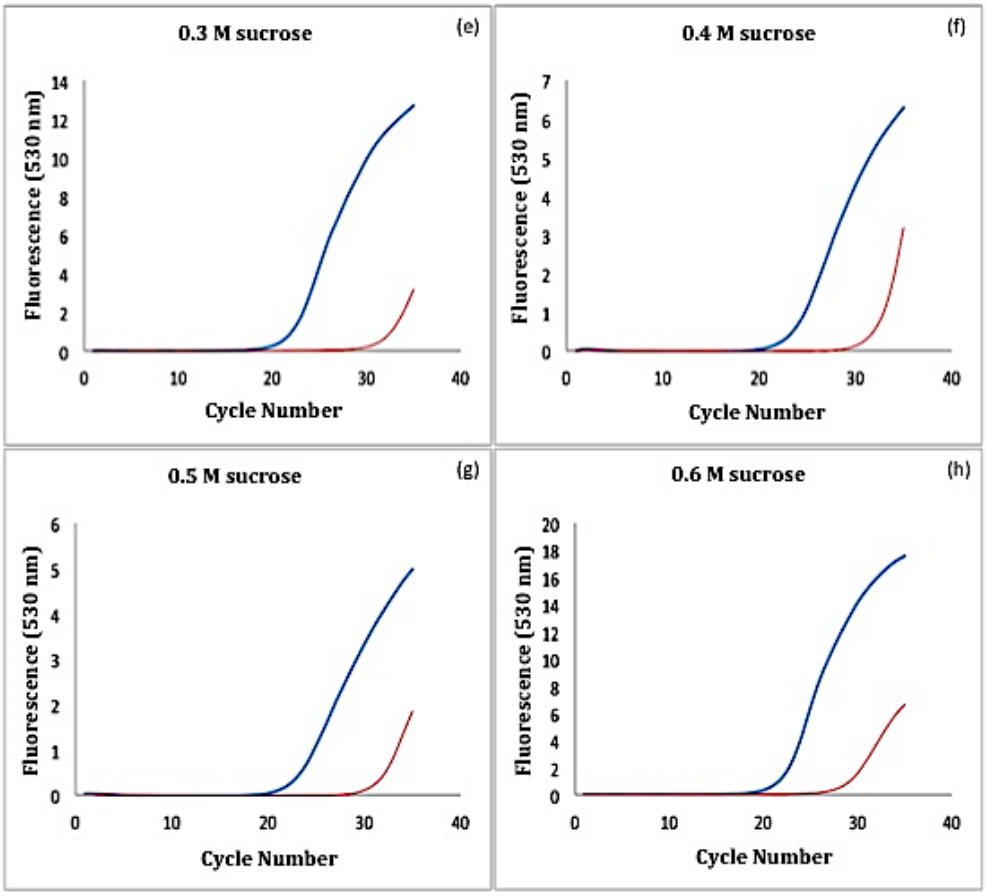


Figure 2.3: Lightcycler PCR curves with increasing concentrations of sucrose. (a) PCR curves corresponding to varying sucrose concentrations. (b) PCR curve representing the control sample (no sucrose) (Cp=22), the lower curve represents the negative controls (no template) (Cp>30). (c) PCR curves representing the sample with 0.1 M sucrose (Cp=21). (d) PCR curves representing the sample with 0.2 M sucrose (Cp=22). (e) PCR curves representing the sample with 0.3 M sucrose (Cp=22). (f) PCR curves representing the sample with 0.4 M sucrose (Cp=23). (g) PCR curves representing the sample with 0.5 M sucrose (Cp=22). (h) PCR curves representing the sample with 0.6 M sucrose (Cp=22).

The plot of the computed mean Cp values vs the sugar concentrations obtained from several replicates (n=7) for both trehalose and sucrose is presented in Figure 2.4. In the case of trehalose, there is a significant difference between the mean Cp values obtained from samples with 0.1 M and 0.3 M trehalose ($p<0.05$). However, there is no significant difference between the mean Cp values obtained from the positive controls (no trehalose) and the rest of the samples with different trehalose concentrations. In the case of sucrose (Figure 2.4), there is no significant difference between the mean Cp values obtained from the positive controls (no sucrose) and the rest of the samples containing different concentrations of sucrose.

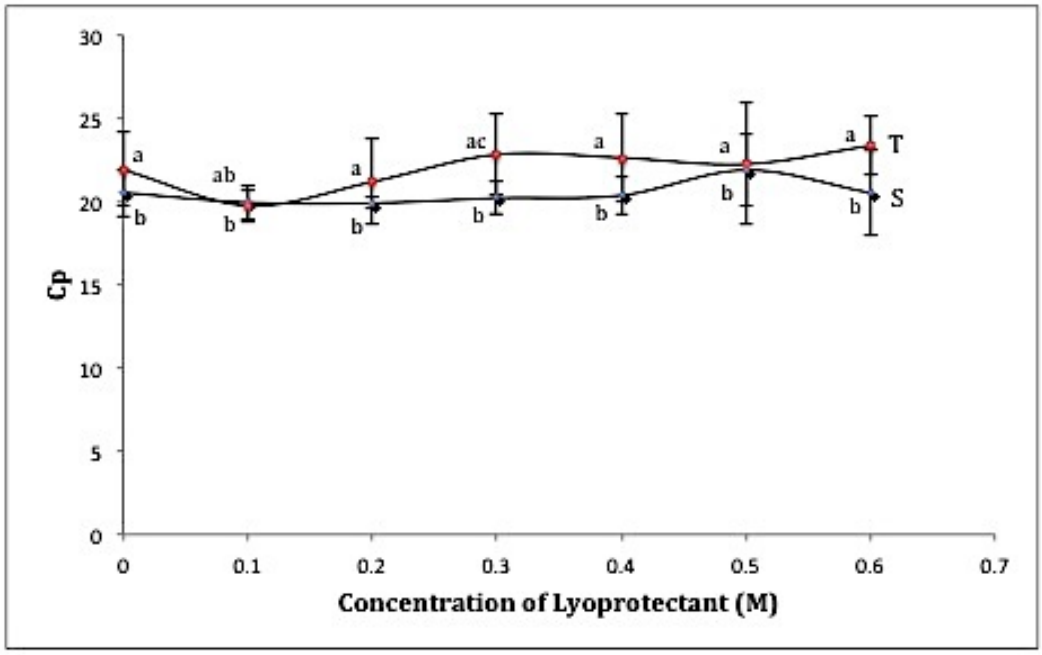
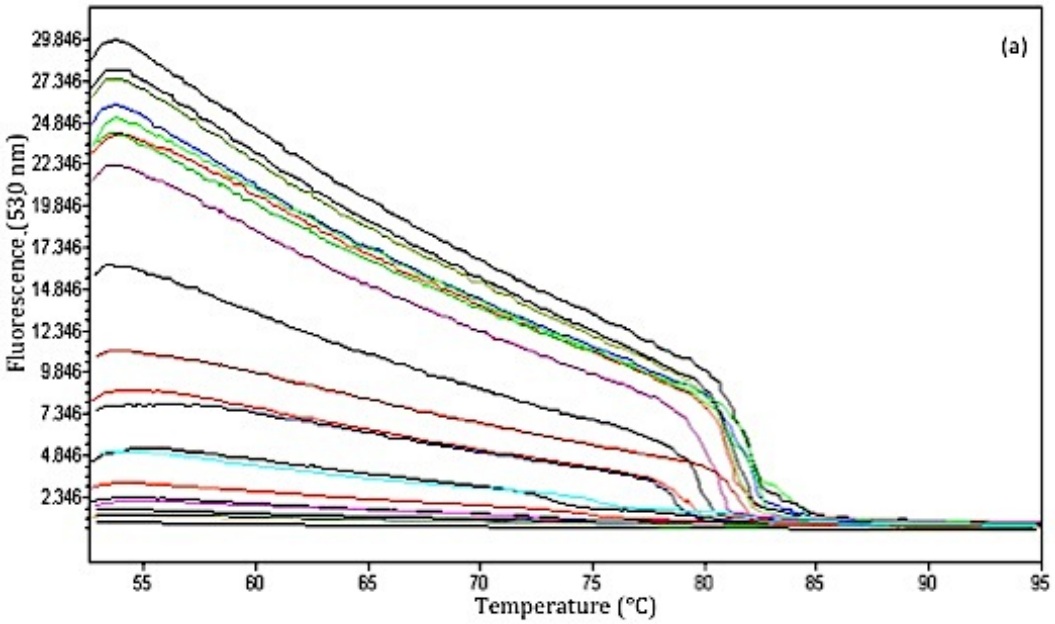


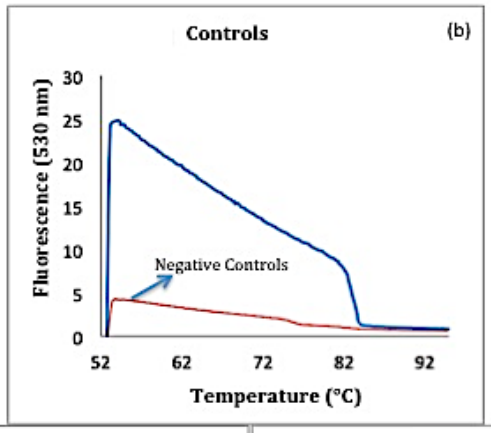
Figure 2.4: A comparative analysis of the Cp values obtained with (a) varying concentrations of trehalose and (b) varying concentrations of sucrose. The statistical significance of the data sets are represented by a,b and c which indicate significantly different data sets. For example, in case of trehalose, the data points represented by 'ab' are significantly different ($p<0.05$) from those represented by 'a'. T-trehalose, S-sucrose.

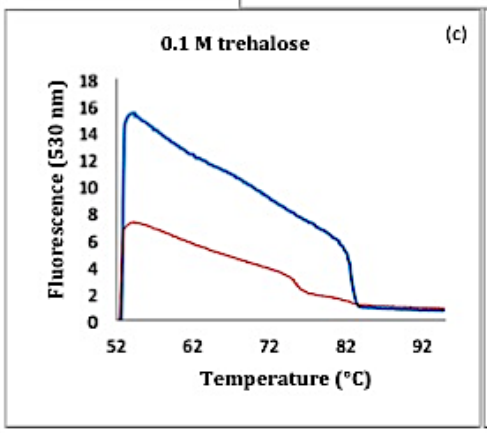
2.4.2 LightCycler Melt Curve Analysis

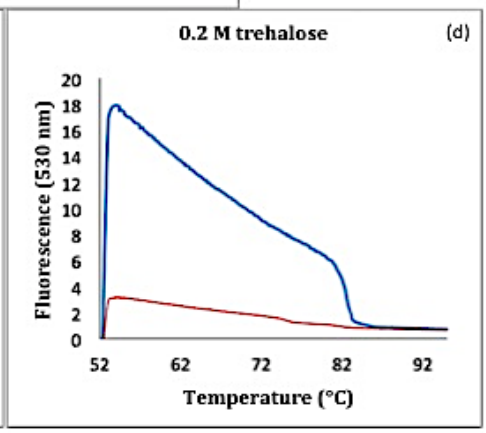
The temperature vs the fluorescence intensity plotted by the software during MCA for different samples pertaining to trehalose and sucrose for a single experiment are shown in Figures 2.5 and 2.6. As shown, there is large decrease in the fluorescence intensity for the control samples at approximately 82 ^{o}C (Figures 2.5b and 2.6b). This point shifts towards decreasing temperatures with increase in concentrations of trehalose (Figures 2.5 c-h) and sucrose (Figures 2.6 c-h) in the formulations indicating that the addition of sugars to PCR formulations has a profound influence on the melt temperatures. The figures also show that the decrease in fluorescence intensity is very minimal in the negative controls (no template DNA) indicating the absence of an amplified product (Figures 2.5 b-h and Figure 2.6 b-h).

Trehalose Melt Curves









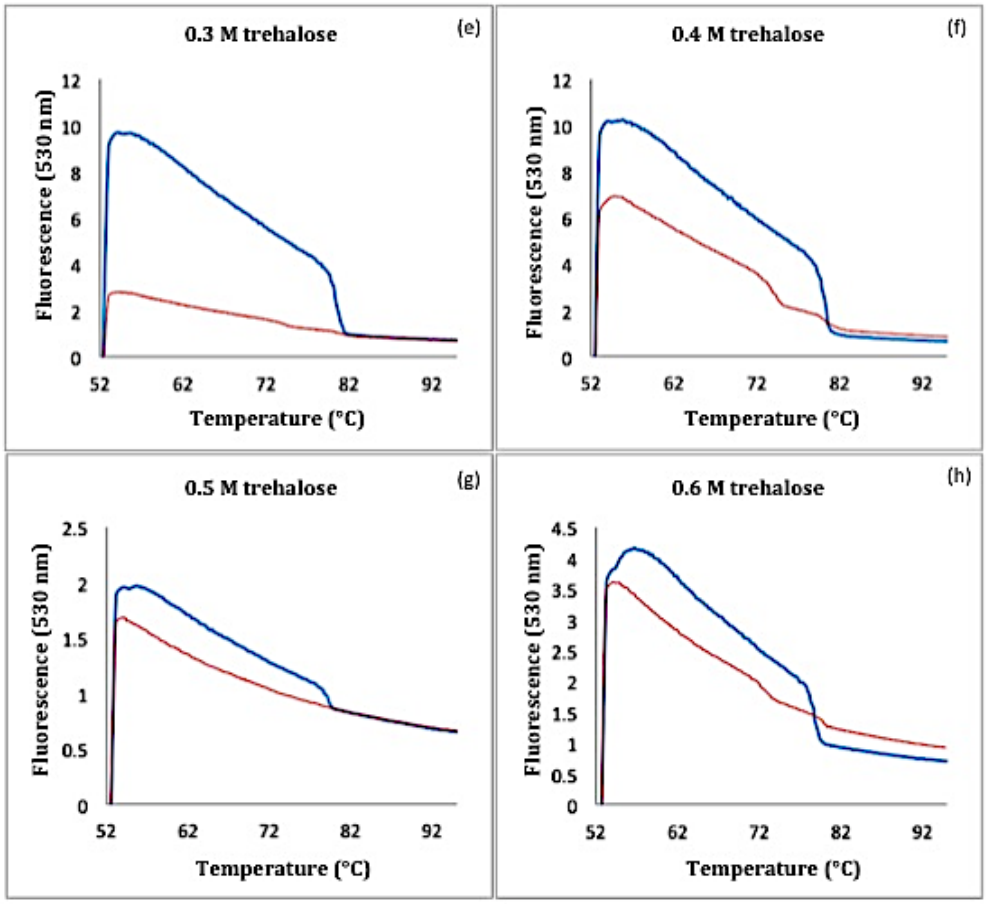
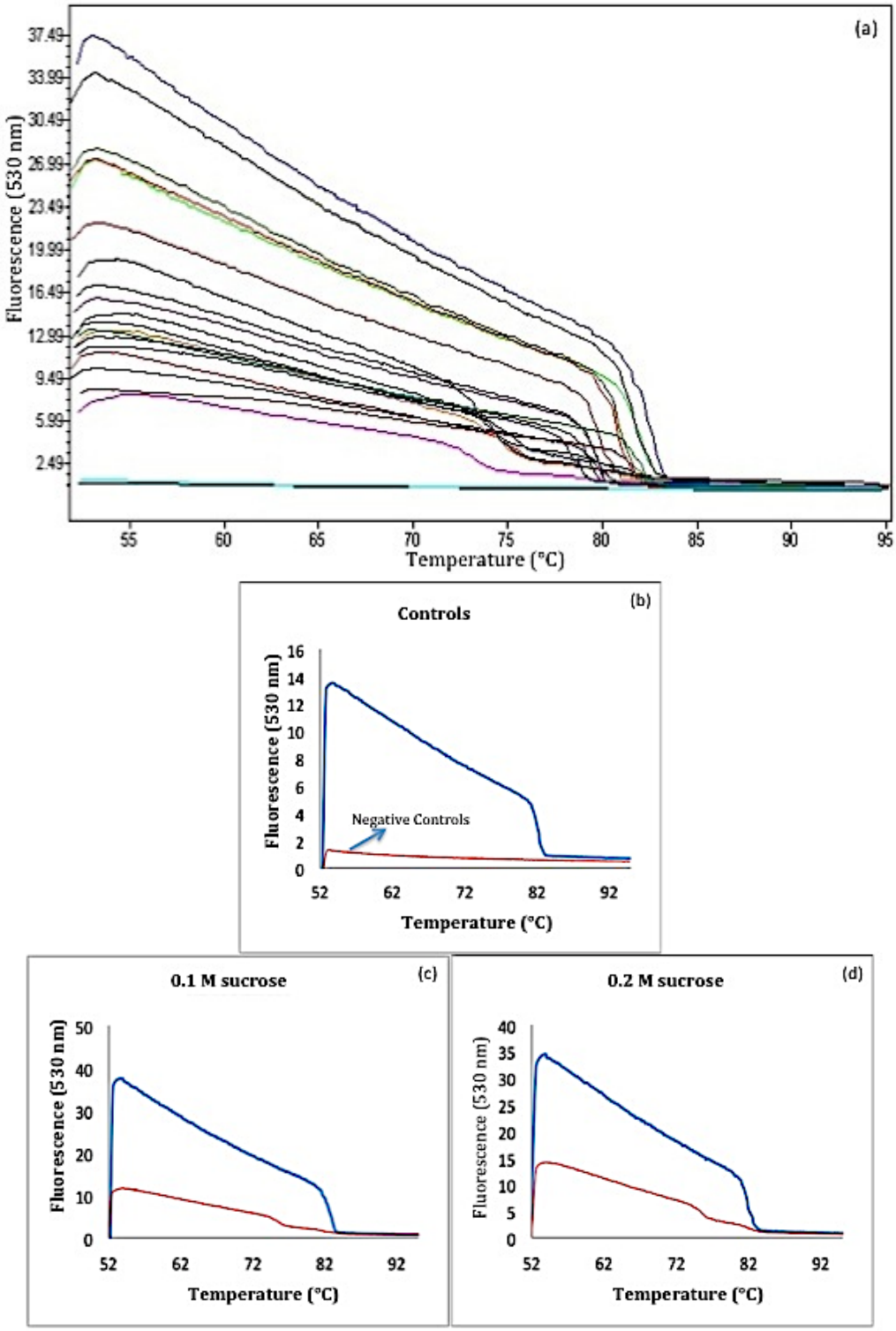


Figure 2.5: Lightcycler Melt curves generated with increasing concentrations of trehalose. (a) Melt curves corresponding to varying trehalose concentrations. (b) Melt curve representing the control sample (no trehalose), the lower curve represents the negative controls (no template). (c) Melt curves representing the sample with 0.1 M trehalose. (d) Melt curves representing the sample with 0.2 M trehalose. (e) Melt curves representing the sample with 0.3 M trehalose. (f) Melt curves representing the sample with 0.4 M trehalose. (g) Melt curves representing the sample with 0.5 M trehalose. (h) Melt curves representing the sample with 0.6 M trehalose.

Sucrose Melt Curves



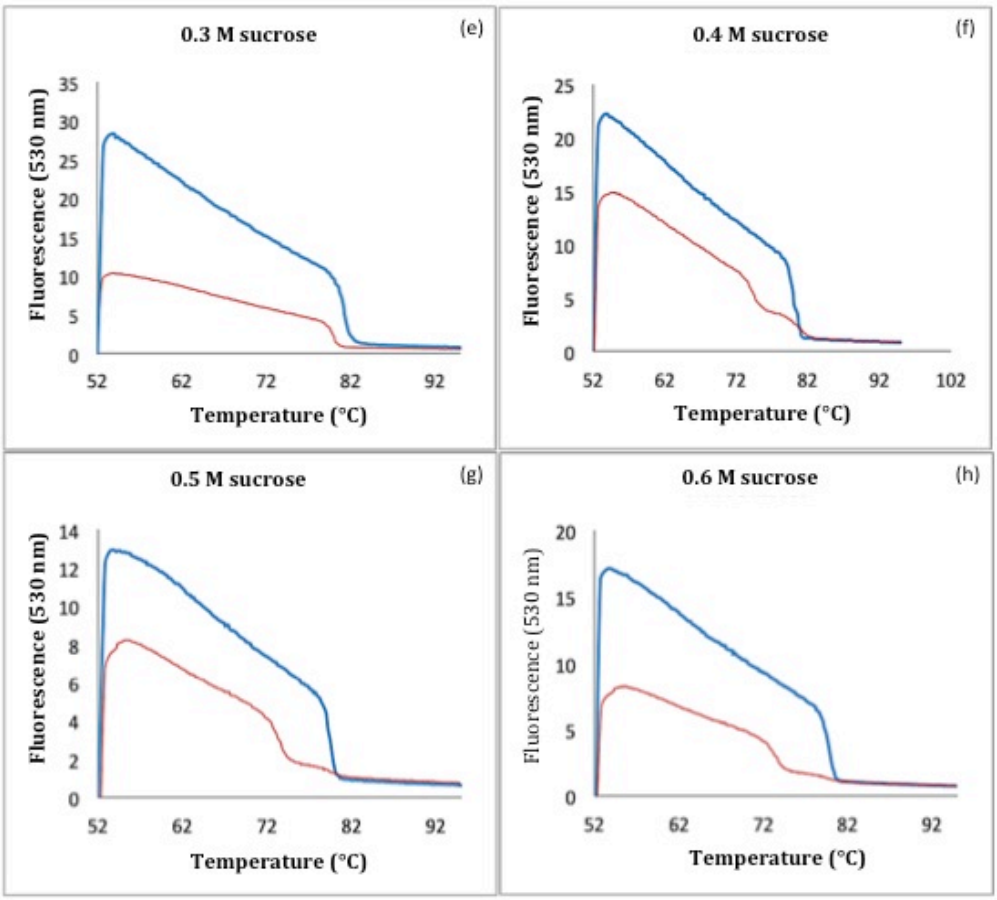
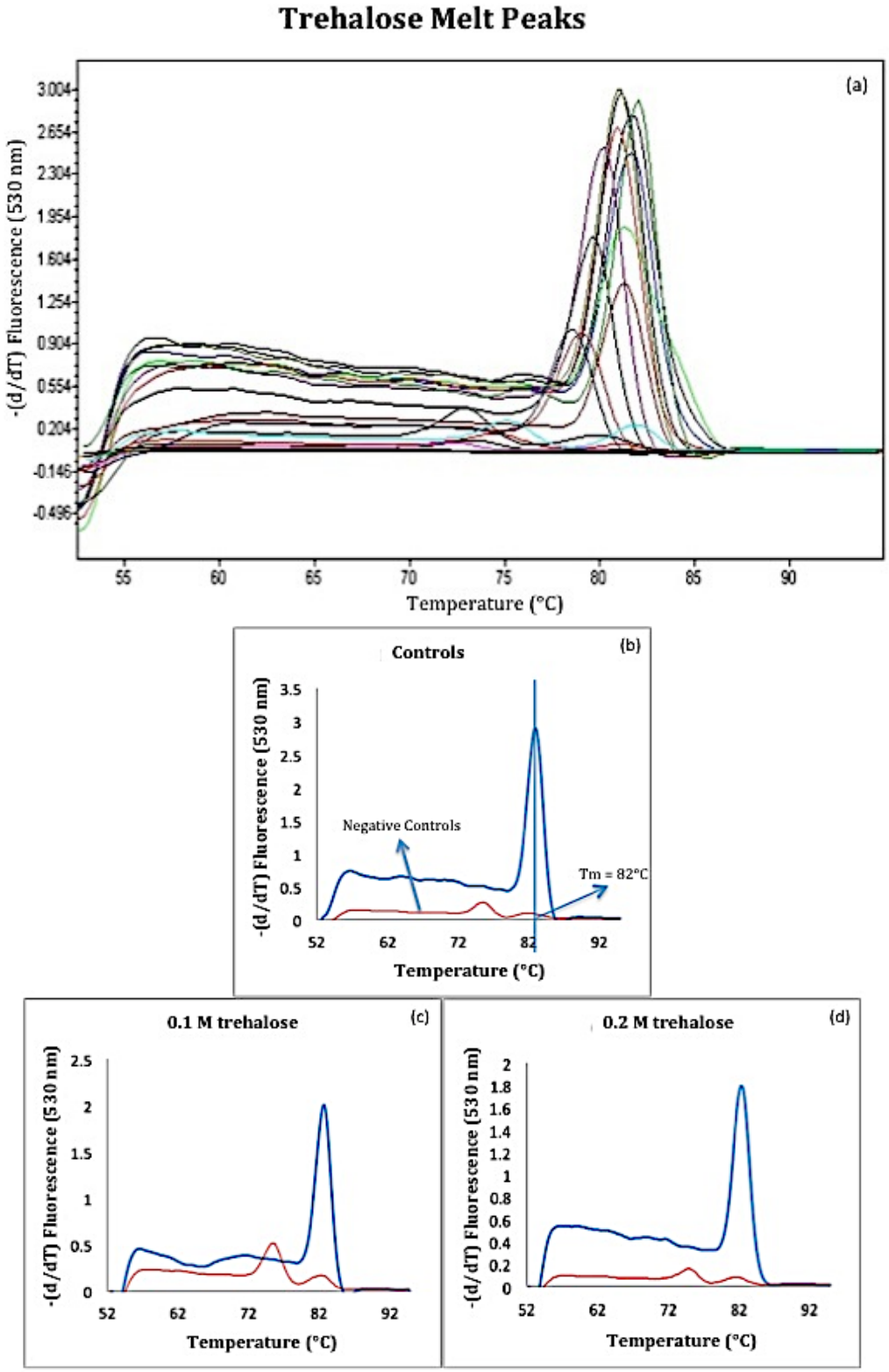


Figure 2.6: Lightcycler Melt curves generated with increasing concentrations of sucrose. (a) Melt curves corresponding to varying sucrose concentrations. (b) Melt curve representing the control sample (no sucrose), the lower curve represents the negative controls (no template). (c) Melt curves representing the sample with 0.1 M sucrose. (d) Melt curves representing the sample with 0.2 M sucrose. (e) Melt curves representing the sample with 0.3 M sucrose. (f) Melt curves representing the sample with 0.4 M sucrose. (g) Melt curves representing the sample with 0.5 M sucrose. (h) Melt curves representing the sample with 0.6 M sucrose.

The temperature vs melt peaks plotted by the Lightcycler software are shown in Figures 2.7 and 2.8. The melt peaks, as compared to the melt curves, more precisely indicate the melt temperature at which 50% denaturation of DNA occurs. As shown in the figure the melt temperature of the positive controls (samples with no trehalose or sucrose) is 82 ^{o}C (Figures 2.5b and 2.6b) . The melt peaks shift towards decreasing temperatures from 82 ^{o}C to 78 ^{o}C with the increase in concentrations of sugars from 0 – 0.6 M in the PCR formulations (Figures 2.5 c-h and 2.6 c-h). The melt peak heights indicate maximum fluorescence intensity emitted by the dissociation of DNA. As shown in the figures, the melt peaks of the positive controls (Figures 2.5b and 2.6b) are high (≈3) and those of the negative controls (Figures 2.5b and 2.6b) are very low (≈0.2).


Trehalose Melt Peaks
(a)
-(d/dT) Fluorescence (530 nm)
Temperature (°C)
(b)
Controls
Negative Controls
Tm = 82°C
(c)
0.1 M trehalose
(d)
0.2 M trehalose

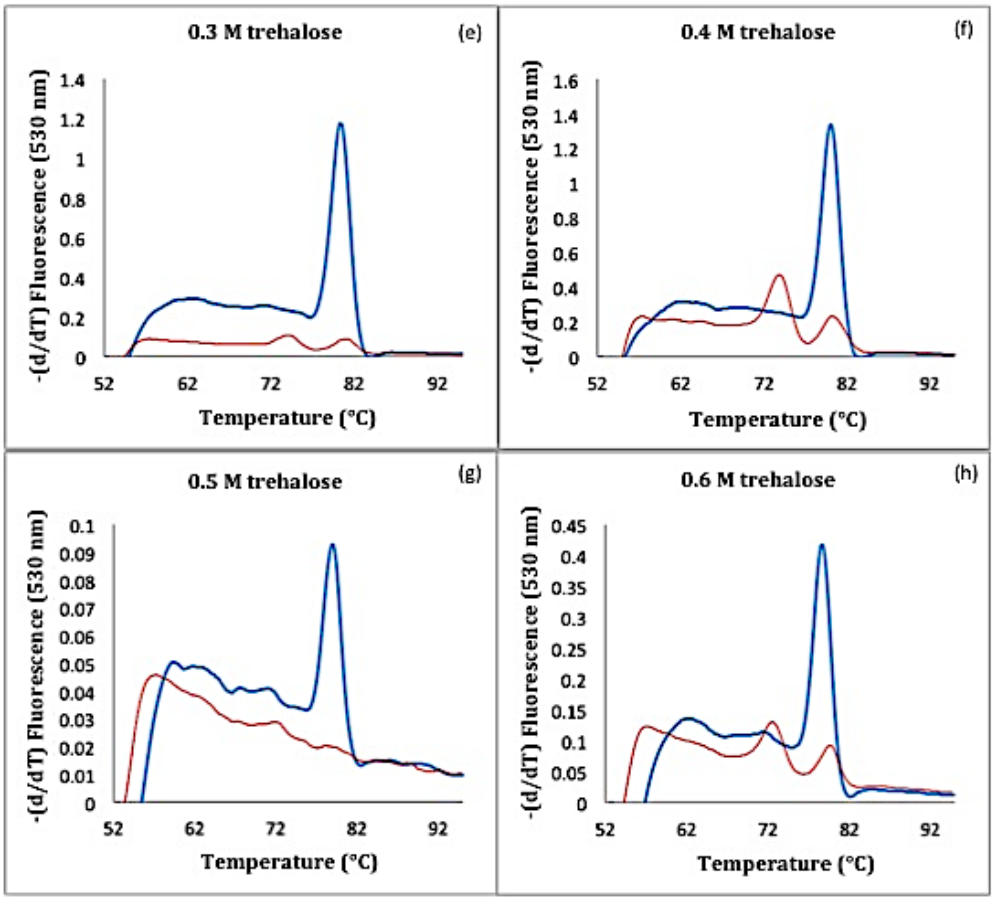
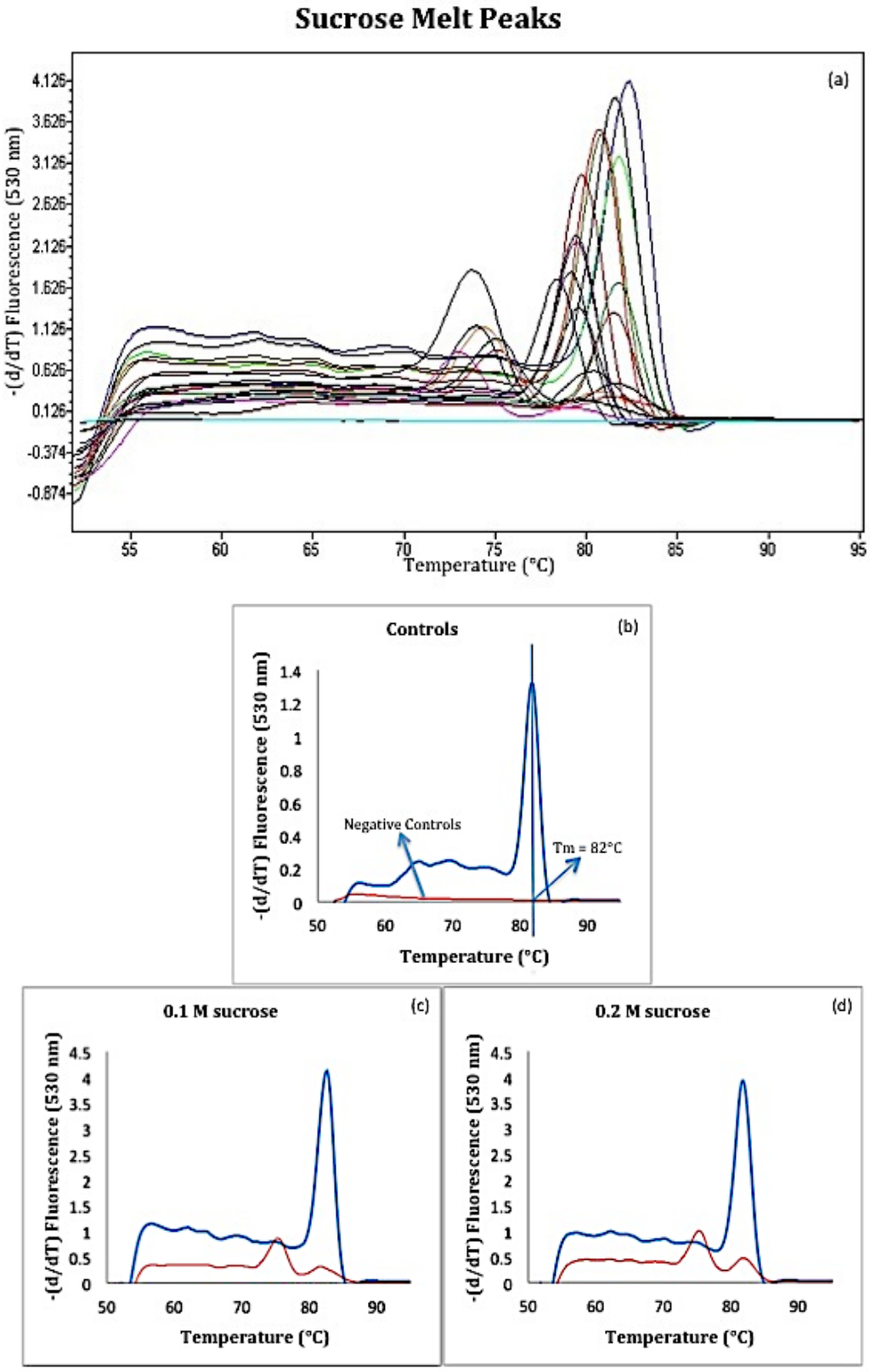


Figure 2.7: Lightcycler Melt peaks generated with increasing concentrations of trehalose. (a) Melt peaks corresponding to varying trehalose concentrations. (b) Melt peak representing the control sample (no trehalose) (Tm=82°C, peak height≈3), the smaller peaks represent the negative controls (no template) (peak height≈0.2). (c) Melt peak representing the sample with 0.1 M trehalose (Tm=81.6°C). (d) Melt peak representing the sample with 0.2 M trehalose (Tm=82.3°C). (e) Melt peak representing the sample with 0.3 M trehalose (Tm=80.3°C). (f) Melt peak representing the sample with 0.4 M trehalose (Tm=82°C). (g) Melt peak representing the sample with 0.5 M trehalose (Tm=79.8°C). (h) Melt peak representing the sample with 0.6 M trehalose (Tm=78.5°C). A shift in the melt peaks from 82°C - 78°C occurs with the increase in trehalose concentration from 0 – 0.6M.


Sucrose Melt Peaks
(a)
-(d/dT) Fluorescence (530 nm)
Temperature (°C)
Controls
(b)
Negative Controls
Tm = 82°C
0.1 M sucrose
(c)
0.2 M sucrose
(d)

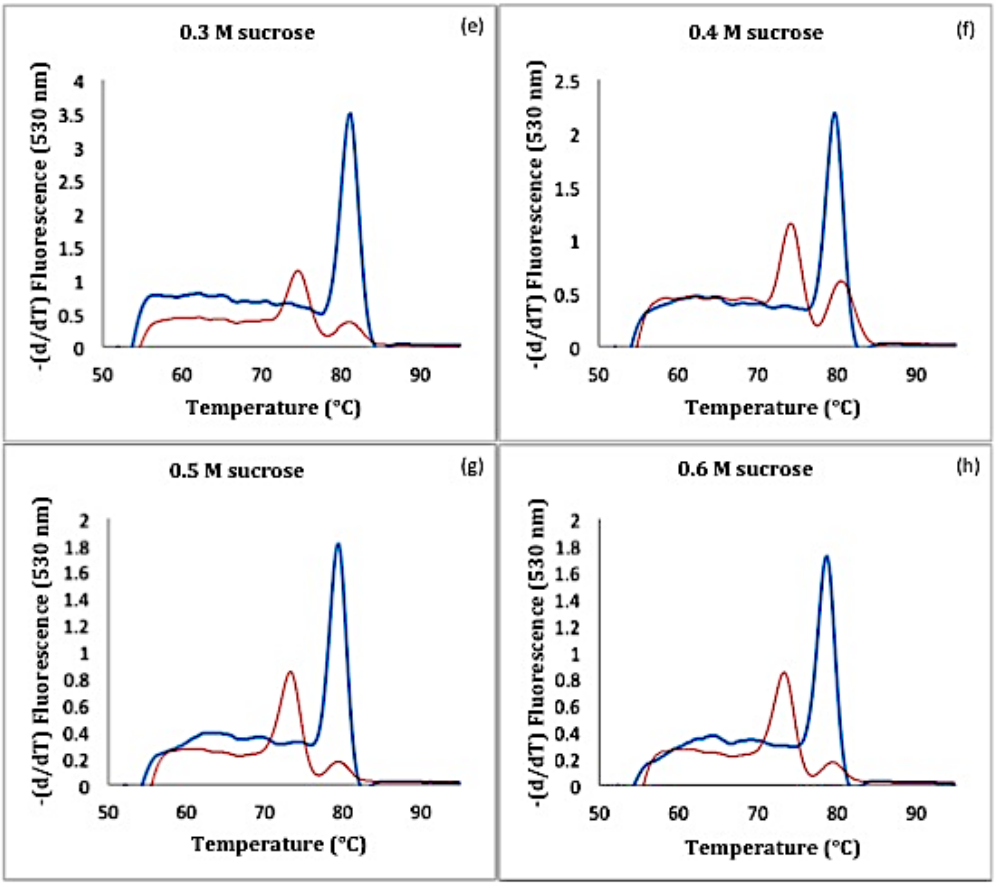


Figure 2.8: Lightcycler Melt peaks generated with increasing concentrations of sucrose. (a) Melt peaks corresponding to varying sucrose concentrations. (b) Melt peak representing the control sample (no sucrose) (Tm=82°C, peak height≈3), the smaller peaks represent the negative controls (no template) (peak height≈0.2). (c) Melt peak representing the sample with 0.1 M sucrose (Tm=82°C). (d) Melt peak representing the sample with 0.2 M sucrose (Tm=81.9°C). (e) Melt peak representing the sample with 0.3 M sucrose (Tm=81.1°C). (f) Melt peak representing the sample with 0.4 M sucrose (Tm=80.7°C). (g) Melt peak representing the sample with 0.5 M sucrose (Tm=79.9°C). (h) Melt peak representing the sample with 0.6 M sucrose (Tm=78.5°C). A shift in the melt peaks from 82°C - 78°C occurs with the increase in sucrose concentration from 0 – 0.6M.

The plot of the computed mean melt peak heights vs the sugar concentrations obtained from several replicates for both trehalose and sucrose is presented in Figure 2.9. In case of trehalose, there is a significant difference between the mean melt peak heights of the positive controls (no trehalose) and that of samples containing 0.5 and 0.6 M trehalose ($p<0.05$). However, there is no significant difference between the mean melt peak heights of the positive controls and that of samples containing trehalose concentrations in the range of 0.1- 0.4 M ($p>0.05$). In the case of sucrose, there is a significant difference between the peak heights of 0.2 M sucrose and 0.5, 0.6 M sucrose ($p<0.05$). There is also a significant difference between the peak heights of 0.1 M and 0.5 M sucrose ($p<0.05$). However, there is no significant difference between the melt peak heights of the positive controls (no sucrose) and that of the samples containing sucrose concentrations in the range of 0.1-0.6 M. The trend in the sucrose concentration vs mean melt peak heights plot shows that the peak heights increase with increase in sucrose concentrations in formulations upto 0.2 M. However, the peak heights decrease with increase in sucrose concentrations in the formulations from 0.2 – 0.6 M.

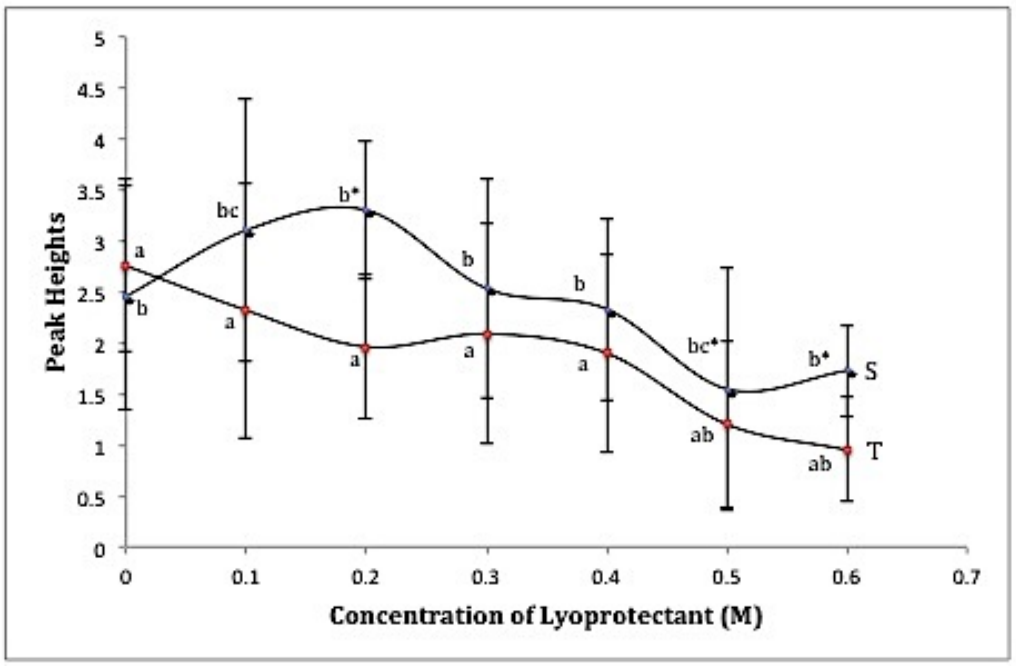


Figure 2.9: A comparative analysis of the melt peak heights obtained with (a) varying concentrations of trehalose and (b) varying concentrations of sucrose. The statistical significance of the data sets are represented by a,b and c which indicate significantly different data sets. For example, in case of trehalose, the data points represented by 'ab' are significantly different ($p<0.05$) from those represented by 'a'.

The plot of the computed mean melt temperatures vs the sugar concentrations obtained from several replicates for both trehalose and sucrose is presented in Figure 2.10. In the case of trehalose, there is no significant difference between the mean melt temperatures of the positive controls (no trehalose) and that of the samples containing 0.1, 0.2 and 0.3 M trehalose ($p>0.05$). However there is a significant difference between the mean melt temperatures of positive controls (no trehalose) and that of the samples containing 0.4, 0.5 and 0.6 M trehalose. The trend observed in the plot shows that the melt temperatures decrease with increasing concentrations of trehalose in the formulations indicating that trehalose lowers the template melting temperature. Similar observations of a decrease in the melt temperature with increase in concentrations of trehalose were also reported by Ivel et al [16]. In the case of sucrose, there is no significant difference between the mean melt temperatures of the positive controls (no sucrose) and that of the samples containing 0.1 and 0.2 M sucrose ($p>0.05$). However there is a significant difference between the mean melt temperatures of positive controls and that of the samples containing sucrose concentrations in the range of 0.3 - 0.6 M sucrose ($p<0.05$). The trend observed in the plot shows that the mean melt temperatures increase with an increase in sucrose concentrations in the formulations up to 0.1 M and decreases with increase in concentrations of sucrose from 0.1 – 0.6 M.

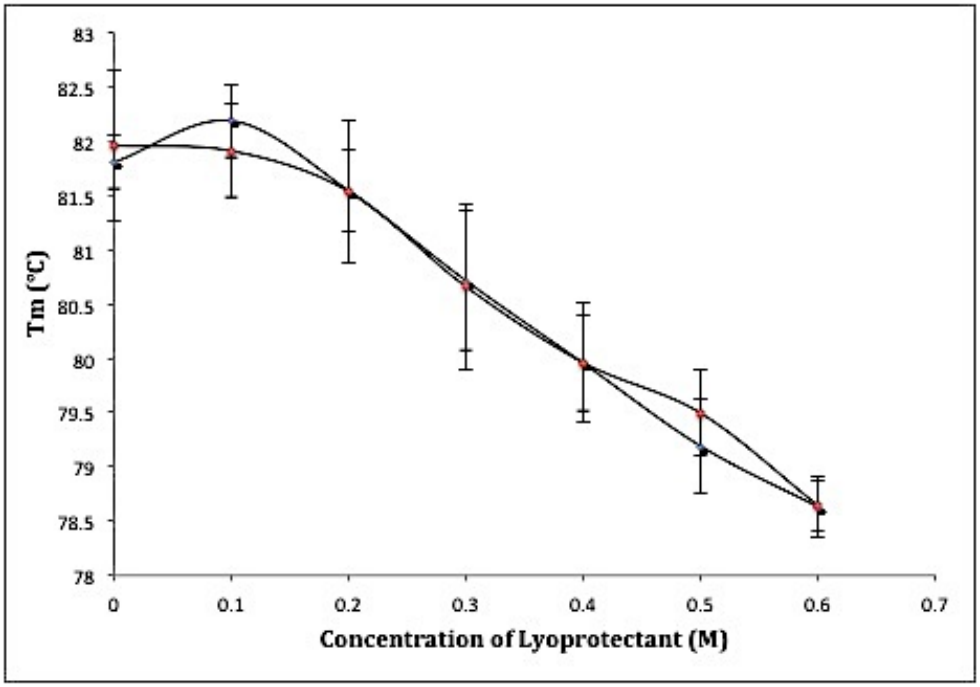


Figure 2.10: A comparative analysis of the melt temperatures (Tm) obtained with (a) varying concentrations of Trehalose and (b) varying concentrations of sucrose

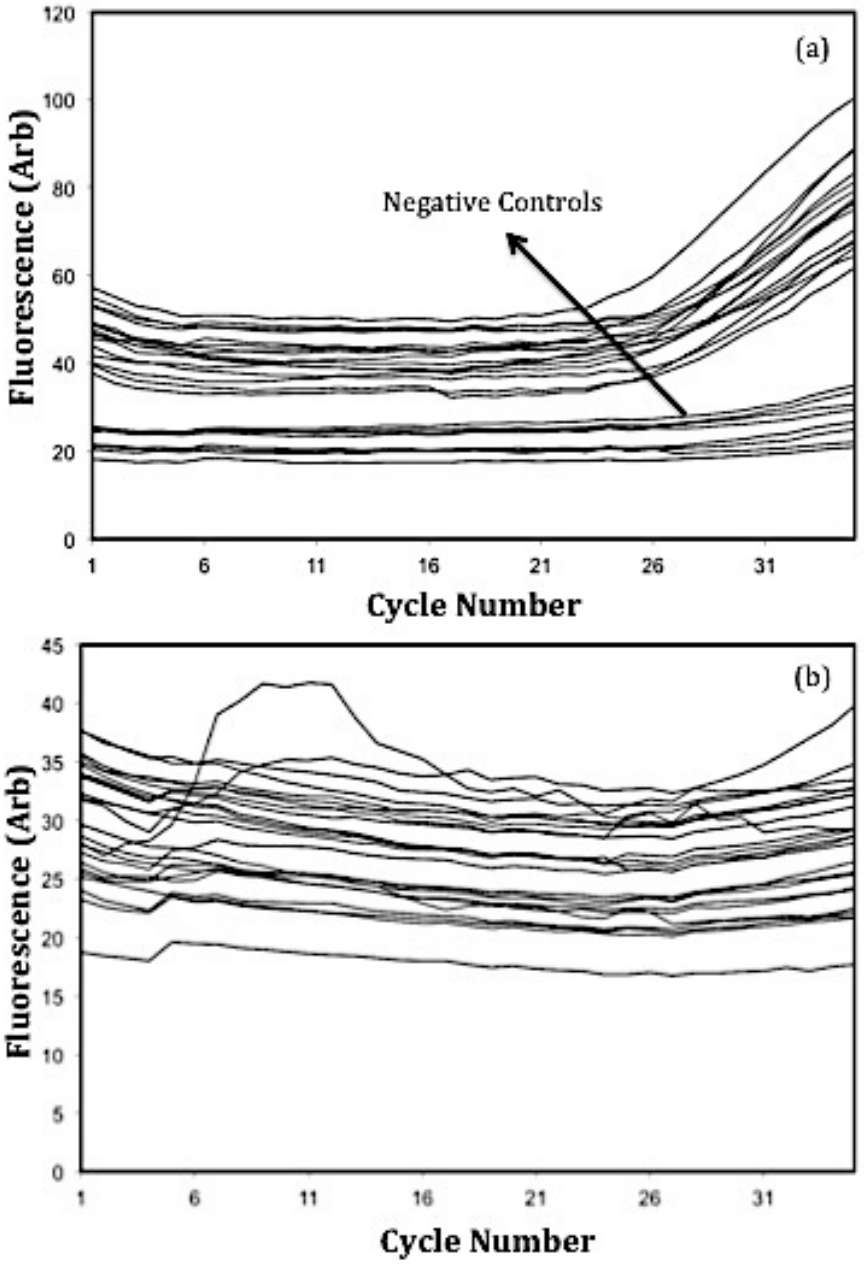
2.4.3 In-Gel PCR and Melt Curve Analysis

In order to study the influence of trehalose and sucrose on in-gel PCR, several experiments were conducted with different concentrations of trehalose and sucrose. Taking into account, the results obtained from the LightCycler experiments, trehalose concentrations in the range of 0.1 – 0.4 M and sucrose concentrations in the range of 0.1 – 0.2 M were considered for the in-gel PCR experiments. However, there were several problems with acrylamide polymerization when the sugar concentrations in the gel PCR formulations were greater than 0.4 M. Taking this factor into account, 0.3 M trehalose and 0.1 M sucrose were chosen as a compatible combination of sugars to conduct the in-gel PCR experiments. A higher concentration of trehalose was preferred due to its high glass transition temperature [1, 21]. Table 2.1 gives an insight into the variation of glass transition temperature of both trehalose and sucrose with increasing concentration of water in the formulation. The glass transition temperature decreases with increasing concentrations of water in the formulation. Hence it is preferable to have a lyoprotectant with a higher glass transition temperature. A higher glass transition temperature will ensure that the enzyme formulations are maintained in a vitrified state which would facilitate a better stability for the PCR formulations during long durations of storage [22].

Tg (°C)/c (%)	trehalose	sucrose	Water
100 (w/v)	100-115 [23, 24]	≈ 67 [25]	≈ 138 [23]
≈ 50 (w/v)	≈ -88 [25]	≈ -110 [25]	

Table 2.1: Comparison of glass transition temperatures of pure and aqueous trehalose and sucrose. 'c' represents the concentration of sugar in the solution and Tg represents the glass transition temperature

The results shown in figure 2.11 were obtained with PCR formulations containing 0.3 M trehalose and 0.1 M sucrose and formulations containing 0.3 M trehalose. The fluorescence intensity of the PCR curves of the control sample (with no trehalose or sucrose) exponentially rises after cycle 22 whereas no exponential rise in the fluorescence intensity of the sample with 0.3 M trehalose and 0.1 M sucrose is observed indicating that the PCR efficiency of this sample was too low. The fluorescence intesity of the sample with 0.3 M trehalose rises after cycle 23.


(a)
Negative Controls
Fluorescence (Arb)
Cycle Number
(b)
Fluorescence (Arb)
Cycle Number

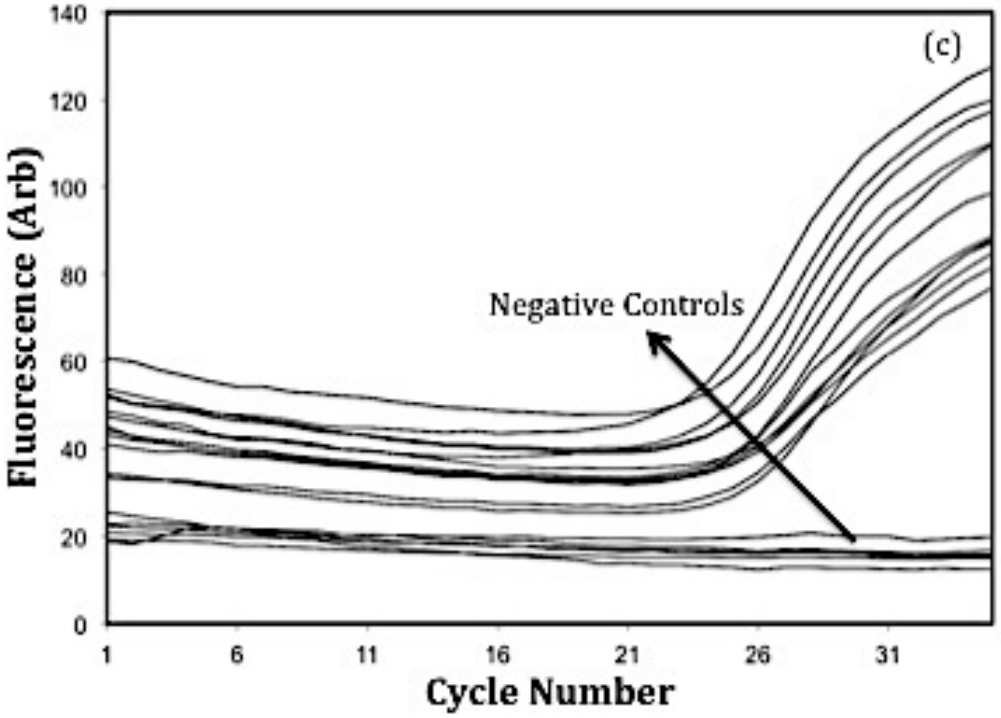
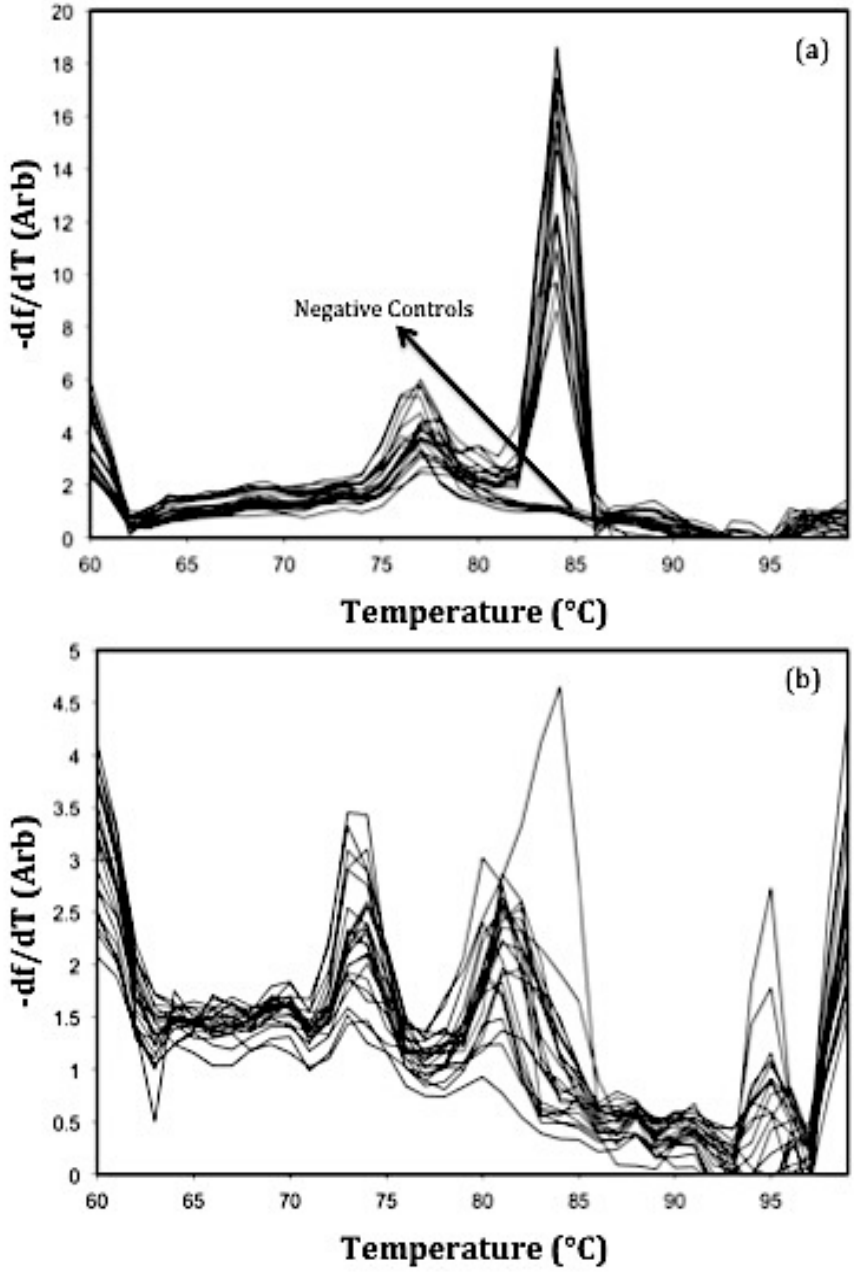


Figure 2.11. A comparison of Viriloc6 PCR curves generated with (a) PCR formulations with no trehalose or sucrose (controls), (b) PCR formulations with 0.3 M trehalose and 0.1M sucrose as the final concentration and (c) PCR formulations with 0.3 M trehalose as the final concentration.

The results shown in figure 2.12 represent the melt peaks obtained with PCR formulations containing 0.3 M trehalose and 0.1 M sucrose and formulations containing 0.3 M trehalose. The melt peak heights of the sample with 0.3 M trehalose and 0.1 M sucrose are very low (≈4) compared to the melt peak heights of the control sample (≈20). The melt peak height of the control sample (≈20) is lower compared to melt peak height of the sample with 0.3 M trehalose (≈30). The negative controls of the control sample (no trehalose) have resulted in a small primer-dimer peak whereas no primer dimer peaks were observed in the negative controls of the sample with 0.3 M trehalose. Taking this factor into account, and considering the fact that the PCR buffer used in the formulations contains 40% sucrose, further In- gel PCR experiments were conducted by including only trehalose in the formulations at a final concentration of 0.3 M.


(a)
-df/dT (Arb)
Negative Controls
Temperature (°C)
(b)
-df/dT (Arb)
Temperature (°C)

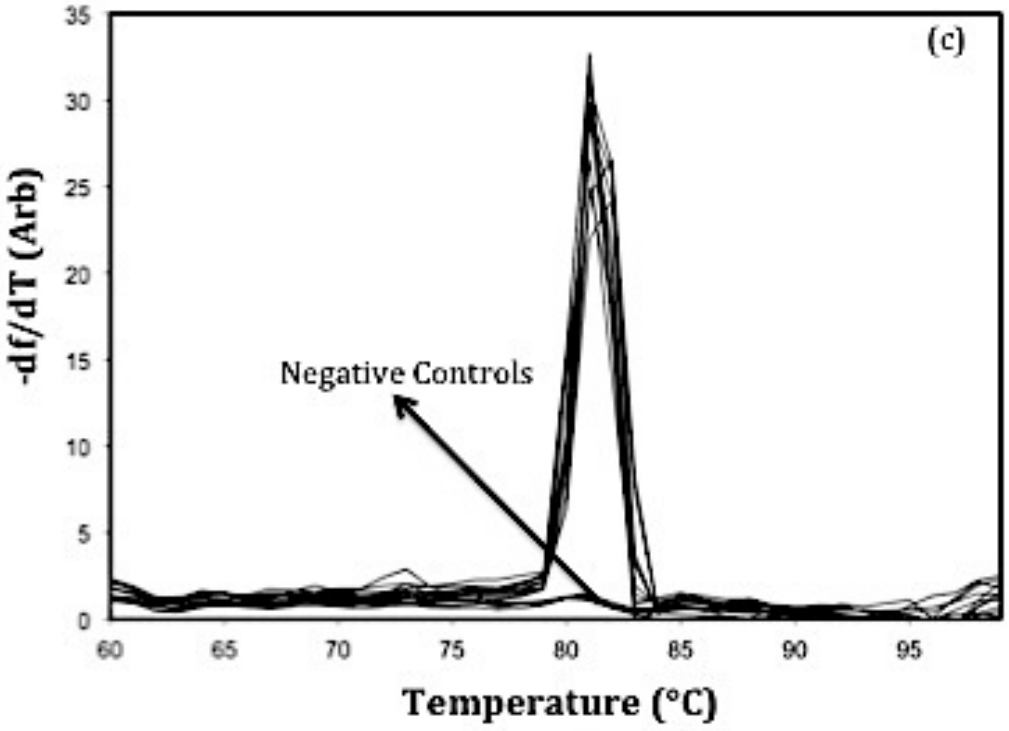


Figure 2.12. A comparision of Viriloc6 melt peaks generated with (a) PCR formulations with no trehalose or sucrose (controls), (b) PCR formulations with 0.3 M trehalose and 0.1M sucrose as the final concentration and (c) PCR formulations with 0.3 M trehalose as the final concentration.

2.5 Discussion

A comparative analysis of the lyoprotectant concentration versus the Cp values indicates that increasing concentrations of either trehalose or sucrose in the PCR formulations have very little or no influence on the Cp values (Figure 2.4). However, a considerable decrease in peak heights of formulations with trehalose concentrations above 0.4 M can observed in the trehalose concentration vs mean melt peak height plot (Figure 2.9). This indicates that increasing trehalose concentrations in PCR formulations above 0.4 M has a profound effect on the PCR. In the case of sucrose, an increase in melt peak heights is observed with the increase in concentration of sucrose upto 0.2 M and decreases with further increases in sucrose concentration (Figure 2.9). The melt peak heights obtained with 0.3 M (≈2.5) and 0.4 M (≈2.4) sucrose are comparable to the melt peaks obtained with the control samples (≈2.5) with no sucrose. However, the melt peak heights decrease with further increase in sucrose concentrations above 0.4 M (peak height < 1.6) indicating that sucrose concentrations in PCR formulations above 0.4M have a profound effect on reaction. Increasing sugar concentrations in the PCR formulation also leads to the decrease in the melt temperatures of amplified target.

The results obtained with increasing sugar concentrations in gel PCR formulations indicate that the PCR efficiency drops with the increase in total sugar concentration above 0.3M. Severe problems with gel polymerization

also occur when the sugar concentrations are increased above 0.4M. This behaviour may be attributed to the fact that the addition of sugars in such high concentrations results in a great increase in gel density thereby reducing the interaction of reagents entrapped within the gel. The use of 0.3 M trehalose in the gel PCR formulations had improved PCR efficiency indicating that an increase in concentration of sugars in the gel PCR formulations up to 0.3 M increases PCR efficiency. However, a further increase in sugar concentrations leads to a decrease in PCR efficiency.

Taking all these factors into account together with the results obtained from in-gel PCR experiments conducted with formulations containing 0.3 M trehalose, we find that the most favourable stabilizer to be used in the desiccation of PCR reagents entrapped within polyacrylamide gels, was trehalose at a final concentration of 0.3M.

2.6 References:

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Chapter 3: Desiccation of In-gel PCR system

3.1 Introduction

The lyoprotectants that are compatible with amplification of DNA in gels during and after desiccation were determined in chapter 2. The next part of the problem was to characterize the process of vacuum desiccation of the in-gel PCR system. Residual water content is one of the most important factors to be considered while drying a protein formulation. Water can affect the protein stability in several ways both indirectly and directly. Indirectly, water can act as a plasticizer, which reduces the glass transition temperature of the protein formulation thereby reducing the stability of the formulation during long-term storage at higher temperatures [1-3]. It can also act as a reaction medium, thereby increasing the interaction between different components of the protein formulation, which is undesirable. Directly, water can be a reactant or a product in the protein formulations.

Residual moisture can be responsible for several instabilities in dried proteins and the effects are complex in many cases. An increase in the residual water content of dried protein formulations increases the deterioration rate of proteins. Hsu et al [4] have observed that increasing moisture content of lyophilized tissue plasminogen activator (tPA) from 4.6% to 7.6% resulted in an increased loss of protein activity during storage. An increase in relative humidity accelerated the loss in activity of vacuum dried restriction enzyme EcoRI during storage at 45°C [2]. Higher residual

moisture contents also result in protein aggregation in many cases. The loss of activity of bovine pancreas RNase induced by aggregation was higher at 9.8% moisture than 1.9% [5]. An increase in protein aggregation with increasing relative humidity was also observed in case of insulin [6]. An increase in moisture content also increases the mobility of proteins, thereby increasing the rate of chemical reactions in the formulation [3, 7, 8], which leads to chemical degradations. Strickley et al [9] have observed that an increase in the moisture content of lyophilized formulations of insulin from 3% to 52% resulted in the formation of degradation products of insulin. However, lower moisture content does not always ensure a stable formulation. The stability of lyophilized rhIL-1ra was higher at moisture content of 3.2% than 0.8% [10]. The effects of residual water also depend on many other factors such as temperature, lyoprotectant composition and concentration and moisture distribution within a protein [3]. The aggregation of lyophilized rGH in the presence of glycine and mannitol increased with an increase in moisture content from 0.7% to 2.5% at 40°C. However, protein aggregation first increased and then decreased with the increase in moisture content at 25°C [11]. All these factors must be taken into consideration when determining the residual moisture content for a protein formulation.

There are several methods to determine the water content of dried formulations such as:

- *Karl Fisher Titration [12]:* The Karl Fischer titration is a technique developed by Karl Fischer to determine the moisture content in a sample. It involves a base, which reacts with water and converts the water into a non-conductive chemical. There are two methods to perform the Karl Fischer titration.

  - Volumetric titration: In this method, the water content is determined based on the amount or volume of reagent consumed to convert the water to a non-conductive chemical. The samples are initially dissolved in a solvent and a reagent is added until the water is removed. The reagent concentration at which all water is removed is directly proportional to the amount of water present initially.
  - Coulometric titration: In this method, the reagent and solvent are both combined in the titration cell. The reagent is released by the induction of an electrical current when the sample is added. The moisture content in the sample is determined by the amount of current required to convert the water.

Gravimetric analysis [13]: Gravimetric analysis is a method to determine the moisture content in a sample based on the weight of sample before and after desiccation.

Gas chromatography [14]: In this method, the sample is passed in a gas stream through a narrow tube known as the column. The different components of the sample pass through the column at different rates based on their physical and chemical properties, and are detected at the end of the column. The results are obtained in the form of a plot known as the chromatrogram, which has the detector response on the y-axis and the retention time on the x-axis. Each component of the sample is presented as a peak in the plot and the area under the peak is directly proportional to the quantity of the component present in the sample.

However, because our formulations are semi-solid in nature, only Karl Fischer titration and gravimetric analysis methods are applicable. Though Karl Fischer titration is a very efficient method to determine the moisture content of a sample, it is extremely complicated to use this technique for our applications due to the minute sample volumes. Hence, gravimetric analysis is the only method that can be followed to determine the moisture content in the gel PCR formulations.

3.2 Objective

To study the desiccation kinetics and residual water content of the gel PCR formulations, which contain trehalose as the lyoprotectant.

3.3 Overall Approach

To determine the residual water content several measurements were required. We determined the weight of the glass molds prior to filling them with gel, the weight of the same molds filled with polymerized gels and the weight of the molds with gels that were desiccated for a predetermined time period. At the end, gels were dried to an anhydrous condition to determine the weight of mold plus anhydrous gel. The calculations to then determine residual water are described in the results section. Although the effect of residual water will not be tested in Chapter 3, its impact in desiccated gels will ultimately be tested in PCR reactions, to be described in Chapter 4. The primers included in the gel reaction mixture are those for amplifying BKV DNA.

3.4 Materials and Methods

3.4.1 Characterization of the Desiccation Process

In order to characterize the desiccation process, 20 molds (with 6x8 array of wells) with geometries shown in Figure 3.1 were selected. Each of these molds, were weighed 30 times on a balance (Mettler-Toledo International Inc.) to determine initial weight of each mold by itself and the error in the weighing process that is introduced by the balance.

3.4.1.1 Gel PCR mix preparation

Four hundred microliters of gel PCR mix was prepared with 16 µl Taq Polymerase (20 units/ µl), 8 µl each of 10 µM BKV forward and reverse primers (refer section 2.3.1 for primer sequences) to produce 100 bp product, 8 µl 10mM dNTP, 8 µl 1% BSA (Sigma), 80 µl 5 X PCR buffer (333 mM tris-sulfate, pH 8.6, 83 mM $(NH4)_2SO_4$ (Sigma) and 40% sucrose (Sigma, Lot#BCBB7807)), 16 µl 50mM $MgCl_2$, 40 µl 10xLC Green Plus (Idaho Technology Inc., Salt Lake City, Utah), 40 µl of 40% acrylamide (Sigma, cat no. A9099) + 4% bis-acrylamide aqueous solution (N,N-methylene bis-acrylamide, BioRad, Hercules, CA, cat no. BA05-1610201), 8 µl of 3% azobis (Wako, Richmond, cat no. VA-086) and 4 µl of 10% TEMED (N,N,N',N'-tetramethyleneethylenediamine, Sigma, cat no. T7024), 80 µl 1.5M trehalose (Cargill Foods Inc., Cat#2K141) and 84 µl water. The final concentration of trehalose in the formulation is 0.3 M as discussed in chapter 2. This formulation was then de-gassed under vacuum.

3.4.1.2 Gel Casting

The formulations containing all the reagents except the template DNA, were loaded into 24 wells within each mold according to the spacing shown in Figure 3.1. A 22x22mm cover slip was slid on top of the mold. The idea was to add the template DNA-water mixture to the positive controls section of the mold during rehydration and water without the template to the negative controls section. The molds were then introduced into the gel polymerization chamber where it was exposed to the 405 nm laser for 24 minutes to photopolymerize the acrylamide mix.

The weights of the polymerized gel molds were then weighed by three replicate measurements and the molds were introduced five at a time into the Vacuum Oven (Fisher Scientific) for 15 minutes. The time taken to weigh the molds was approximately five minutes during which the gels lost approximately 10% of their total water content. The pressure in the vacuum chamber fluctuated between 0 – 0.1 mm of Hg and the chamber temperature varied from 15 – 17°C. After desiccating the molds for 15 minutes, the weights of the molds were again determined by three replicate weight measurements for each mold. The molds containing the desiccated gels were then left in the vacuum oven overnight in order to dry the gels until they were anhydrous. The weights of the molds with anhydrous gels were again determined by three replicate weight measurements. Our preliminary desiccation experiments, all conducted in the same manner indicated that it

takes 15 minutes for the gels to lose approximately 90% of their total water content. Our target was to achieve less than 10% residual water content in the gel PCR formulations. Hence, in order to study the drying rate of the gels, our experiments were conducted with an initial drying time of 15 minutes and the subsequent experiments were conducted by increasing the drying time by 3 minutes for each experiment.

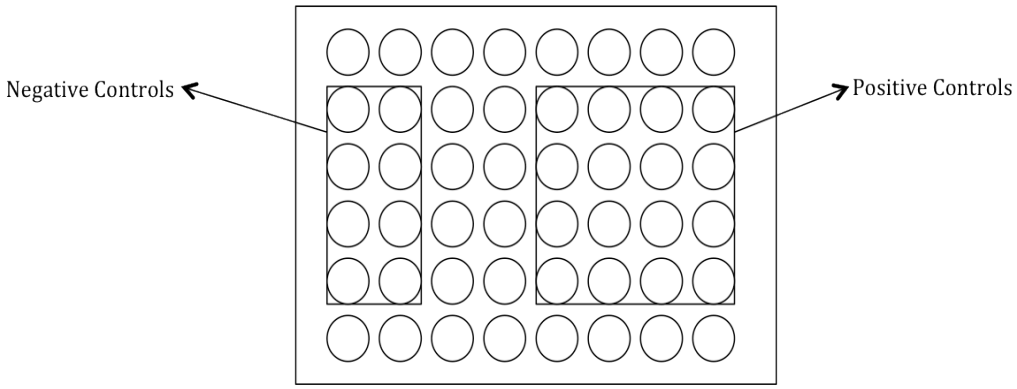

Figure 3.1: Schematic of a 6x8 mold.

The following equations were applied in order to calculate the residual moisture percentages in the gel after 15 minutes of Vacuum Desiccation.

Weight of the mold (g)= A

Weight of the mold + Fresh gels (not desiccated) = X

Weight of the molds + Desiccated gels = Y

Weight of the molds + Anhydrous gels = Z

Weight of water in the gels initially = X-Z = B

Weight of the water in the gels after 15 minutes of Vacuum Desiccation = Y – Z = C

Residual water content (%) = C/B * 100

This process was repeated three times for 15 minutes of vacuum desiccation for each mold to determine the variations in the residual water percentages between multiple repetitions for each mold and between all the molds. The residual water percentages were also calculated for 18, 21, 24 and 30 minutes of vacuum desiccation following the same protocol. After obtaining all required measurements, a plot of residual water versus time was generated at the end of these series of experiments. The molds were cleaned and decontaminated between each process according to the protocols described in section 2.3.2.3. The pictures of fresh, desiccated and rehydrated gels are presented in figure 3.2.

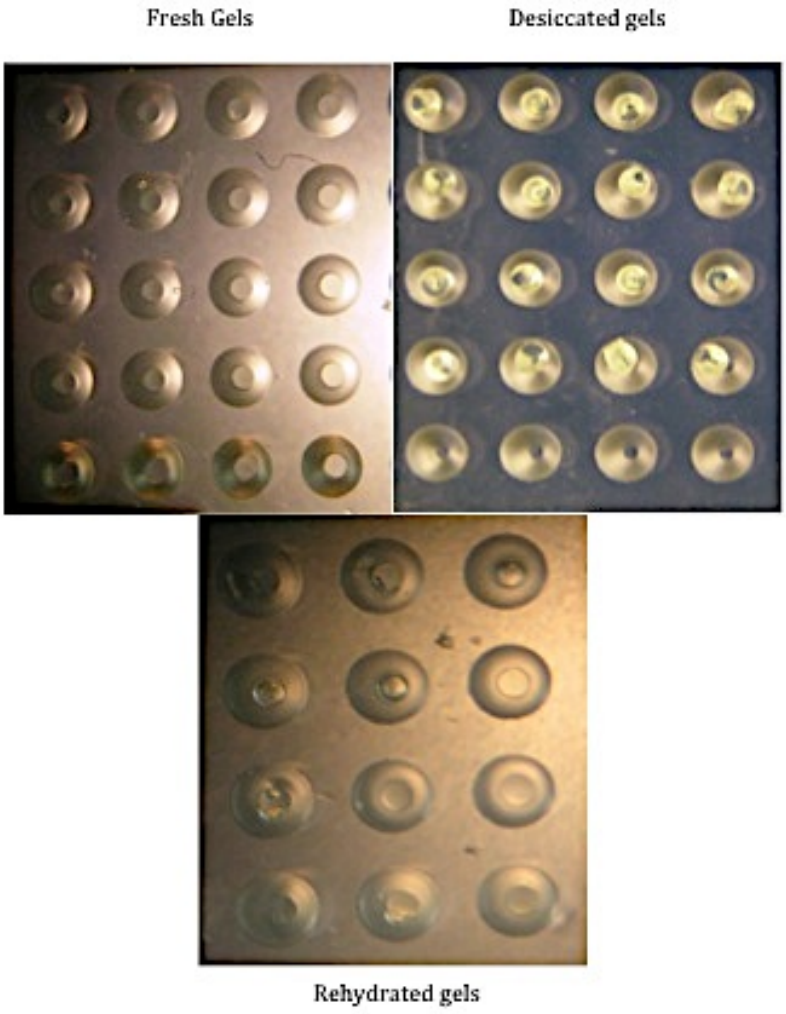

Figure 3.2: Pictures of Fresh, Desiccated and Rehydrated gels.

An experiment was also designed in order to estimate the time taken by the gels to become anhydrous. The PCR formulation was prepared according to the previous protocols. The formulation was loaded into the molds following the methods described in the previous section. The weights of these molds were measured in triplicate after gel polymerization and the molds were then introduced into an oven set at 100°C for 24 hours to bake the gels. The weights of the molds with the baked gels were then measured in triplicate to determine the weights of the anhydrous gels. Once the anhydrous weights were obtained, a fresh set of gel molds were prepared according to the protocols described above. The weights of the molds with fresh gels were again measured in triplicate and the molds were then introduced into the vacuum oven to desiccate the gels. The weights of the molds along with the desiccated gels were measured every 30 minutes until the gels were anhydrous. The total water content that was present initially in the gels (parameter 'B' in the equations mentioned above) was also plotted for each mold. The time taken by the gels to become completely anhydrous ranged from 120 to 150 minutes depending on the size of wells in the mold.

3.4.2 Scanning electron microscopy images of the desiccated Gel PCR system

In order to study the affects of the desiccation process on the structure of the gels, desiccated gel PCR systems (desiccated for 30 minutes) were prepared according the methods outlined in the previous sections. To acquire the scanning electron microscopy images of the gels, it is necessary for them to be conductive. In order to increase the conductivity of the gels, they were coated with gold particles using a sputter coating machine to cover the surface of the desiccated gels with a thin layer of gold. The gels were then introduced onto the microscope and several images of the gels at various magnifications were acquired. The gold particles are coated on the gels in a vacuum chamber and hence SEM images of fresh gels could not be obtained [15].

3.5 Results and Discussion

3.5.1 Characterization of the desiccation process

Figure3.3 a. shows the variation of residual moisture (%) with time (min). The gels lose up to 90% of their water content in the first 15 minutes of desiccation. The gels retain upto 7.9 ± 0.77% of their water content after 24 minutes of desiccation, 7.5 ± 1.11% after 30 minutes of desiccation and it takes approximately 150 minutes under vacuum for the gels to lose all their moisture and become anydrous as shown in Figure 3.3b. The error bars in the figure represent the cumulative error that accounts for the error introduced by the balance during weight measurements, the variation (standard deviation) in residual water content for a single mold between the three consecutive experiments for a single drying time and the variation (standard deviation) in the residual water content between molds for a single drying time. The total water content that was present initially in the gels, for each mold is presented in Figure 3.4. The error bars in the figures represent the cumulative error that accounts for the error introduced by the balance during weight measurements and the variation (standard deviation) in the total water content present in the gels for a single mold between multiple experiments. However, the errors introduced by the balance are quite minute compared to the variations in moisture contents between different molds, presumably due to variations introduced by the manufacturing process for the molds.

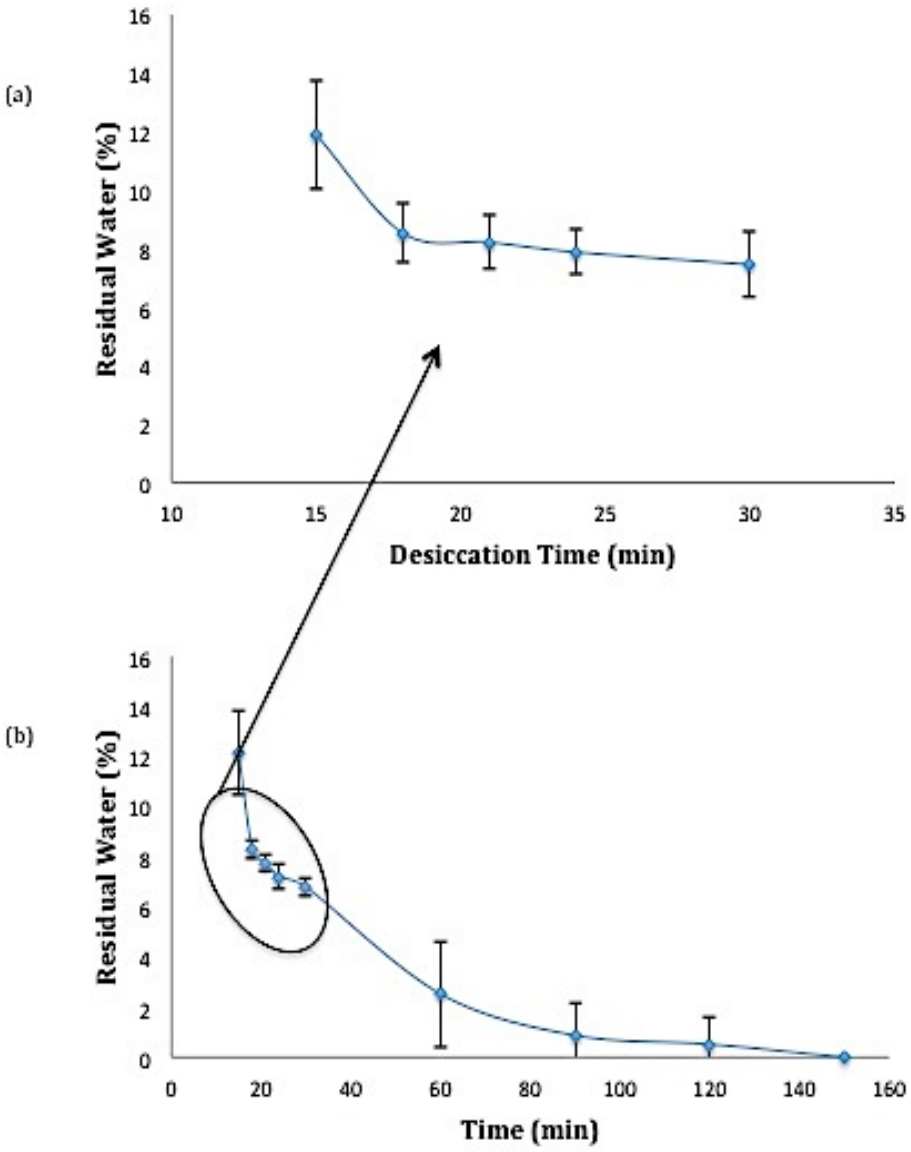

Figure 3.3: The variation of residual moisture with desiccation time (combined data obtained from all the molds) (a) during the first 30 minutes of desiccation (b) until the posts are anhydrous (≈150 minutes).

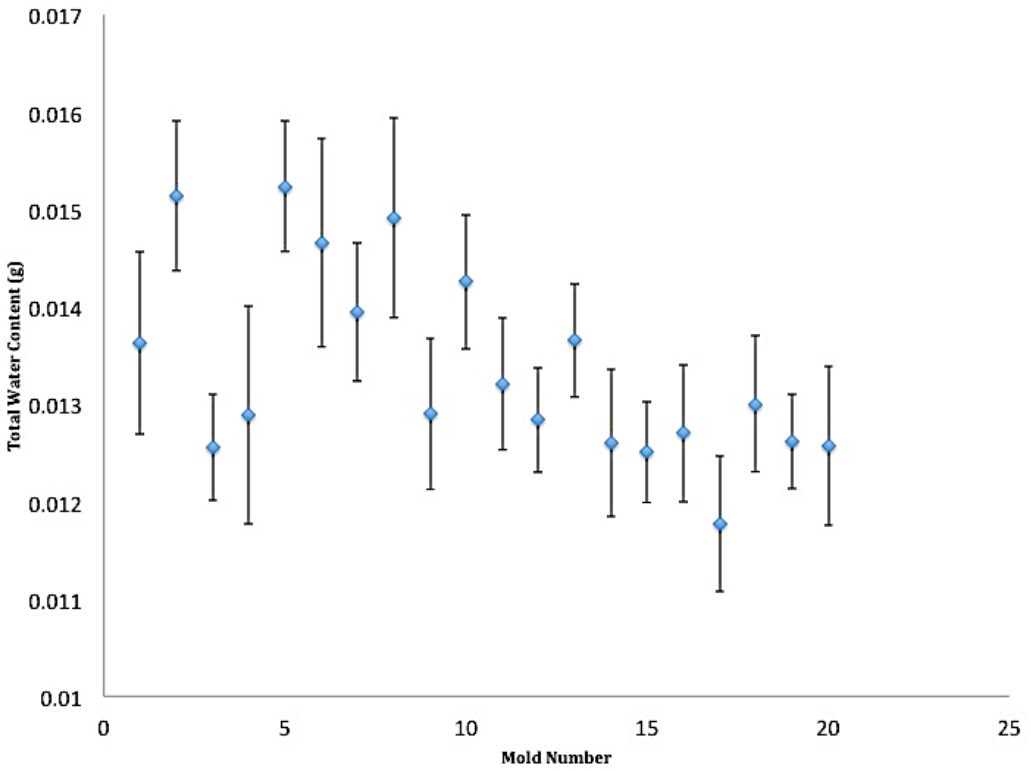

Figure 3.4: Variation in the total water content between different molds. The x-axis represents the number assigned to the mold and the y-axis represents the weight of the total water content present initially in the molds calculated from the beginning weights and the anhydrous weights. The error bars represent the standard deviations in the total water content between different experiments.

3.5.2 Scanning Electron Microscopy images of the desiccated Gel PCR system

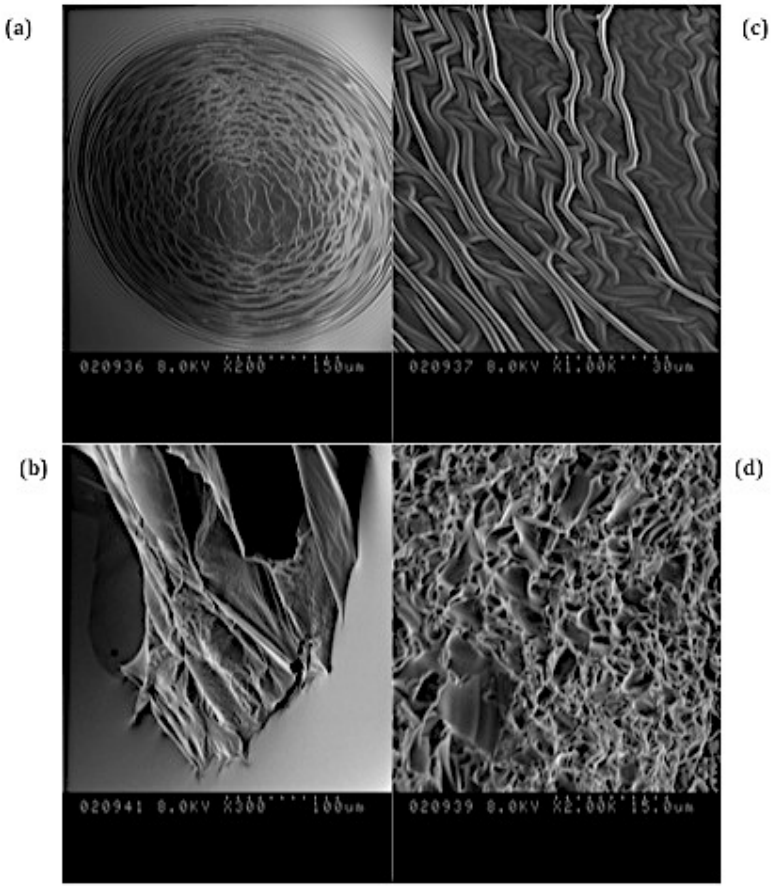

Figure 3.5: Scanning Electron Microscopic images of the desiccated gel PCR system. (a) Top view with 200x magnification (b) Top view with 1000x magnification (c) Side view with 300x magnification (d) Surface of the gels with 2000x magnification.

Figure 3.4 shows the polymer network of the desiccated gels at 200x magnification. The polymer network shrinks due to the desiccation process. Figure 3.4c shows the gel structure at 1000x magnification. The gels in each

well of the mold dry in a different manner in the vacuum oven depending on the air bubbles or unpolymerized monomer present in the gels. Some of the gels just shrink and settle at the bottom of the well in a mold whereas other gels stick to the walls of the well. Figure 3.4b shows a gel that is stuck to the wall of the well in the mold.

3.6 Discussion

The gel PCR formulations have both strong and weak binding sites to accommodate water. The drying rate is very high initially until the gels lose weakly bound water which amounts to approximately 90% of total water content. However, the removal of the strongly bound water molecules takes more time. There is a slight decrease in the drying rate from 15 to 18 minutes and it further decreases from 18 – 21 minutes (Figure 3.3a). There is very little difference between the residual water content of the gels desiccated for 24 minutes and that of the gels desiccated for 30 minutes.

Though all molds have similar dimensions in terms of length and width, there are variations in the size and diameter of the wells between different molds due to the error introduced while drilling the molds. Different molds have different drying rates depending on the size and dimension of their wells. There is also a difference in the total water content of the gels of a single mold between different experiments. This difference may arise due to the presence of unpolymerized monomer in the wells or air bubbles present in the gel or both. There are several reasons for the occurrence of these issues. The molds are made of glass and over time they may start microscale chipping and may also develop small cracks in wells due to the vigorous cleaning and decontamination procedures applied between each experiment. The small cracks may introduce air bubbles in the gels during polymerization. Human error while loading the gel PCR formulations may also lead to the

formation of air bubbles in the gels thereby subsequently increasing the amount of unpolymerized monomer in the wells. Hence the drying rate of gels for a give mold depends on all these factors and heterogneity among molds is to be expected.

The SEM images obtained with the desiccated gels indicate that vacuum desiccation does not effect the porosity of the gels. Takahashi et al. [16] have presented a comparison of the effects of different drying methods on gels with SEM imaging and our results are in concordance with their results obtained with vacuum desiccation. The change in porosity of the gels may lead to an increased uncontrollable interaction between the PCR reagents which is undesirable.

In conclusion, vacuum desiccation may not have a negative effect on the structure of the gels and can be considered as an apt method for drying gel PCR formulations. In the context of developing these formulations to be used at the point of care, we must assume that the desiccated reagents may be subjected to different conditions of temperature and humidity. There may be a further decrease or increase in the residual water content of these formulations depending on the packaging efficiency. Hence, an optimum residual water content is necessary to ensure a longer shelf-life for the gel PCR formulations. However, the only way to determine the optimal residual water content for a protein formulation is to conduct long-term stability studies with different moisture contents.

3.7 References

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Chapter 4: Analysis of stability in desiccated gels used for PCR amplification

4.1 Introduction

The activity of dried formulations may be high immediately after drying, but their stability decreases with time of storage. For example, there was 70% loss in the activity of lyophilized porcine pancreatic elastase after 2 weeks of storage at 40°C and 79% RH [1]. Schebor et al [2] reported a significant loss in the activity of lyophilized invertase after 7 days of storage at 95°C. The objective of Chapter 4 is to evaluate the stability of PCR reagents in desiccated gels for the miniaturized PCR system described here and in chapters 1-3 and to determine the time frame within which these reagents retain their activity.

4.1.1 Mechanism of protein formulation instability during storage

There are several pathways, which lead to the instability of protein formulations during storage such as protein aggregation, chemical degradations, deamidation and hydrolysis [3].

4.1.1.1 Protein Aggregation

Protein aggregation (clumping together) is one of the most common complications that lead to the instability of dried protein formulations during storage [4]. Examples of proteins that aggregate under accelerated conditions include BSA at 37 or 60°C ([5]; [6]), rHA at 37°C and 96% RH ([7]),

porcine pancreatic elastase at 40°C and 79% RH ([1]), *Humicola lanuginose* lipase at 40 or 60°C [8]. A few proteins also aggregate under ambient conditions [3] such as b-galactosidase [9] and hGH ([10];[11]). Protein aggregation may also occur due to physical (non-covalent) interactions such as in the case of tetanus toxoid [12] and glucose oxidase [5]. Another pathway that leads to protein aggregation is the disulfide bond formation [3] and this mechanism is predominant in the case of b-galactosidase [9], insulin ([13], [12, 14]), BSA and b-lactoglobulin ([5], [6]).

4.1.1.2 Chemical degradations

Chemical degradations such as oxidation may not be predominant in solid formulations when compared to liquid formulations. However, there are some examples where, chemical degradations lead to the instability of a protein formulation during storage such as irreversible aggregation of lyophilized rGH during storage [10].

4.1.1.3 Deamidation

Deamidation is a chemical reaction that leads to the removal of amide group and is one of the major causes for the degradation of protein formulations during storage [3]. Strickley et al [14] have reported that lyophilized insulin deamidated via a cyclic anhydride intermediate at C-terminal AsnA21 during storage. Deamidation of lyophilized rGH during storage was reported by Pikal et al [10].

4.1.1.4 Hydrolysis

Hydrolysis can lead to the instability of protein formulations during storage, even though the moisture content is low in dried formulations. Hydrolysis of bFGF in a lyophilized sugar formulation was observed by Wang et al [15].

4.1.2 Factors affecting dried protein formulation stability during storage

There are several factors that affect the stability of dried protein formulations during storage such as storage temperature, glass transition temperature, residual water content, combination and concentration of excipients, and crystallization of amorphous lyoprotectants.

4.1.2.1 Storage Temperature

Storage temperature is one of the most important factors that effects dried protein formulations. Storage of protein formulations at high temperatures leads to an increased mobility of protein molecules, thereby facilitating protein – protein interactions [3]. An increased loss of activity of the restriction enzyme PstI in a trehalose formulation was observed between 37°C and 70°C [16]. An increase in protein aggregation was observed in the case of vacuum-dried LDH between 3°C and 60°C [17]. Chemical degradations are also accelerated at high temperatures such as the dimerization of TNF at temperatures between 25°C and 80°C [18].

4.1.2.2 Glass Transition temperature

Glass transition temperature (Tg) is one of the most important parameters that affect the stability of dried formulations [19]. The viscosity of a dried formulation decreases with the increase in temperature and above Tg, the state of the formulation is altered from glassy to rubbery. Below the glass transition temperature, individual atoms have restricted movement about their equilibrium positions. However, an increase in temperature above Tg, the atoms have a higher degree of both translational and rotational freedom, thereby increasing the mobility of the protein and decreasing the stability of the formulation [20, 21]. Hence, it is always better to have a higher glass transition temperature for the desiccated formulation. It has been recommended that the glass transition temperature be at least 20°C above the ambient storage temperature [19, 22]. There are several factors that affect Tg such as residual water content (increase in water content decreases the Tg of a formulation), composition of the formulation and lyoprotectants involved in the formulations [19, 23]. Hence, an approximate estimation of the formulation glass transition temperature is necessary before conducting any long-term stability studies [2, 24]. Detailed differential scanning calorimetry [21] studies on the glass transition temperatures of trehalose were conducted by Simperler et al. [25]. Their studies indicate that the glass transition temperature of trehalose decreases with increase in the moisture content of the formulation. The drop in the glass transition temperature is

approximately 8 K for every 1% rise in the moisture content. Trehalose is the major component of the gel PCR formulation and assuming that the drop in Tg is linear with increase in the moisture content, the Tg of the gel PCR formulation may range from 31-38°C. Hence, long term stability studies for 10 weeks were conducted by storing the desiccated gels at 40 °C, which is above the glass transition temperature, as well as at temperatures that are below the Tg.

4.1.2.3 Lyoprotectants

A detailed description of the importance and effects of lyoprotectants on the stability of a dried formulation is presented in chapter 2. In that chapter, different combinations of lyoprotectants were tested and the optimal combinations of lyoprotectants were determined. It was determined that trehalose at a final concentration of 0.3 M was a compatible lyoprotectant for preserving in-gel PCR reagents.

4.1.2.4 Residual Water Content

A detailed description of the effects of residual water content on the stability of dried formulations is presented in chapter 3. The desiccation kinetics of the gel PCR formulations were studied and the time of desiccation to achieve the required range of residual water content was determined.

4.2 Objective

To conduct long-term stability studies on the desiccated gel PCR formulations and estimate the shelf life of the formulations.

4.3 Materials and Methods

The residual water content in the dried formulations should be less than 10% [3]. Hence, Stability studies were conducted with dried gel PCR formulations containing 0-2% and 6-10% residual water content.

4.3.1 Estimation of Residual Water Content

Once we perform PCR with the desiccated gels, the molds are completely covered with oil and the gels cannot be further dried to determine the anhydrous weights. Hence, only an estimate of the residual water content can be obtained. To estimate the residual water content, the weight of the molds were measured in triplicate prior to conducting the stability studies. The weights of the molds with freshly polymerized gels and the weights of the molds with the desiccated gels were also measured prior to storing the molds. Before each PCR experiment, the weights of the molds with desiccated gels were again noted. Since the same molds were used for characterizing the desiccation process in chapter 3, all these weight measurements were compared to the weight measurements taken for studying the desiccation kinetics and the range of residual water content was estimated. This range was also correlated with the time of desiccation/residual water content plot.

4.3.2 Stability studies with gels containing 0-2% residual water content

Two hundred fifty microliters of gel PCR mix was prepared with 10 µl Taq Polymerase (20 units/ µl), 5 µl each of 10 µM BKV forward and reverse primers (refer section 2.3.1 for primer sequences) to produce 100 bp product, 5 µl 10mM dNTP, 5 µl 1% BSA (Sigma), 50 µl 5 X PCR buffer (333 mM tris-sulfate, pH 8.6, 83 mM $(NH4)_2SO_4$ (Sigma) and 40% (w/v) sucrose (Sigma, Lot#BCBB7807)), 10 µl 50mM $MgCl_2$, 25 µl 10xLC Green Plus (Idaho Technology Inc., Salt Lake City, Utah), 25 µl of 40% acrylamide (Sigma, cat no. A9099) + 4% bis-acrylamide aqueous solution (N,N-methylene bis-acrylamide, BioRad, Hercules, CA, cat no. BA05-1610201), 5 µl of 3% azobis (Wako, Richmond, cat no. VA-086) and 2.5 µl of 10% TEMED (N,N,N',N'-tetramethyleneethylenediamine, Sigma, cat no. T7024), 50 µl 1.5M trehalose (Cargill Foods Inc., Cat# 2K141) and 62.5 µl water. The final concentration of trehalose in the formulation is 0.3 M as discussed in chapter 2.

The formulation was then de-gassed under vacuum and loaded onto 8 different molds. Twenty-four wells of each mold were loaded with the formulation. Refer to section 2.3.2.2 for gel casting procedures. After polymerization, template DNA was then introduced into one of the molds by adding 10 µl of a mix containing 9.5 µl water and 0.5 µl BKV DNA on top of the gels. This mold, which serves as the fresh control was then left on the bench for approximately 5 minutes to allow diffusion of the DNA into the gels. Another cover slip was then slid on top of the mold to remove the extra liquid.

The mold was then inverted and immersed in mineral oil (Sigma, cat no. M5904), in an anodized aluminium (23x23mm) pan, which was then placed on the peltier element of the Viriloc instrument. PCR was then performed with these gels, with the following parameters: Predenaturation at 94 ^{o}C for 120 s, denaturation at 94 ^{o}C for 10 s, anneal at 58 ^{o}C for 20 s, extension at 72 ^{o}C for 20 s for 35 cycles. The final elongation step was performed at 72 ^{o}C for 120 s. MCA within the temperature range of 55 ^{o}C - 95 ^{o}C , was also performed on these samples. The peak heights obtained during MCA were considered for analysis.

The weights of the rest of the molds with polymerized gels were then noted and the molds were introduced into the vacuum oven for 90 minutes after which the weights of the molds with desiccated gels were noted again. Because the molds were to be used for performing PCR, it was impossible to directly determine the anhydrous weight so this value was inferred for calculations of residual water. From figure 3.2, we can infer that the residual water content in the molds ranged from 0-2%. One of the molds was then rehydrated by adding 10 µl of a mix containing 9.5 µl water and 0.5 µl BKV DNA on top of the gels. This mold, which serves as the desiccated control was then left on the bench for approximately 5 minutes to allow diffusion of the DNA into the gels. A cover slip was then slid on top of the mold to remove the extra liquid. The mold was then prepared for carrying out PCR following the same procedures as the fresh control. The PCR and MCA parameters are also

same as that used for the fresh controls. The rest of the molds were stored at 4°C (refrigerator) and room temperature for 48 hours. Every 24 hours, one mold was selected from each temperature condition. The weight of the mold was measured and the mold was then subjected to rehydration with the template DNA. PCR and MCA were then performed with these molds.

4.3.3 Stability studies with gels containing 6-10% residual water content

In an attempt to introduce negative controls on same mold, the rest of the experiments were conducted to detect BKV 150bp product as the molds were contaminated with BKV 100bp product. However, I found that contamination of the negative controls could not be avoided with the present geometry of the molds.

Two hundred and fifty microliters of gel PCR mix was prepared with 10 µl Taq Polymerase (20 units/ µl), 5 µl each of 10 µM BKV forward and reverse primers to produce 150 bp product, 5 µl 10mM dNTP, 5 µl 1% BSA, 50 µl 5 X PCR buffer, 10 µl 50mM $MgCl_2$, 25 µl 10xLC Green Plus, 25 µl of 40% acrylamide + 4% bis-acrylamide aqueous solution, 5 µl of 3% azobis and 2.5 µl of 10% TEMED, 50 µl 1.5M trehalose and 62.5 µl water.

Primer Sequence:

Forward Primer: BKV -F 150bp 5'- TAT TTT AAG ATC CGC CTG A -3'

Reverse Primer: BKV- Rani 5'-TCA AAC ACC CTA ACC TCT TCT ACC TG-3'

The formulation was then de-gassed under vacuum and loaded onto 8 different molds. Twenty-four wells of each mold were loaded with the formulation. Please refer to section 2.3.2.2 for gel casting procedures. After polymerization, template DNA was then introduced into one of the molds by adding 10 µl of a mix containing 9.5 µl water and 0.5 µl BKV DNA on top of the gels. This mold, which serves as the fresh control was left on the bench for approximately 5 minutes to allow diffusion of the DNA into the gels. A cover slip was then slid on top of the mold to remove the extra liquid. The mold was then prepared for carrying out PCR following the same procedures outlined in the previous section. The PCR and MCA parameters are the same as those used in the previous section.

The weights of the rest of the molds with polymerized gels were then measured and the molds were introduced into the vacuum oven for 24 minutes (refer to Chapter 3). The weights of the molds with desiccated gels were again measured. From Figure 3.2, we can infer that the residual water content in the molds ranged from 6-10%. One of the molds was then rehydrated by adding 10 µl of a mix containing 9.5 µl water and 0.5 µl BKV DNA on top of the gels. This mold, which serves as the desiccated control was then left on the bench for approximately 5 minutes to allow diffusion of the DNA into the gels. A cover slip was then slid on top of the mold to remove the extra liquid. The mold was then prepared for carrying out PCR following the same procedures as the fresh control. The PCR and MCA parameters are also

same as those used for the fresh controls. The rest of the molds were stored at 4°C and room temperature 48 hours. Every 24 hours, one mold was selected from each temperature condition, the weight of the mold was measured and the mold was then subjected to rehydration with the template DNA. PCR and MCA were then performed with these molds.

4.3.5 One to ten week stability studies with gels containing 6-10% residual water content

Six hundred microliters of gel PCR mix was prepared with 24 µl Taq Polymerase (20 units/ µl), 12 µl each of 10 µM BKV forward and reverse primers to produce 150 bp product, 12 µl 10mM dNTP, 12 µl 1% BSA, 90 µl 5 X PCR buffer, 24 µl 50mM $MgCl_2$, 60 µl 10xLC Green Plus, 60 µl of 40% acrylamide + 4% bis-acrylamide aqueous solution, 12 µl of 3% azobis, 6 µl of 10% TEMED, 120 µl 1.5M trehalose and 192 µl water.

The formulation was then de-gassed under vacuum and loaded onto 20 different molds. Twenty-four wells of each mold were loaded with the formulation. Refer to section 2.3.2.2 for gel casting procedures. After polymerization, template DNA was then introduced into one of the molds by adding 10 µl of a mix containing 9.5 µl water and 0.5 µl BKV DNA on top of the gels. This mold, which serves as the fresh control was then left on the bench for approximately 5 minutes to allow diffusion of the DNA into the gels. A cover slip was then slid on top of the mold to remove the extra liquid. The mold was then prepared for carrying out PCR following the same procedures

outlined in the previous section. The PCR and MCA parameters are also same as those used in the previous section.

The weights of the rest of the molds with polymerized gels were then noted and the molds were introduced into the Vacuum Oven for 24 minutes. The weights of the desiccated molds were noted. From figure 3.2, we can infer that the residual water content in the molds ranged from 6-10%. One of the molds was then rehydrated by adding 10 µl of a mix containing 9.5 µl water and 0.5 µl BKV DNA on top of the gels. This mold, which serves as the desiccated control was then left on the bench for approximately 5 minutes to allow diffusion of the DNA into the gels. A cover slip was then slid on top of the mold to remove the extra liquid. The mold was then prepared for carrying out PCR following the same procedures as the fresh control. The PCR and MCA parameters are also same as those used for the fresh controls. The rest of the molds were stored at 4°C, room temperature and 40°C (incubator set at 40°C) for 10 weeks. Every week for the first 5 weeks, one mold was selected from each temperature condition, the weight of the mold was measured and the mold was then subjected to rehydration with the template DNA. PCR and MCA were then performed with these molds. The remaining molds were stored for at the same conditions for 5 more weeks before rehydrating them.

4.3.6 Packaging Strategies

The desiccated molds are packaged prior to subjecting them to different temperature conditions for long durations. Current packaging strategies involve inserting the molds with the desiccated gels in a food wrapper and sealing the wrapper using a vacuum food-packaging machine (FoodSaver V3840 vacuum sealing system) . Figure shows a packaged mold.

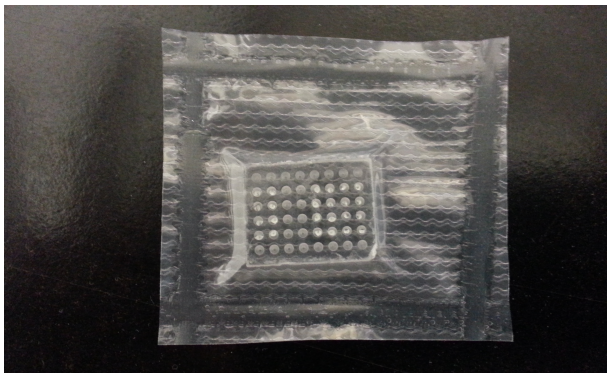


Figure 4.1: Packaged gel PCR chip

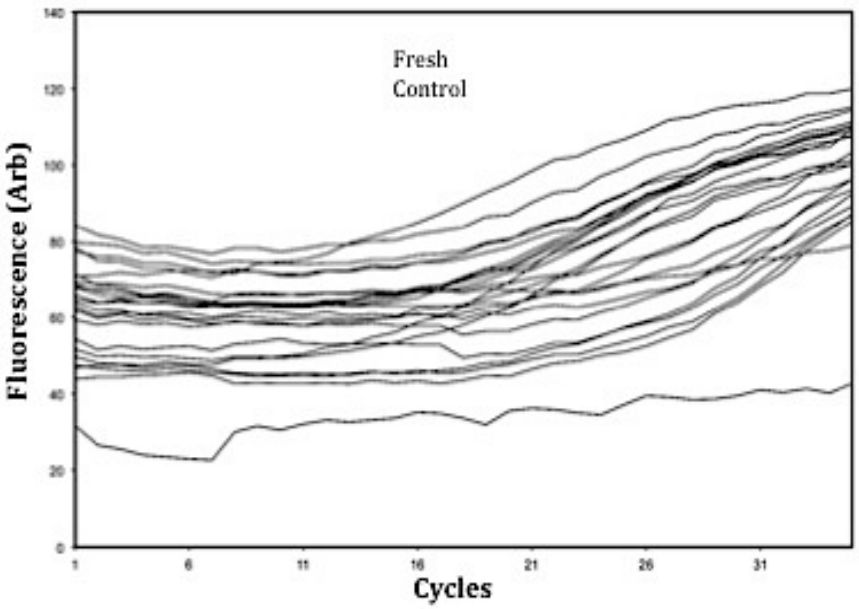
4.3.7 Data Analysis

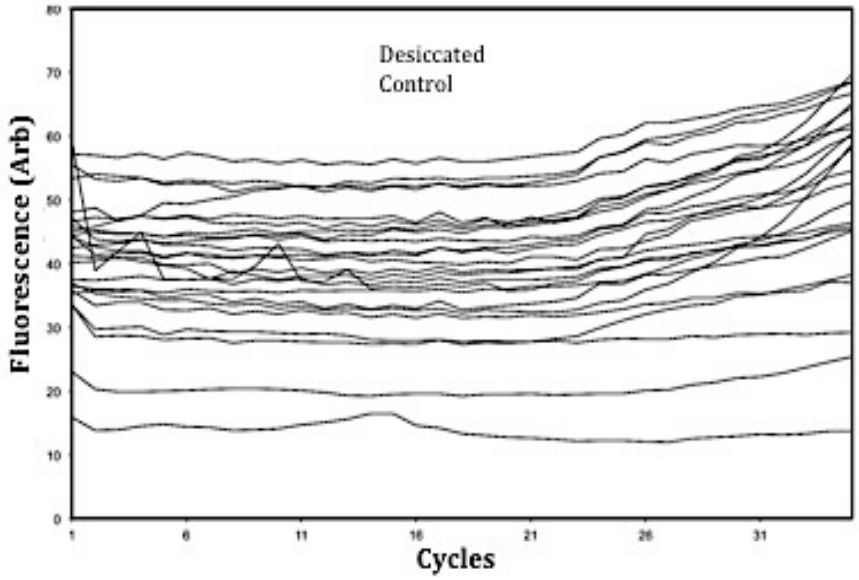
Since our technology was still in the stage of testing during the time of this thesis project and several geometries of the gel PCR chip were being tested here and by others in the team. Hence, there were only a limited number of chips for a particular geometry. This meant that I was able to allocate only one mold per time point and condition tested. While this limits the extent to which certainty can be achieved, it does provide sufficient information for a future design that is more comprehensive. Due to the small sample set, it was very difficult to analyze the data based on peak heights for the stability studies and peak height is in any case not a quantitative measure of PCR

efficiency. Therefore, a different strategy was applied to estimate the shelf life of the desiccated gel PCR reagents. As discussed in the previous sections, 24 wells of each mold contained the gel PCR reagents. During PCR, some wells within a single mold provide positive results in terms of Cp values and some do not depending on the activity of the gel PCR reagents in those wells. Hence, the data was analyzed on the basis of "%POS" (% positive wells), which is determined by the percentage of the number of wells in a mold (of the total 24 wells) that provide positive result which can range from 0 – 100% for 0/24 – 24/24 wells. The mean Cp value and standard deviation, for all the 24 wells in a mold were computed. The wells with Cp values that lie in the range of the 95% confidence interval of the mean Cp values were considered as a positive result and the wells with Cp values outside this range were considered as failures. For example, if the mean Cp value of a particular mold is 24 and the standard deviation is ±2, all the wells with Cp values in the range of 20-28 (24 ± 1.96×(2) (z-value is 1.96 for 95% confidence)) were considered as positive results. Those more than 2SD below the control rejected as failures.

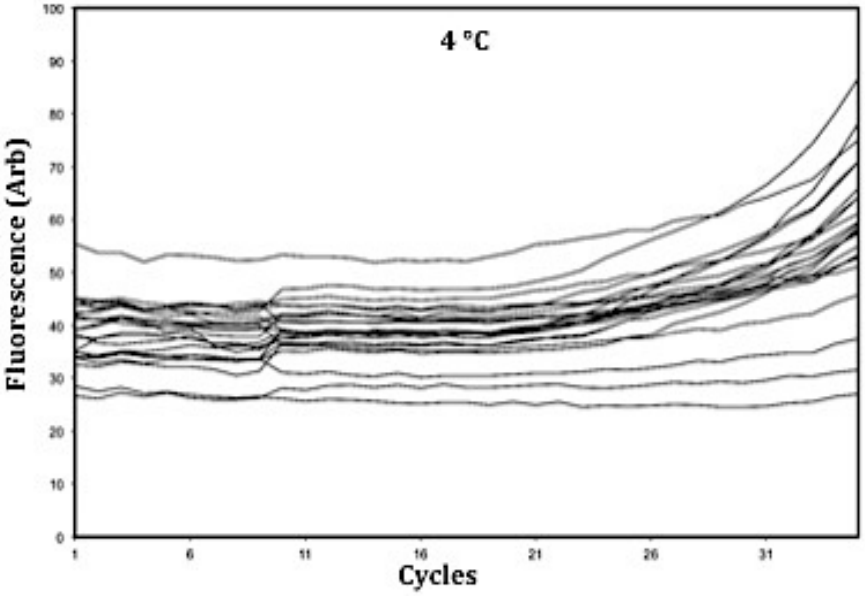
4.4 Results

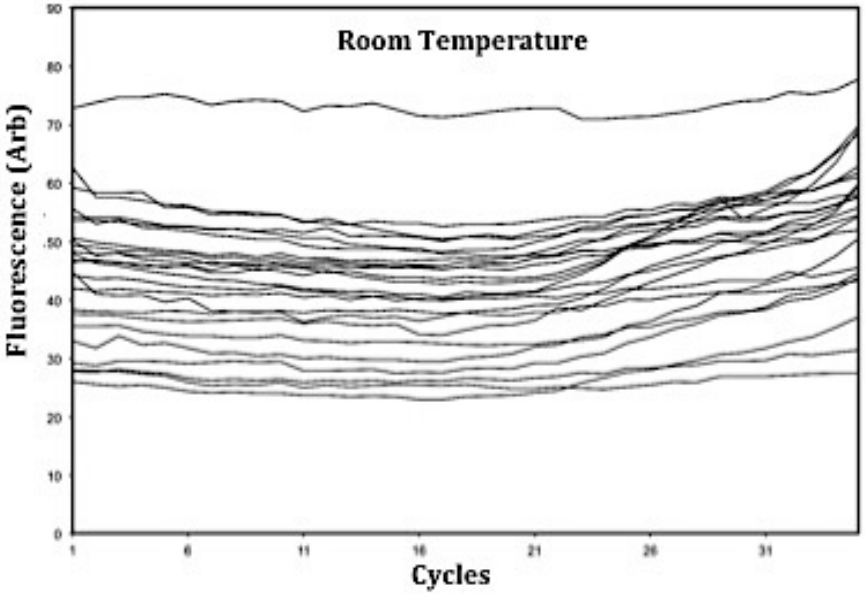
4.4.1 Stability studies with gels containing 0-2% residual water content





24 hours


4 °C
Fluorescence (Arb)
Cycles
0
10
20
30
40
50
60
70
80
90
100
1
6
11
16
21
26
31


Room Temperature
Fluorescence (Arb)
Cycles
0
10
20
30
40
50
60
70
80
90
1
6
11
16
21
26
31

48 hours

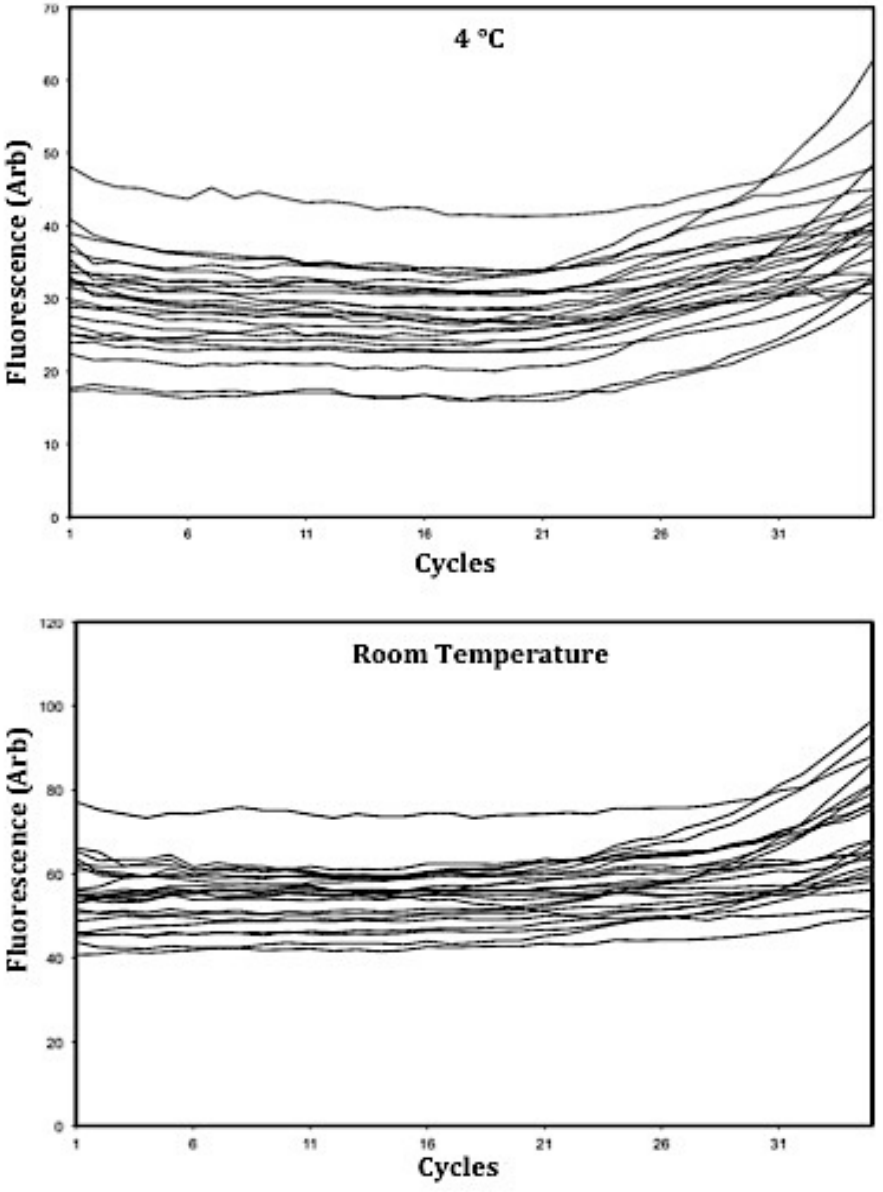
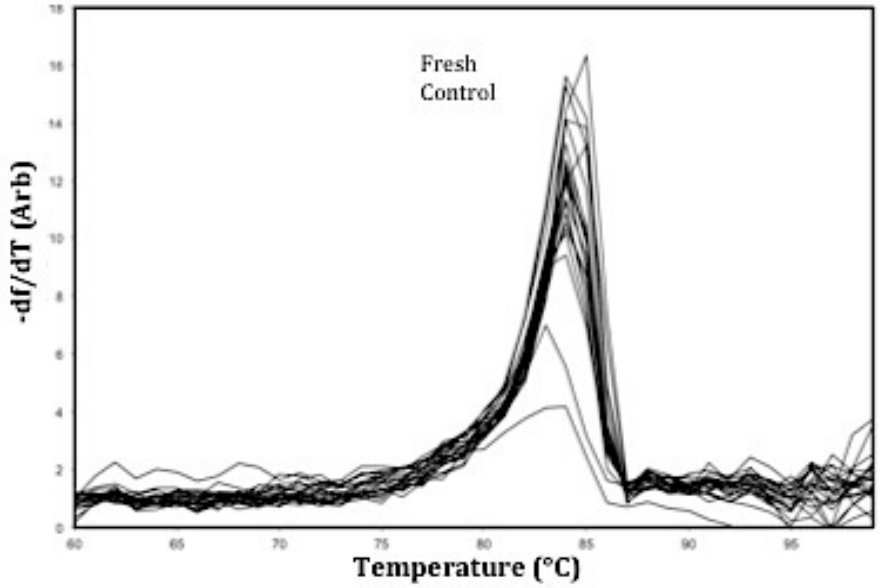
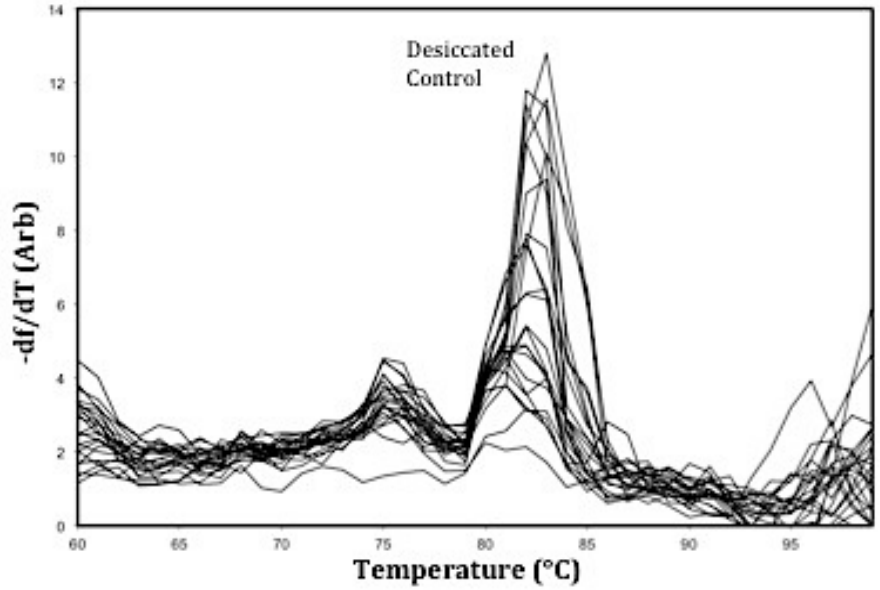


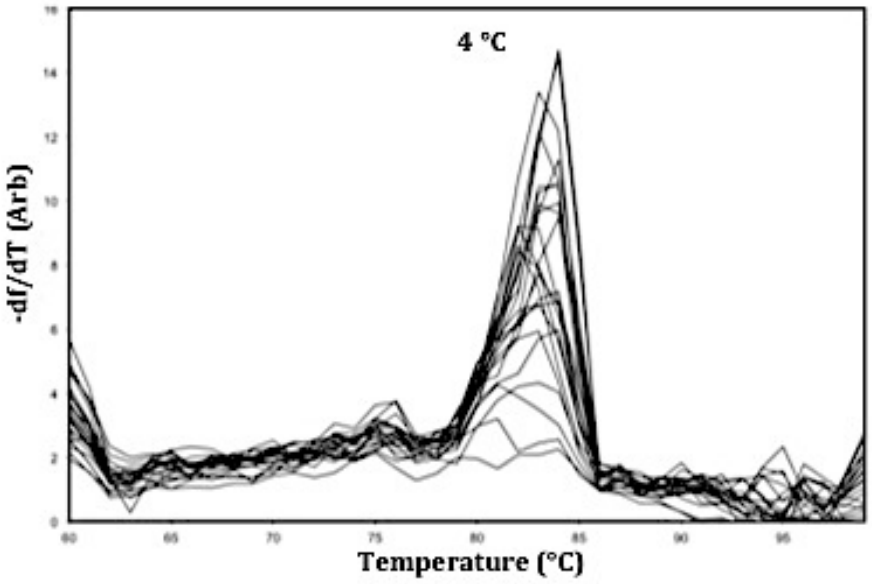
Figure 4.2: PCR curves with formulations containing 0-2% residual water content. A comparison of PCR curves of fresh control samples with desiccated samples containing 0-2%, residual water content, stored at different temperature conditions for 48 hours.

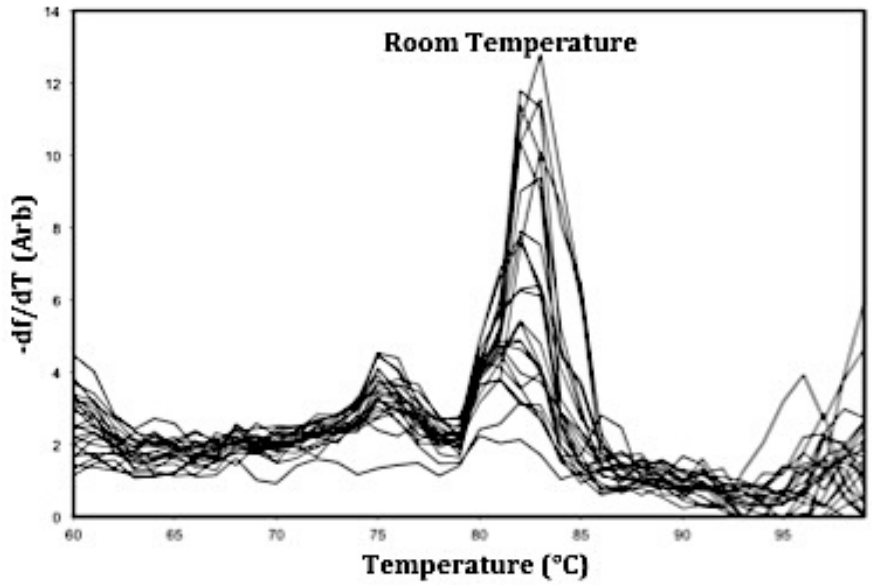
The results shown in Fsigure 4.2 were obtained with PCR formulations containing 0-2%, residual water content. The fluorescence intensity of the PCR curves of the control sample (not desiccated) exponentially rises after cycle 22 whereas the exponential rise in the fluorescence intensity of the desiccated control sample is observed only after cycle 29 indicating that the PCR efficiency of this sample was poor, suggesting that in this mold something had gone wrong during the desiccation process as it was expected that the desiccated control should be equivalent to the fresh control. This likely reflects the low residual water content being inappropriate for preservation of Taq polymease activity as discussed below.


Fresh
Control
-df/dT (Arb)
Temperature (°C)


Desiccated
Control
-df/dT (Arb)
Temperature (°C)

24 hours


4 °C
-df/dT (Arb)
Temperature (°C)


Room Temperature
-df/dT (Arb)
Temperature (°C)

48 hours

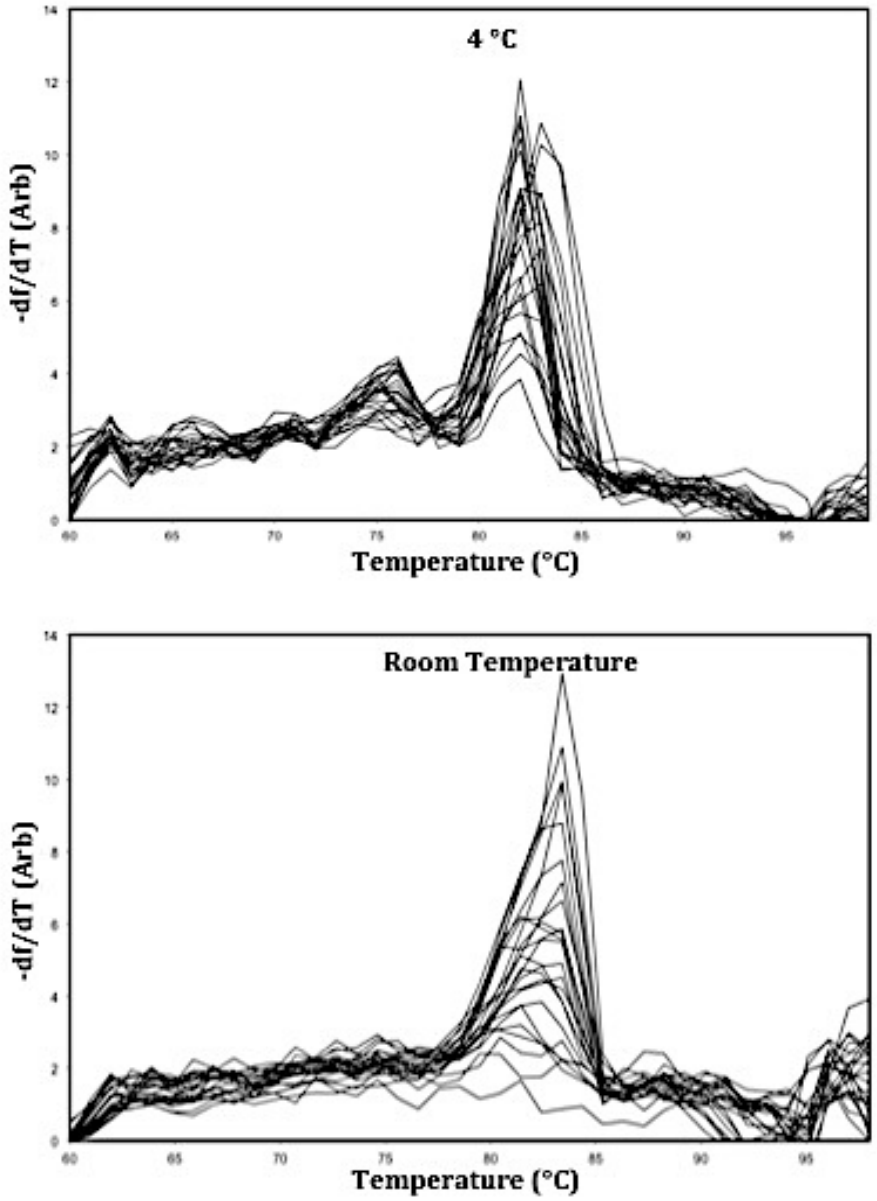


Figure 4.3: Melt peaks with formulations containing 0-2% residual water content. A comparison of Melt peaks of fresh control samples with desiccated samples containing 0-2%, residual water content, stored at different temperature conditions for 48 hours.

The melt peaks obtained with the control and desiccated samples during the 48 hours stability studies are presented in Figure 4.3. The mean melt peak height of the control sample is high (11.7±2.3) when compared to the desiccated controls (5.8±1.7). The desiccated samples that were stored at 4°C have peak heights that range from 7.8-8 and the samples stored at room temperature have even lower peak heights in the range of 5.5-6.5.

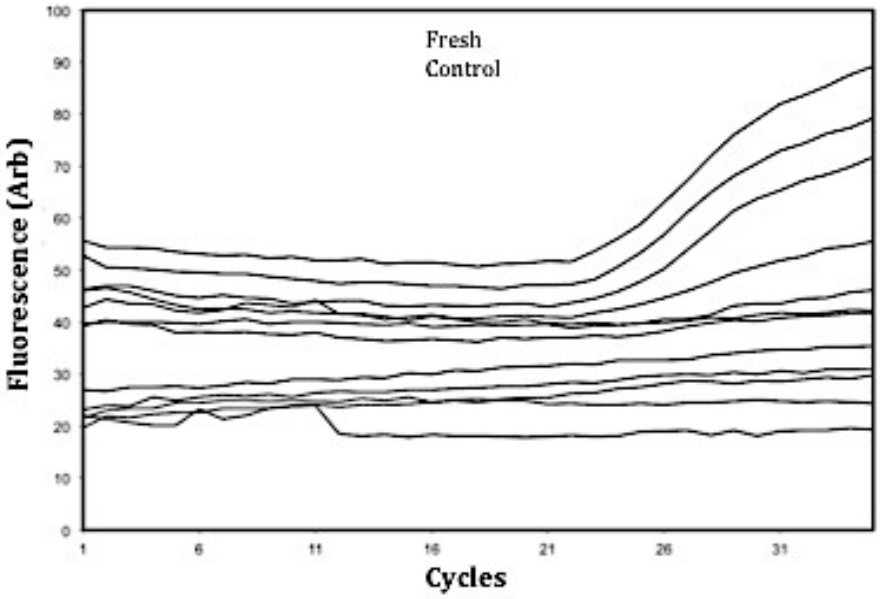
A comparison of the %POS of the control samples and the desiccated samples is presented in Table 4.1. As we can observe from the table, the %POS of the fresh control sample is very high (95.8%) when compared to the %POS of the desiccated control (79.2%). The %POS after 24 hours is 62.5% and 83.3% for the samples stored at 4°C and room temperature respectively. After 48 hours, the %POS is 83.3% and 54.1% samples stored at 4°C and room temperature. Compared to the desiccated control, the %POS is high, even recovering somewhat for samples stored at 4°C for 48 hours, but this comparison is likely to be flawed. It appears that in this experiment, the desiccation process itself failed, because the control evaluated immediately after desiccation is already very low even without any storage time. It appears that low residual water content is immediately damaging to the PCR reagents, likely to reflect loss of activity of the Taq Polymerase, even before any storage occurs.

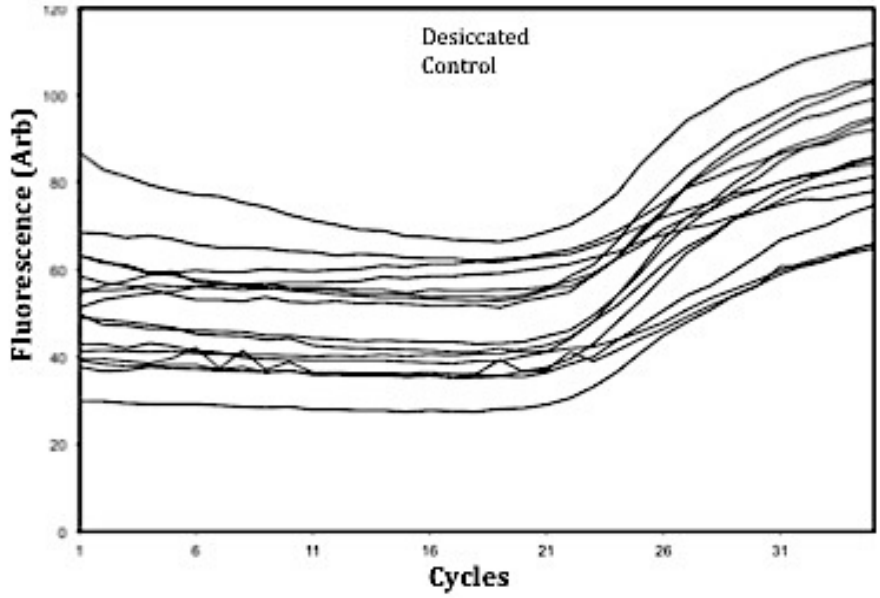
Time/%POS	Fresh Controls	Desiccated Controls	4°C	Room Temperature
0	96	79.2		
24 Hours			62.5	83.3
48 Hours			83.3	54.1

Table 4.1: A comparative analysis of %POS of fresh control samples with desiccated samples containing 0-2%, residual water content stored at different temperature conditions for 48 hours.

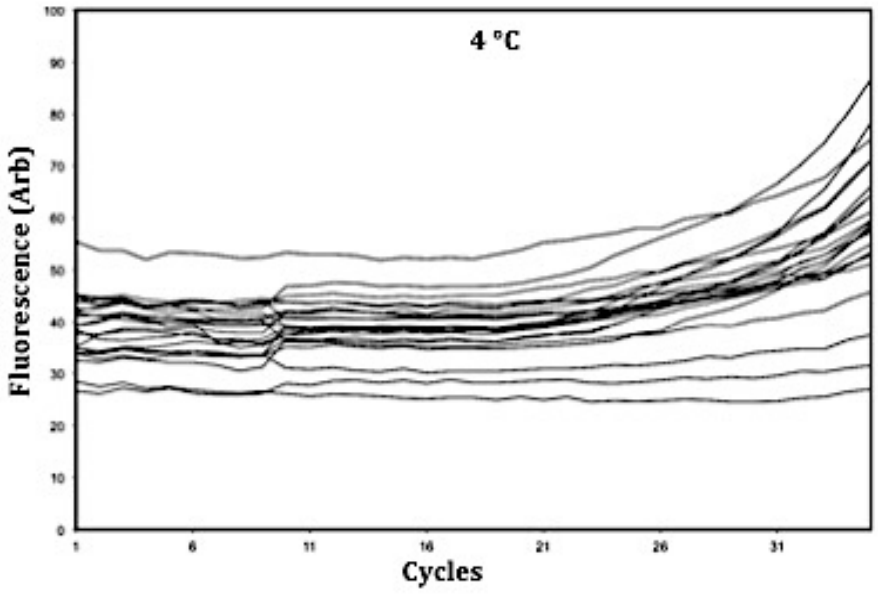
4.4.2 Stability studies with gels containing 6-10% residual water content

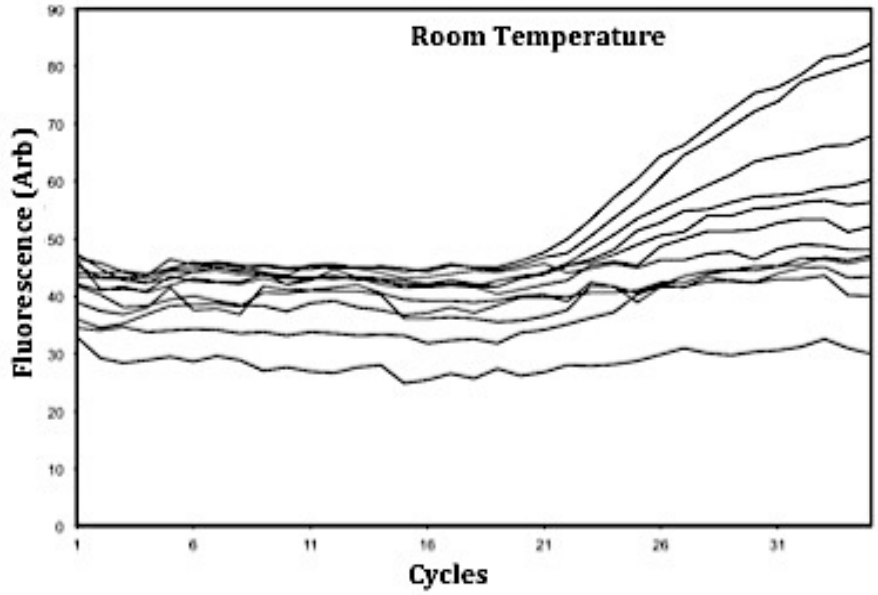
The results shown in Figure 4.4 were obtained with PCR formulations containing 6-10%, residual water content. The desiccated samples were stored at two different temperature conditions of 4°C and room temperature. This preliminary study was conducted to determine the viability of the dried gel PCR formulations after 48 hours. The fluorescence intensity of the PCR curves of the control sample (not desiccated) exponentially rises after cycle 22 and the exponential rise in the fluorescence intensity of the desiccated control sample is observed after cycle 20 indicating that the PCR efficiency of the desiccated sample is comparable to that of the fresh sample. The Cp values of samples that were stored at 4°C ranged from 22-25 and that of the samples stored at room temperature ranged from 22-27.


Fresh
Control
Fluorescence (Arb)
Cycles
0
10
20
30
40
50
60
70
80
90
100
1
6
11
16
21
26
31


Desiccated
Control
Fluorescence (Arb)
Cycles
0
20
40
60
80
100
1
6
11
16
21
26
31

24 hours


4 °C
Fluorescence (Arb)
Cycles


Room Temperature
Fluorescence (Arb)
Cycles

48 hours

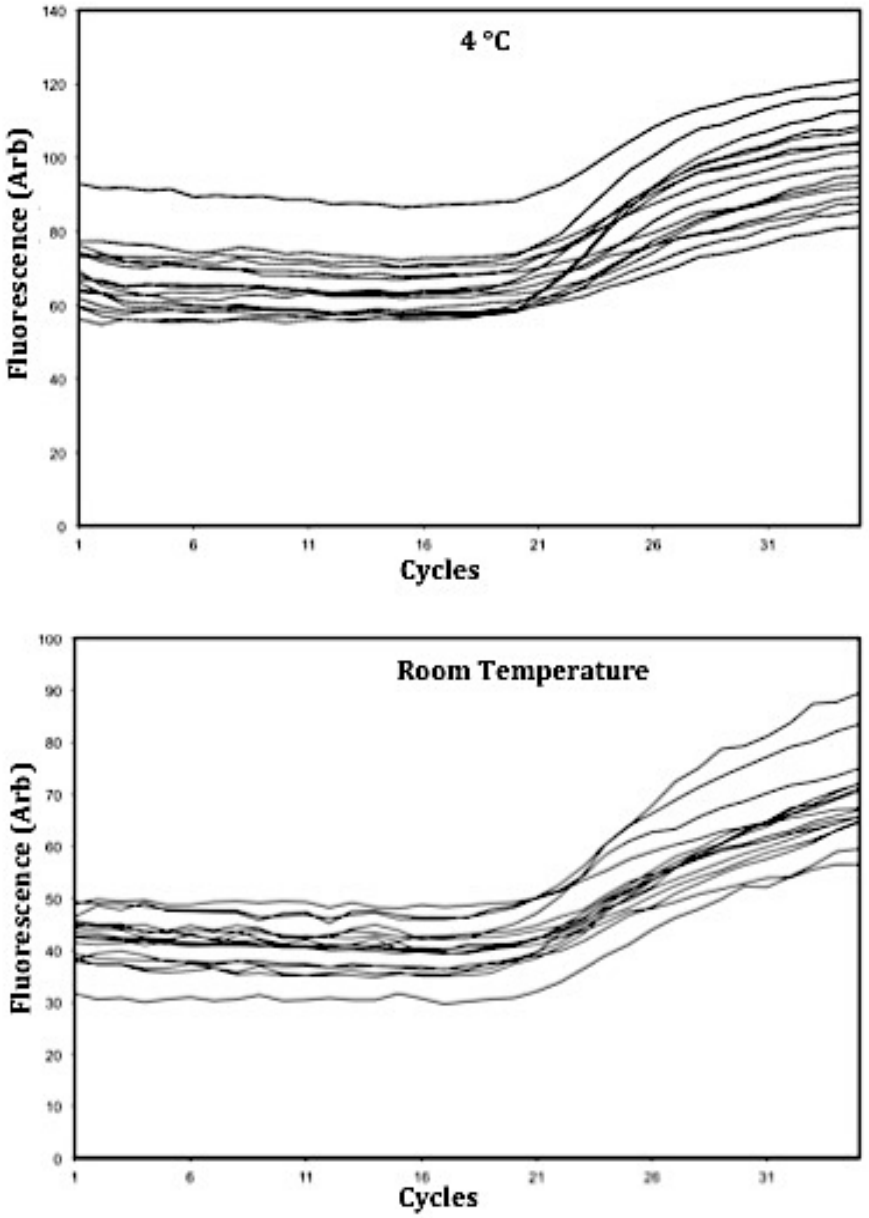
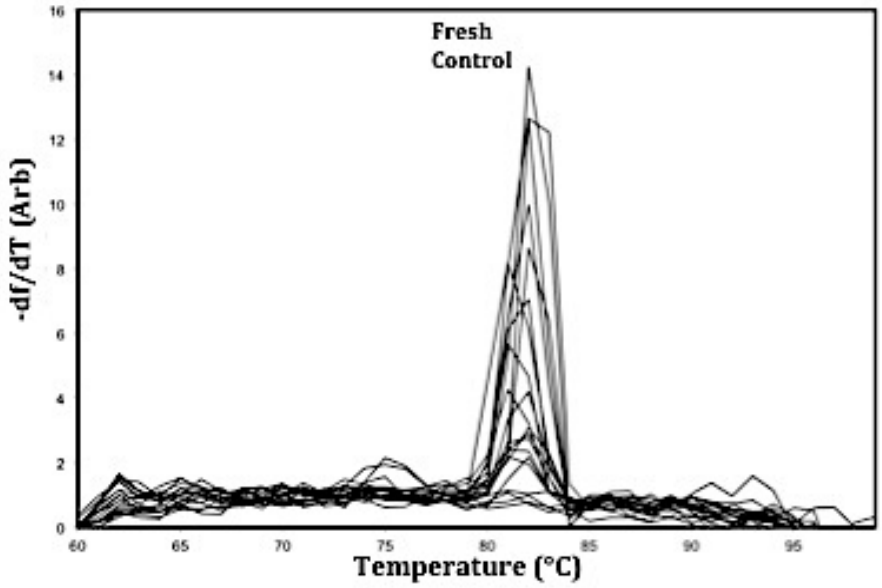
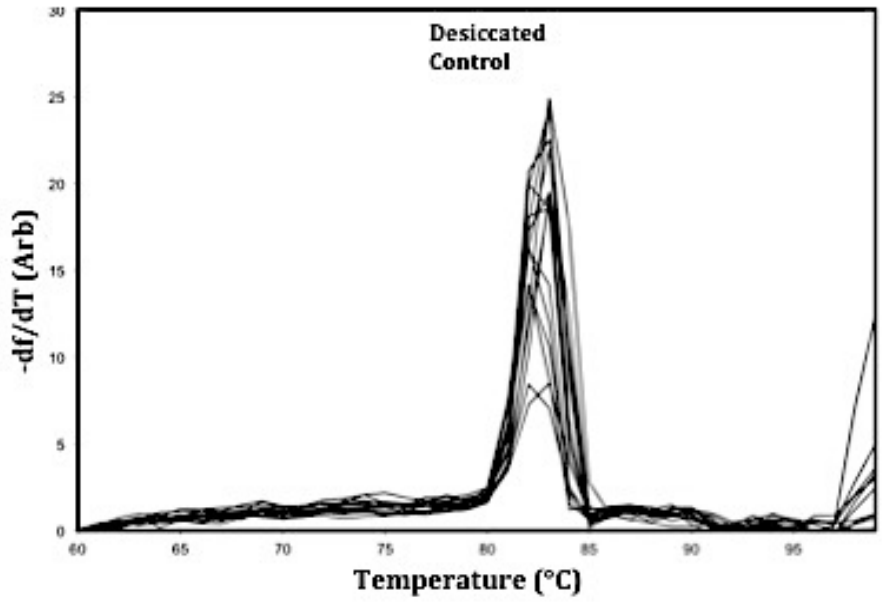


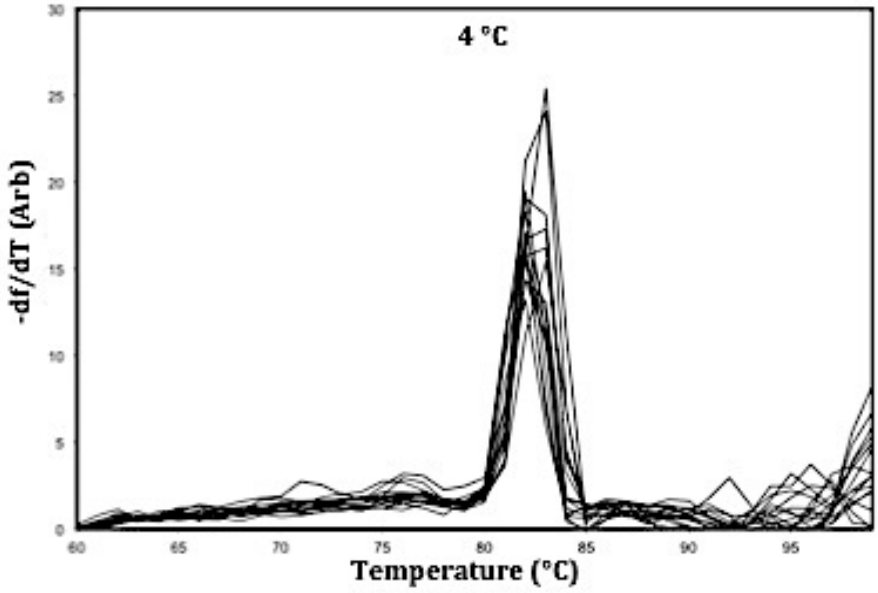
Figure 4.4: PCR curves with formulations containing 6-10% residual water content. A comparison of PCR curves of fresh control samples with desiccated samples containing 6-10%, residual water content, stored at different temperature conditions for 48 hours.

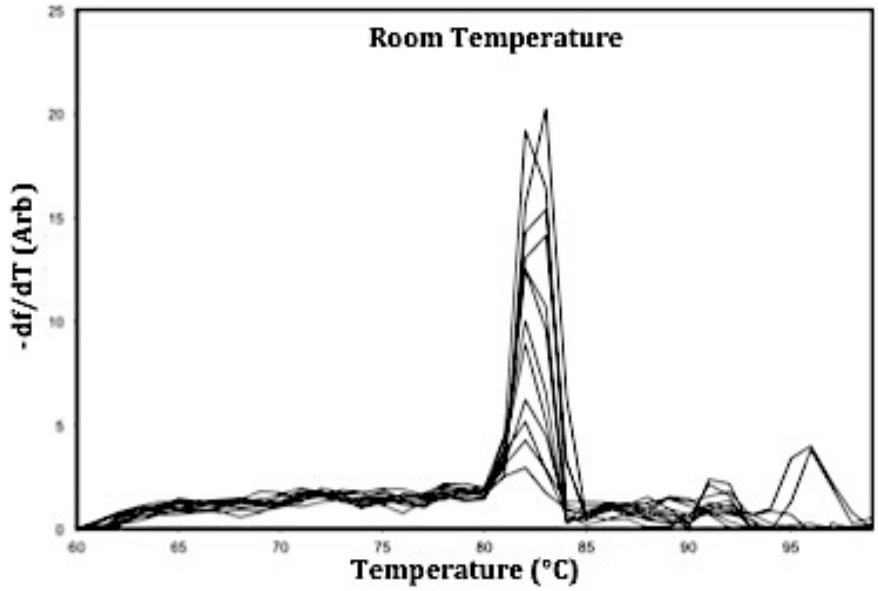
The melt peaks obtained with the control and desiccated samples during the 48 hours stability studies are presented in Figure 4.5. The mean melt peak heights of the fresh and desiccated control samples are 5.1±4.3 and 17.12±6.1 respectively. The mean melt peak heights of the desiccated samples stored at 4°C ranged from 12-17 and that of the desiccated samples stored at the room temperature ranged from 9-17.


Fresh
Control
-df/dT (Arb)
Temperature (°C)


Desiccated
Control
-df/dT (Arb)
Temperature (°C)

24 hours


4 °C
-df/dT (Arb)
Temperature (°C)


Room Temperature
-df/dT (Arb)
Temperature (°C)

48 hours

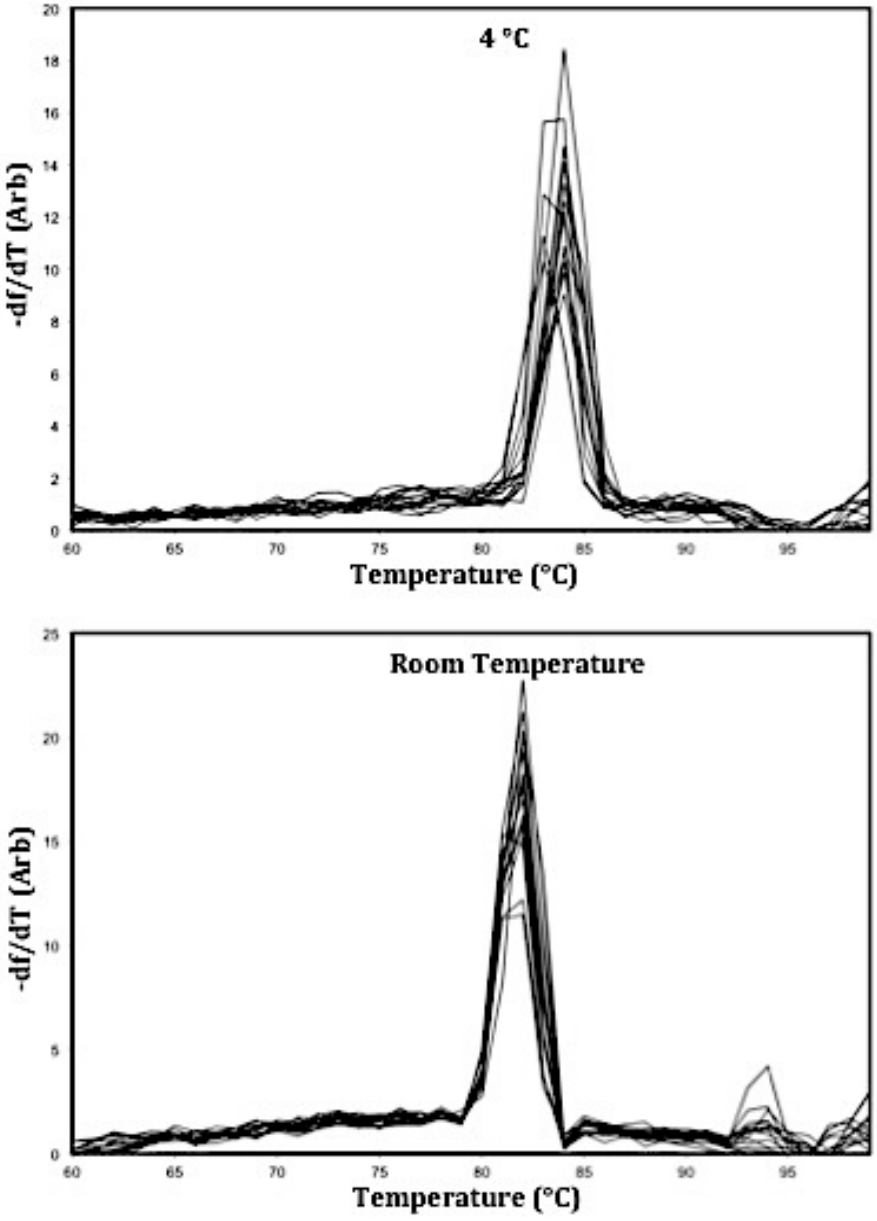


Figure 4.5: Melt peaks with formulations containing 6-10% residual water content. A comparison of melt peaks of fresh control samples with desiccated samples containing 6-10%, residual water content, stored at different temperature conditions for 48 hours.

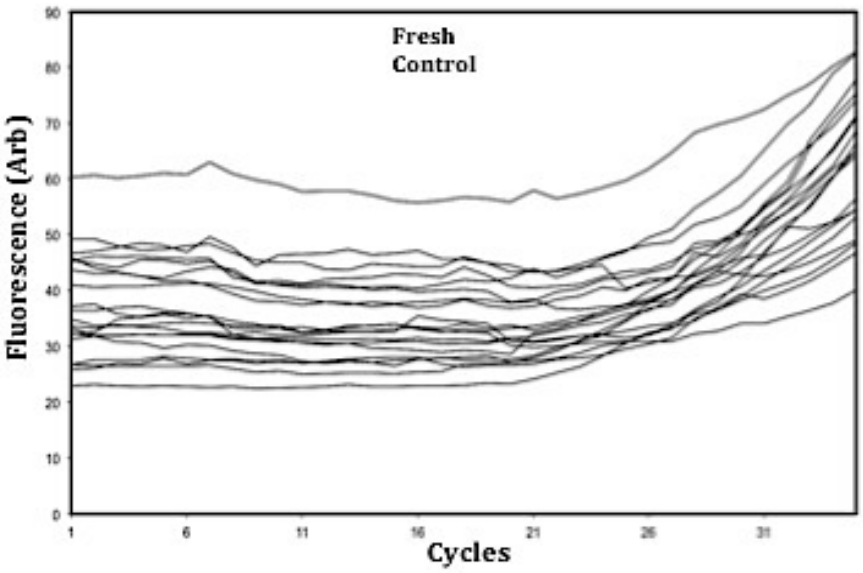
A comparison of the %POS of the control samples and the desiccated samples is presented in Table 4.2. As we can observe from the table, the %POS of the fresh control sample is lower (87.5%) when compared to the %POS of the desiccated control (100%). The %POS after 24 hours is 94% for the all the samples and after 48 hours, the %POS is 100% for all the samples indicating that the range of residual water content present in these samples may be compatible.

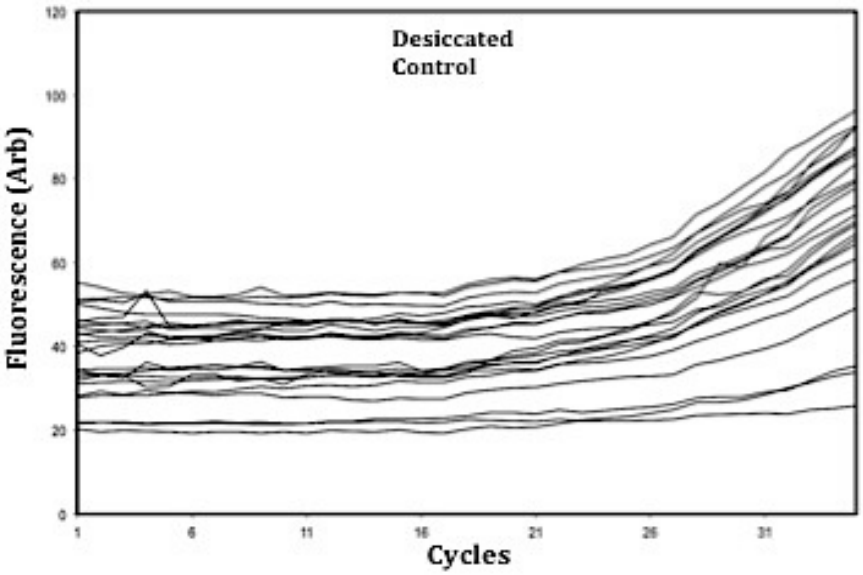
Time/%POS	Fresh Controls	Desiccated Controls	4°C	Room Temperature
0	87.5	100		
24 Hours			94	94
48 Hours			100	100

Table 4.2: A comparative analysis of %POS of fresh control samples with desiccated samples containing 6-10%, residual water content stored at different temperature conditions for 48 hours.

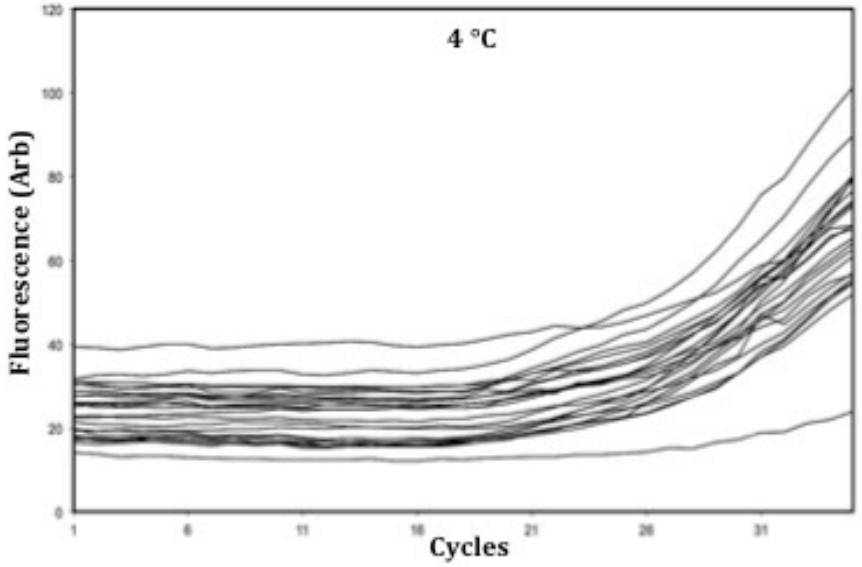
4.4.3 One to ten week stability studies with gels containing 6-10% residual water content

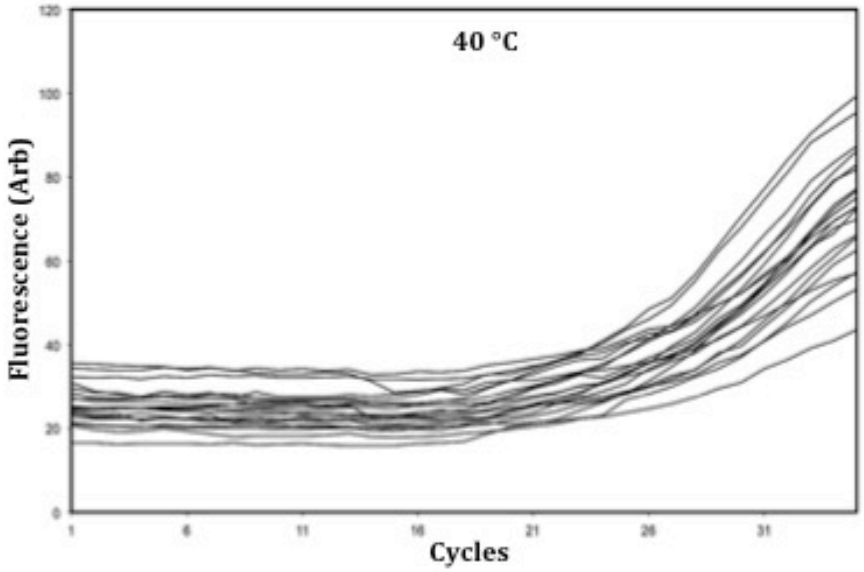
A comparison of the PCR curves generated with fresh control samples and desiccated samples stored for 10 weeks under 3 different temperature conditions of 4°C, room temperature and 40°C are presented in Figure 4.6. It is to be noted that the data for the first week for samples stored at room temperature is not available because of an instrument malfunction during the experiment. The mean Cp value of the fresh control sample is around 28 and that of the desiccated sample is also around 28. For the desiccated samples stored at 4°C, the mean Cp values vary between 26-28. The mean Cp value of these samples is 27 after 10 weeks. For the samples stored at room temperature for 10 weeks, the mean Cp values also range from 26-28. However, for the samples stored at 40°C, the mean Cp values do not vary much during the first 5 weeks (≈28), but the PCR curves for the sample stored for 10 weeks are flat and do not show any exponential rise in fluorescence intensity indicating that there is no amplified product generated with the molds analyzed after week 5.


Fresh
Control
Fluorescence (Arb)
Cycles

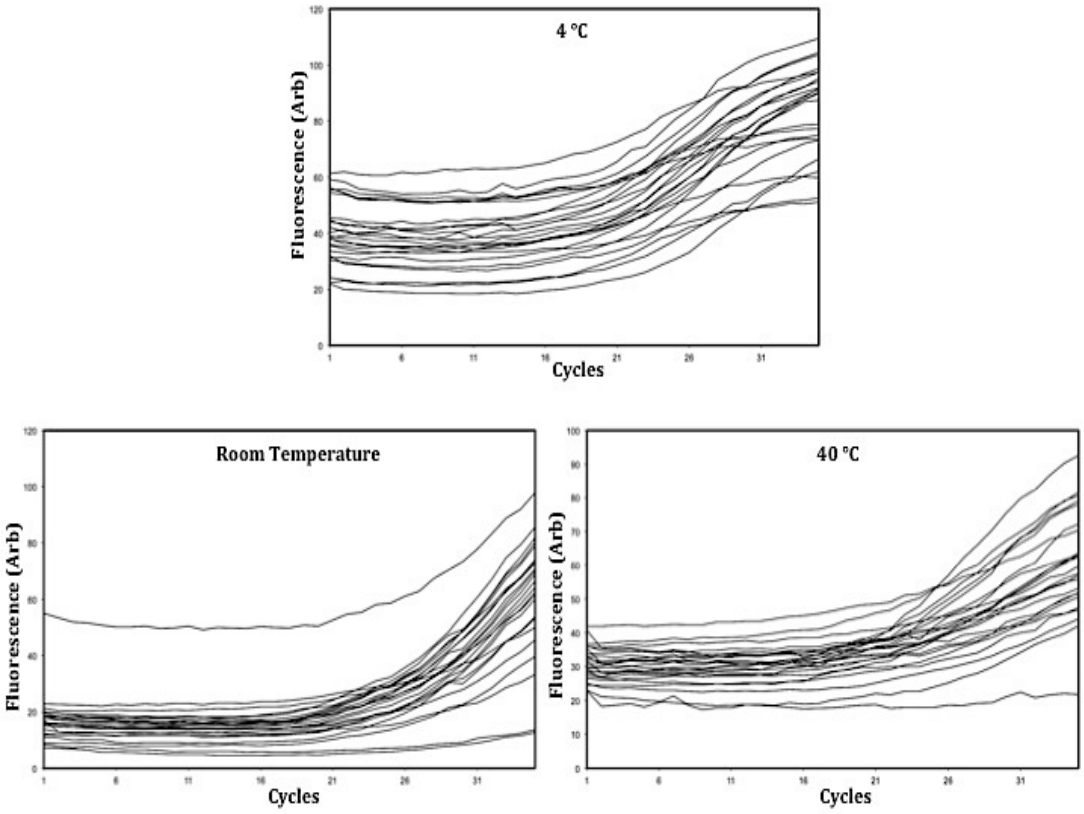

Desiccated
Control
Fluorescence (Arb)
Cycles

Week 1

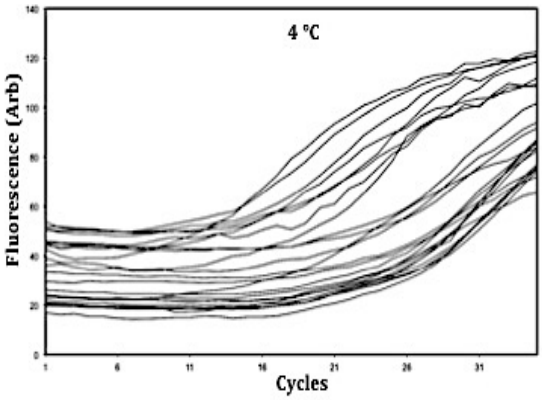

4 °C
Fluorescence (Arb)
Cycles
0
20
40
60
80
100
120
1
6
11
16
21
26
31

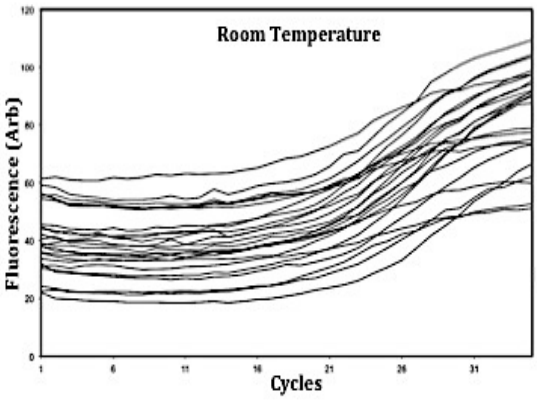

40 °C
Fluorescence (Arb)
Cycles
0
20
40
60
80
100
120
1
6
11
16
21
26
31

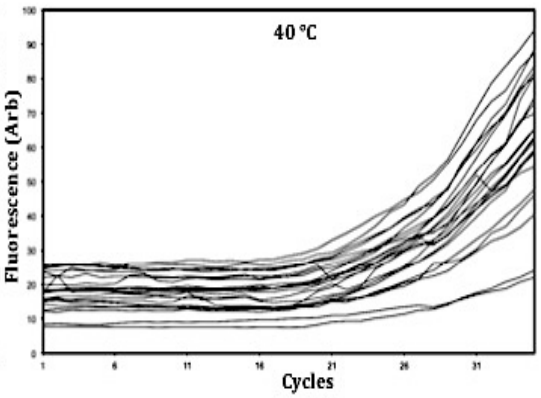
Week 2


4 °C
Fluorescence (Arb)
Cycles
Room Temperature
Fluorescence (Arb)
Cycles
40 °C
Fluorescence (Arb)
Cycles

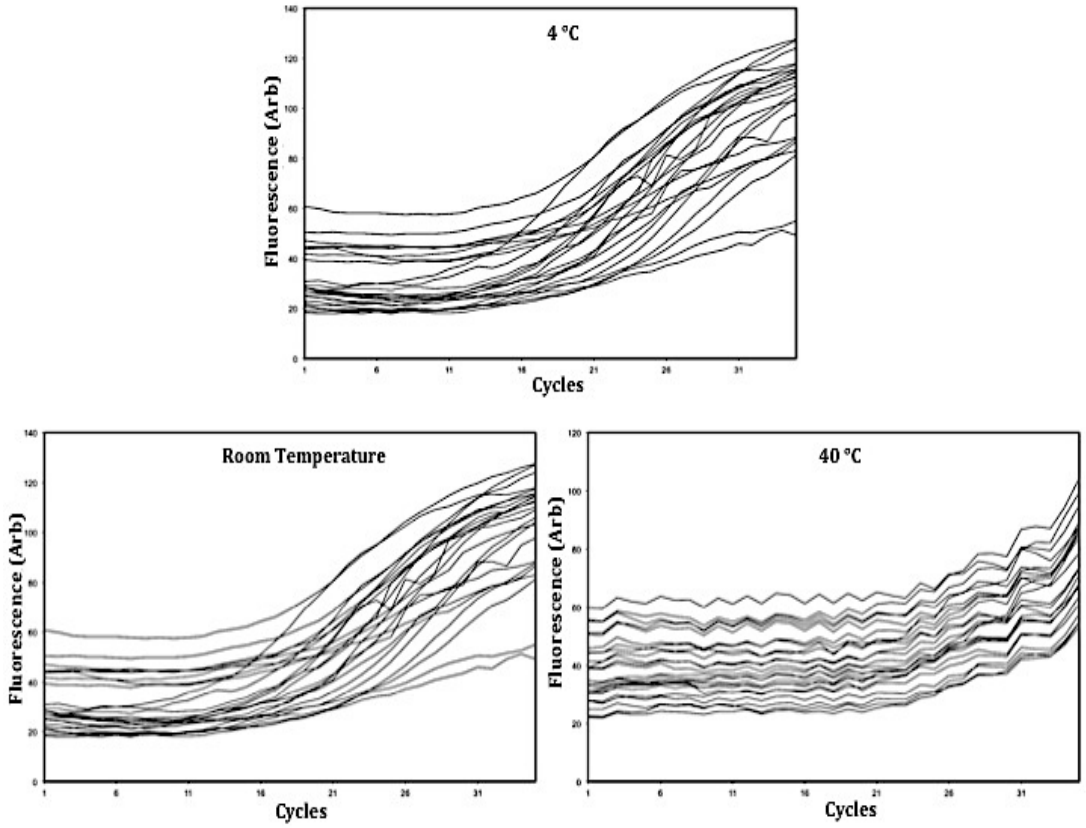
Week 3


4 °C
Fluorescence (Arb)
Cycles

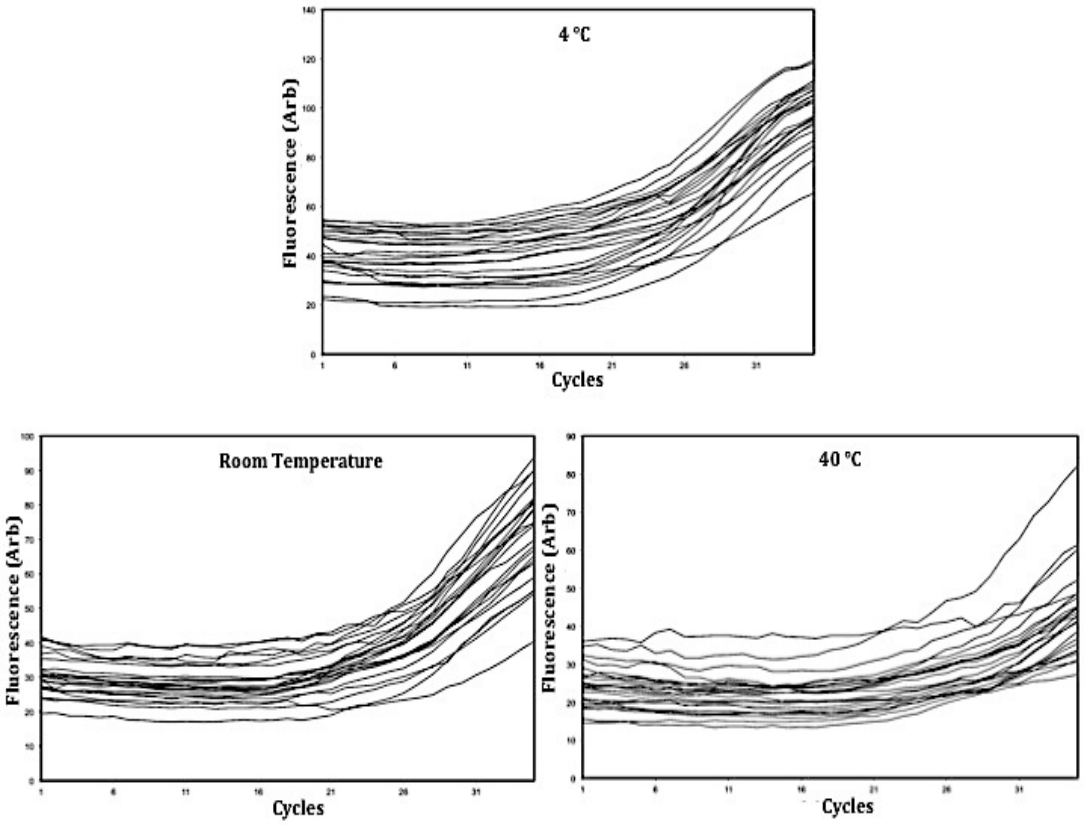

Room Temperature
Fluorescence (Arb)
Cycles


40 °C
Fluorescence (Arb)
Cycles

Week 4


4 °C
Fluorescence (Arb)
Cycles
Room Temperature
Fluorescence (Arb)
Cycles
40 °C
Fluorescence (Arb)
Cycles

Week 5


4 °C
Fluorescence (Arb)
Cycles
Room Temperature
Fluorescence (Arb)
Cycles
40 °C
Fluorescence (Arb)
Cycles

Week 10

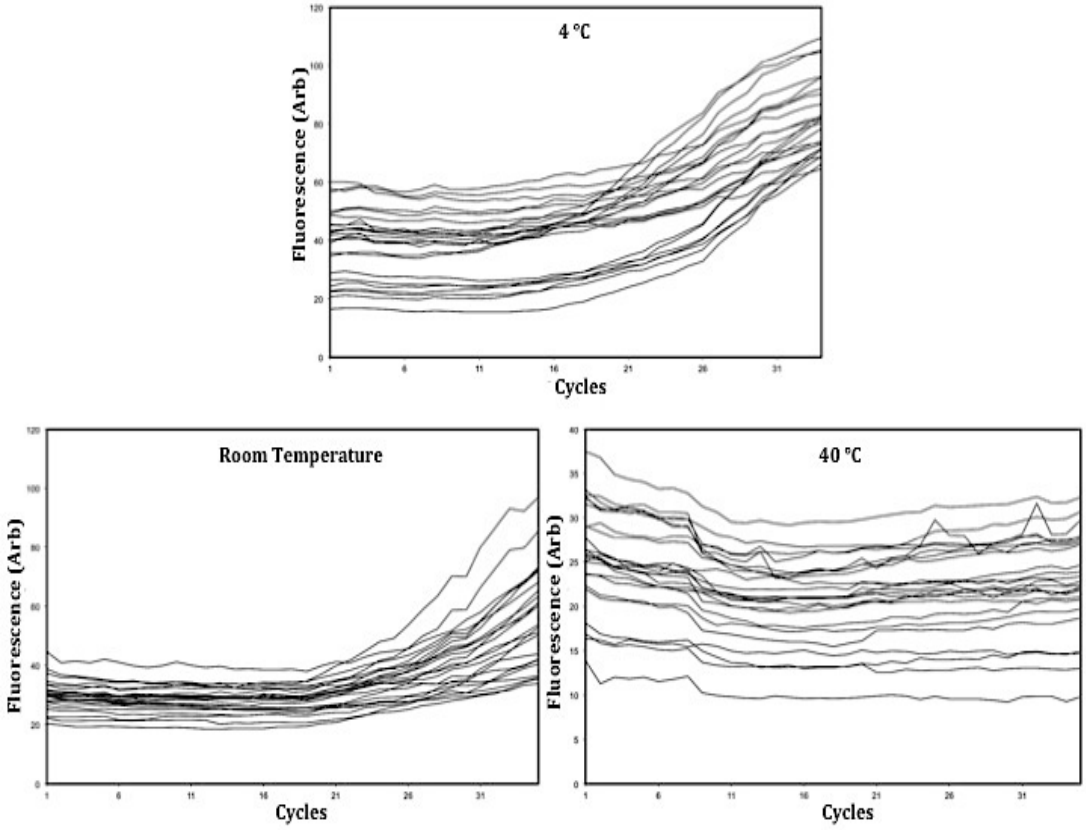
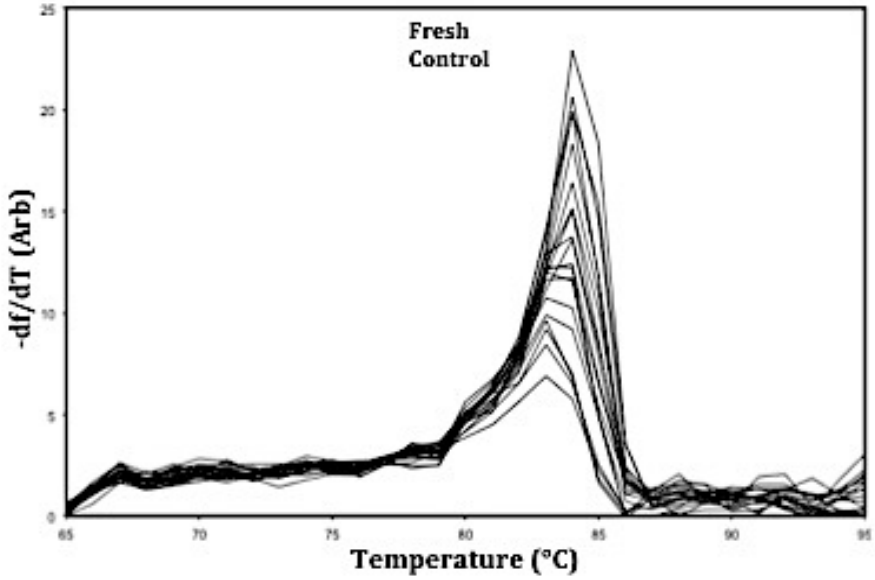
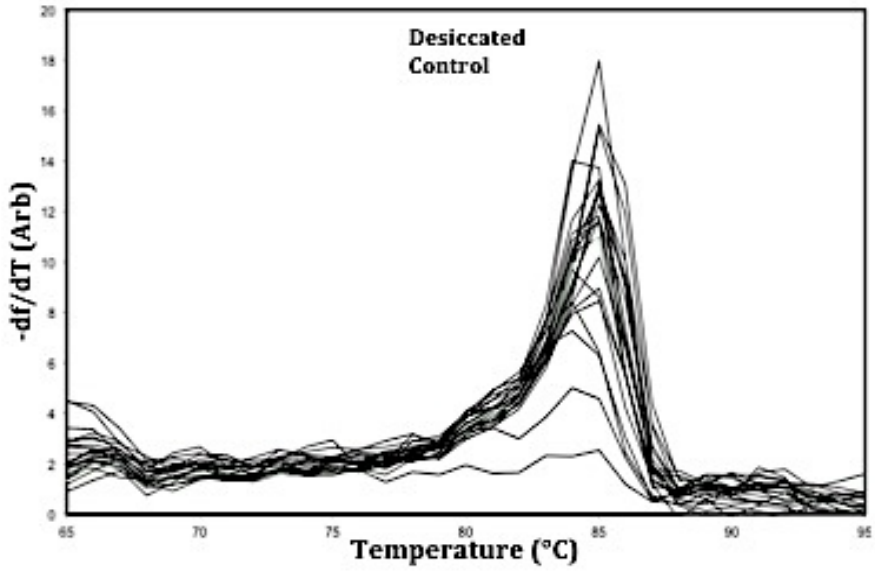


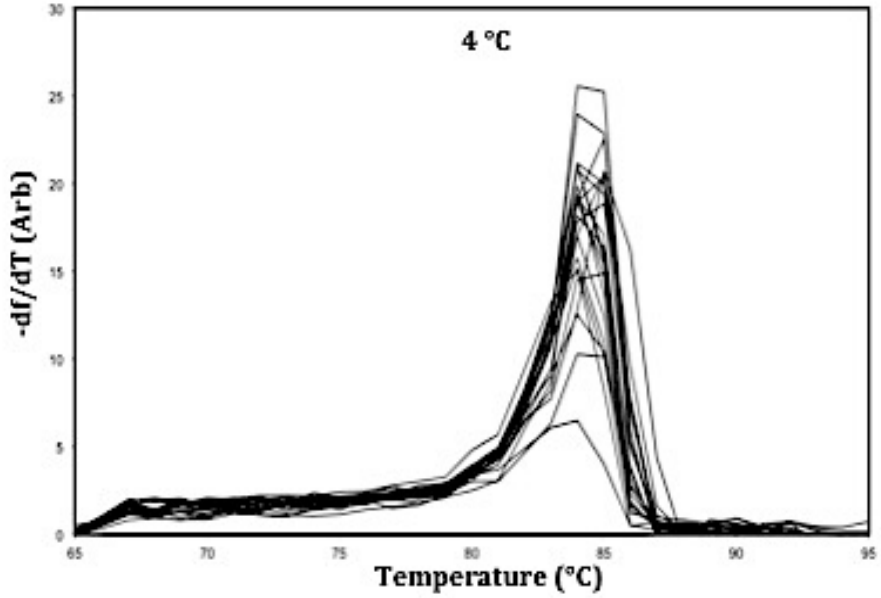
Figure 4.6: PCR curves generated during ten-week stability studies. A comparison of PCR curves of fresh control samples with desiccated samples containing 6-10%, residual water content stored at different temperature conditions for 10 weeks.

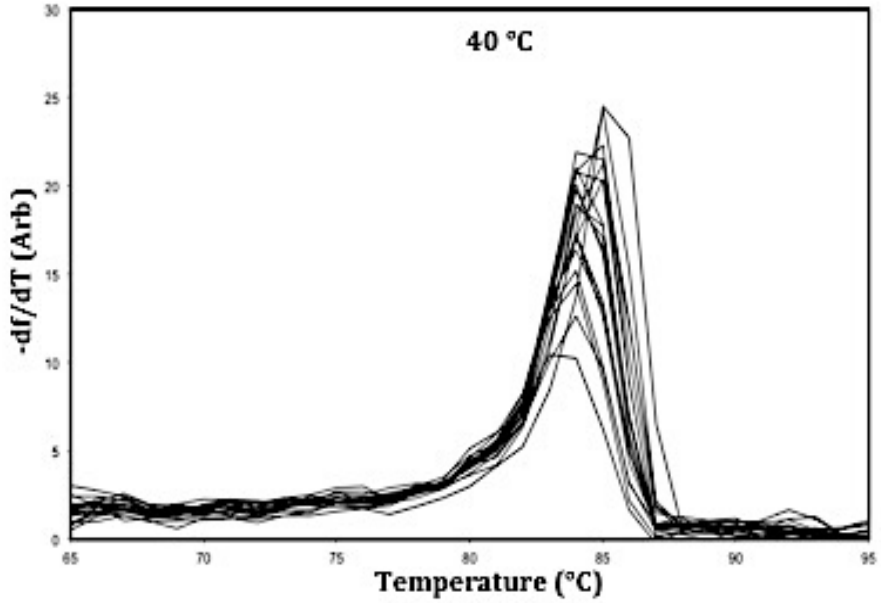
A comparison of the melt peaks generated with the samples is presented in Figure 4.7. The mean melt peak height of the fresh control sample is ≈13.4 and that of the desiccated sample is ≈11. For the desiccated samples stored at 4°C, the mean melt peak height obtained after one week is ≈15.4 and at the end of 10 weeks is ≈13.4 indicating that there is no drop in the activity of the formulation after 10 weeks when stored at 4°C. For the samples stored at room temperature, the mean melt peak height obtained after 2 weeks is ≈18 and at the end of 10 weeks is ≈10 indicating there may be a drop in the activity of the formulation after 10 weeks when stored at room temperature. For the desiccated samples stored at 40°C, the mean melt peak height obtained after 1 week is ≈16, however, no melt peaks are observed at the end of 10 weeks indicating that the desiccated formulations have completely lost their activity.


Fresh
Control
-df/dT (Arb)
Temperature (°C)

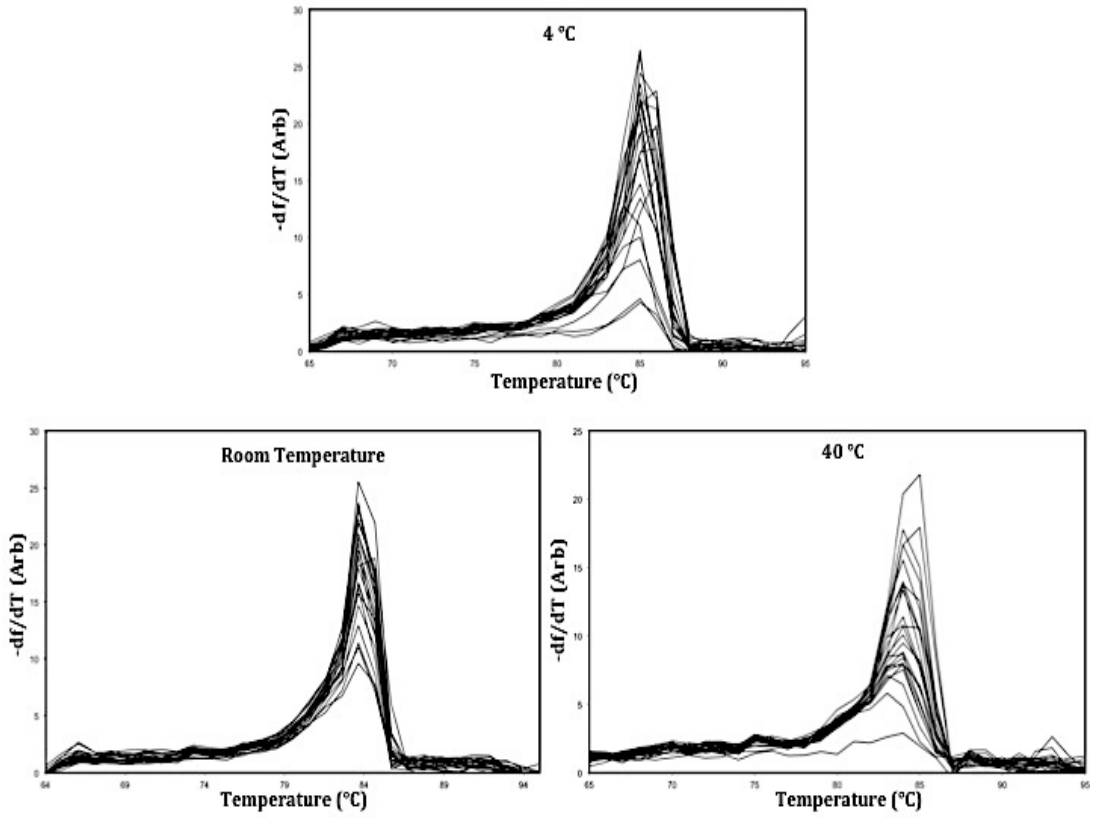

Desiccated
Control
-df/dT (Arb)
Temperature (°C)

Week 1

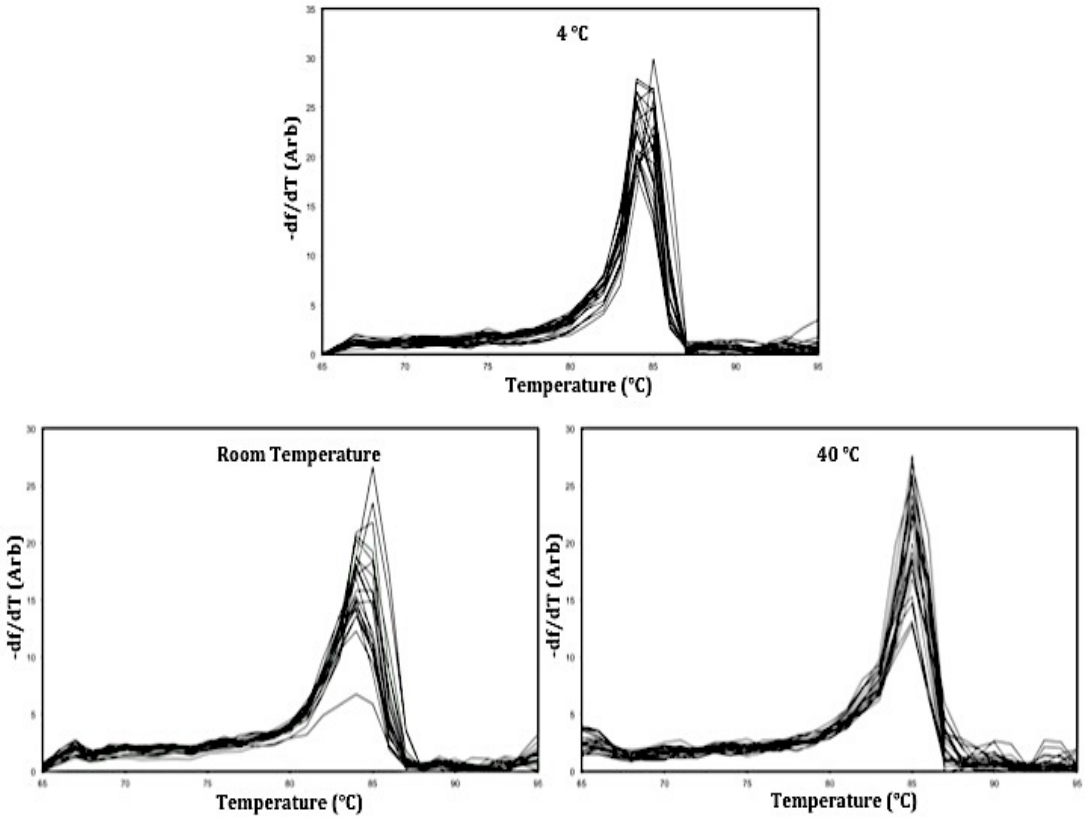




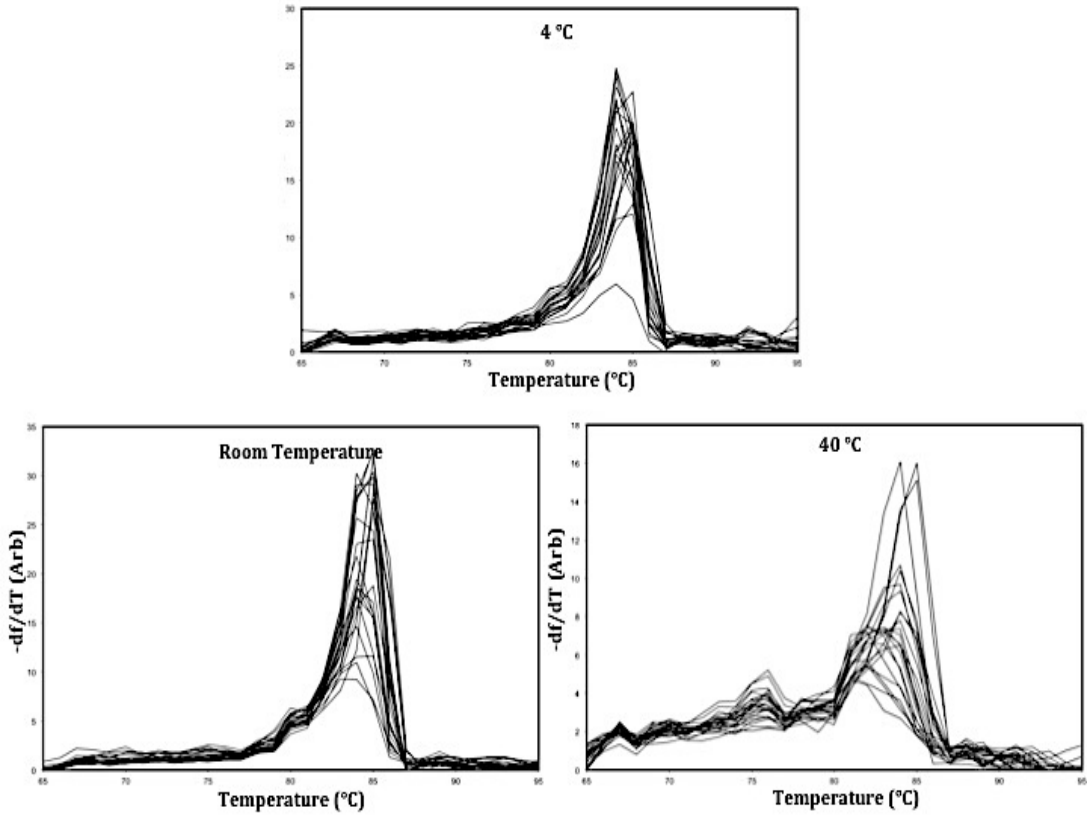
Week 2


4 °C
-df/dT (Arb)
Temperature (°C)
Room Temperature
-df/dT (Arb)
Temperature (°C)
40 °C
-df/dT (Arb)
Temperature (°C)

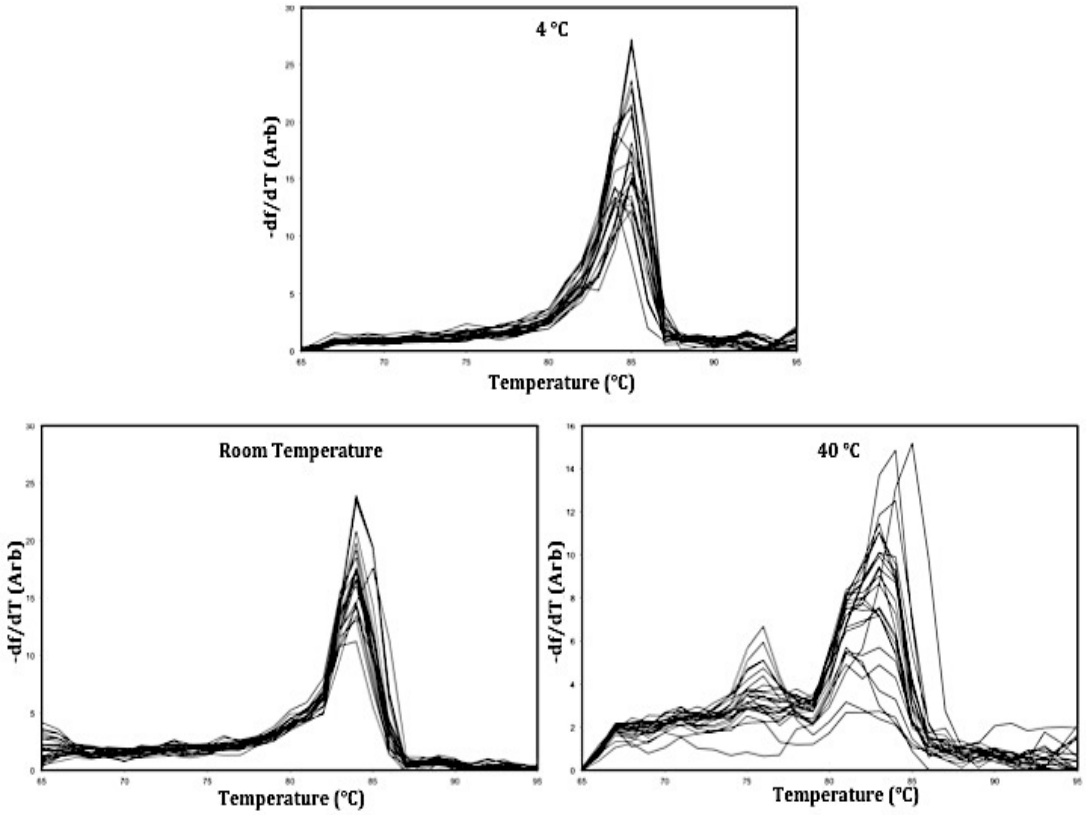
Week 3


4 °C
-df/dT (Arb)
Temperature (°C)
Room Temperature
-df/dT (Arb)
Temperature (°C)
40 °C
-df/dT (Arb)
Temperature (°C)

Week 4


4 °C
Temperature (°C)
Room Temperature
-df/dT (Arb)
Temperature (°C)
40 °C
-df/dT (Arb)
Temperature (°C)

Week 5


4 °C
-df/dT (Arb)
Temperature (°C)
Room Temperature
-df/dT (Arb)
Temperature (°C)
40 °C
-df/dT (Arb)
Temperature (°C)

Week 10

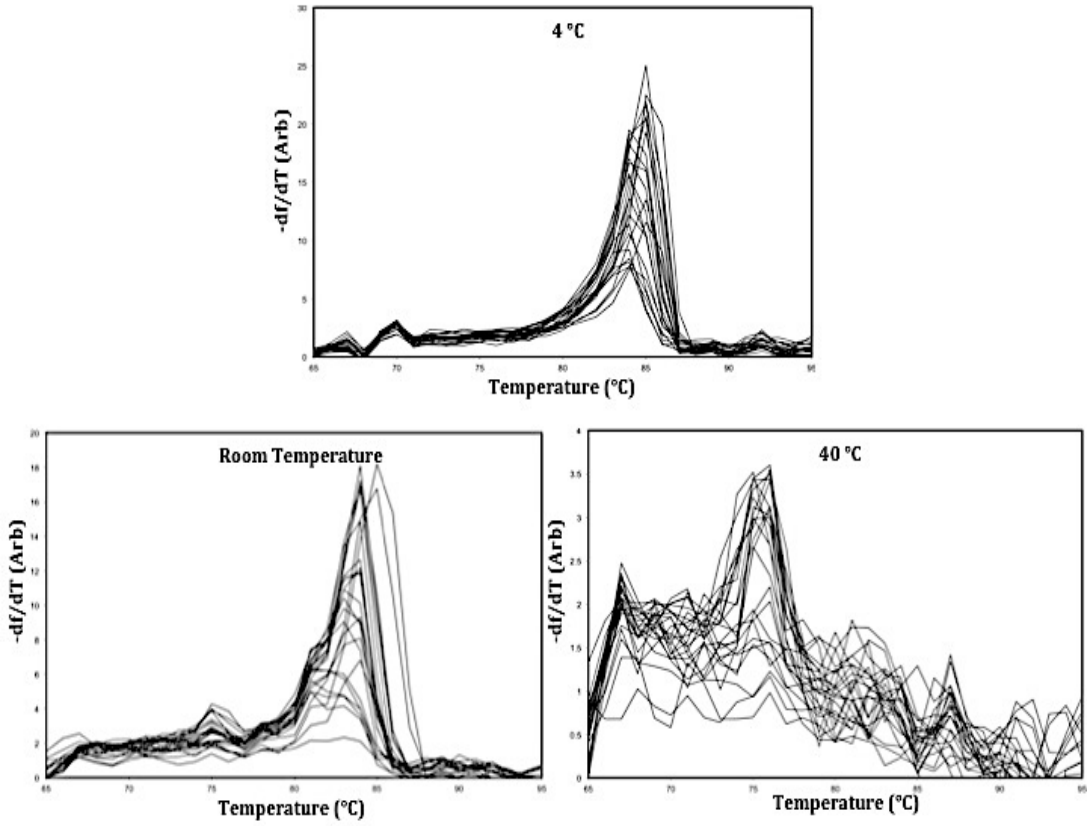


Figure 4.7: Melt peaks generated during ten-week stability studies. A comparison of Melt peaks of fresh control samples with desiccated samples containing 6-10%, residual water content stored at different temperature conditions for 10 weeks.

A comparison of the %POS of the fresh control sample and the desiccated samples stored for 10 weeks under different conditions of temperature is presented in Table 4.3. The %POS of the fresh control sample is 65% and that of the desiccated control is 87.5%. For the samples stored at 4°C, the %POS of samples after 1 week is 91.7% and is 95.8% at the end of 10 weeks indicating that there is a no drop in the activity of the desiccated formulations after 10 weeks when stored at 4°C. For the samples stored at room temperature, the %POS of samples after 1 week is 95.8% and is also 95.8% at the end of 10 weeks indicating that there is no drop in the activity of the desiccated formulations after 10 weeks when stored at room temperature. For the samples, stored at 40°C, the %POS of samples after 1 week is 87.5%, is also 87.5% after 5 weeks and is 4% at the end of 10 weeks indicating that there is a complete loss in the activity of the formulation after 10 weeks, when stored at 40°C. It should be noted that this represents analysis of only one mold.

Time/%POS	Fresh Controls	Desiccated Controls	4°C	Room Temperature	4°C
0	65	87.5			
Week 1			91.7	95.8	100
Week 2			95.8	95.8	100
Week 3			95.8	91.7	91.7
Week 4			95.8	91.7	79.1
Week 5			91.7	91.7	87.5
Week 10			95.8	95.8	4.1

Table 4.3: A comparative analysis of %POS of fresh control samples with desiccated samples containing 6-10%, residual water content stored at different temperature conditions for 10 weeks.

4.5 Discussion

The forty-eight hour stability studies conducted with desiccated gels containing 0-2%, residual water content show that the PCR efficiency is very low when the residual water content is low. Moreover, a further drop in the residual water content was observed during the ten-week stability studies. This further drop is approximately 4% for the desiccated samples stored at 4°C, 6% for the samples stored at room temperature and 8% for the samples stored at 40°C. It is interesting to note that the drop in the residual water content is only dependent on the temperature of storage and is independent of the duration of storage because the drop in residual water content was the same for samples for 24 hours and the samples stored for 10 weeks. The gels containing 0-2%, residual water content already have very low moisture levels and further decrease in the water content may cause a variety of instabilities in the formulation thereby resulting in a lower PCR efficiency.

The stability studies conducted with gels containing 6-10%, residual water content show that there is not much drop in the activity of the desiccated formulations when stored at either 4°C or at room temperature. However, for the desiccated formulations stored at 40°C, there is not much drop in the activity during the first 5 weeks but the samples stored for 10 weeks showed no activity. Since our sample size is small due to the lack of sufficient number of molds and only one set of desiccated samples were available for testing at the end of 10 weeks, it is premature to conclude that the samples stored at 40°C will completely lose their activity in 10 weeks. There may be other

reasons for the drop in PCR efficiency such as, the mold may have been contaminated or there might have been issues with the temperature cycling of the instrument. Hence, extreme conditions may not be the reason for the failure. Further investigation with multiple sample sets and better packaging will be necessary to conclusively find the reason for the loss in activity of the sample. The vacuum generated by the food-packaging machine is not efficient and the packaging strategies employed at present cannot ensure the absence of air in the package. Moreover, the porosity of the food wrapper is also unknown. Hence, further research must be done in terms of packaging material.

Although useful for comparison purposes, the use of "%POS" for the analysis of data may not be ideal. It can be used to accurately estimate the number of gels in the mold in which the amplification has occurred but cannot quantitatively determine amount of amplification achieved. However, due to the small sample set, this was the best strategy that could be used to estimate the shelf life of the gel PCR formulations. Interestingly, in most of our stability experiments, it has also been observed that the desiccated controls show a trend towards a higher %POS compared to the fresh controls. This may be due to the fact that desiccated gels are rehydrated with the template DNA mix and absorb the maximum amount of template possible unlike the fresh controls which are already hydrated and may not absorb the same amounts of the template. However, this aspect cannot be confirmed until the mechanism of DNA diffusion can be further investigated.

The long-term stability studies conducted with gels containing 6-10%, residual moisture content demonstrate, that our choice of lyoprotectants, their concentrations and the residual water content are compatible for the storage of gel PCR reagents.

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Chapter 5: Conclusion

The hypothesis presented at the beginning of this document states that PCR reagents entrapped within polyacrylamide gels can be preserved for long durations under extreme conditions. There is a large literature available that provides information on the different lyoprotectants used to preserve protein or enzyme formulations. However, the literature is limited in the context of preservation of PCR reagents, particularly in the context of a gel-based system. The lyoprotectants used previously for the preservation of PCR reagents deal with liquid PCR systems and the behavior of the gel PCR system is quite different from the liquid PCR systems. For example, our observations have indicated that total lyoprotectant concentration in excess of 0.4M will have a detrimental effect on the gel polymerization. Moreover, the most widely used method for drying PCR reagents is freeze-drying, which cannot be applied to the gel PCR system. The ice crystallization that occurs during the freezing step of lyophilization causes a change in the porosity of the gels. Hence, all these issues were addressed and a compatible combination and concentration of lyoprotectants were determined. The lyoprotectant that was used to preserve the gel PCR system is trehalose at a final concentration of 0.3 M.

Once the combination of lyoprotectants were determined, the next step was to estimate the range of residual water content that can be present in the customized dried gel PCR formulations containing the lyoprotectants, to

ensure their long term stability. The method of drying chosen was vacuum desiccation. However, it is not advisable to suddenly expose the gel PCR formulations to absolute vacuum. Hence, the change in pressure and temperature conditions during desiccation had to be controlled. Several manipulations had to be done to achieve an optimal decrease in pressure over time until the oven attained absolute vacuum. Several experiments were also performed to study the desiccation kinetics of gel PCR formulations and a curve representing the decrease in residual water content with time was generated (Chapter 3). Long-term stability studies were also conducted with gel PCR formulations containing residual water content in the range of 0-2% and 6-10% (Chapter 4). Our results show that PCR efficiency is poor when the residual water content is very low. Moreover, our observations have also indicated that there is further drop in the moisture content of the formulations during storage. This has potentially detrimental effects on subsequent PCR amplification after storage and rehydration. Hence, the gel PCR formulations were desiccated until the residual water content was in the range of 6-10% and this range will ensure that there is no complete loss of moisture even when there is drop in the total residual water content. The particular system chosen for this thesis may also limit the extent of further loss in residual water after desiccation, because gel reaction units are enclosed within a glass mold with only the top surface of a gel post being exposed to post-desiccation drying. It is likely that geometries of gel reaction units that have more exposed surfaces may require considerably more

efficient packaging to prevent further moisture loss. Long-term stability studies have demonstrated that the desiccated gel PCR formulations can retain most of their activity upon rehydration even after 10 weeks, when stored at 4°C or ≈25°C (room temperature). Our studies have also demonstrated that the formulations can retain most of their activity for up to 5 weeks when stored at 40°C. A more extensive study is required to characterize success rates after storage at temperatures between 25°C- 40°C or higher. The shelf life stability of the desiccated gel PCR formulations can be further improved by developing better and enclosed geometries for the desiccated gel posts on chips by implementing better packaging strategies.

The in gel PCR system presented in this document has a great potential to be used at the point of care. The instrument that is presently in use is small, inexpensive and portable. The in gel PCR technology is very sensitive and highly specific. Until my thesis work was done, a central issue was the method for storing gel-based PCR reaction cassettes, particularly for use in resource-poor and often harsh environments. The desiccation strategy developed here provides a means to successfully store the gel-based PCR reagents in essentially any geometry at ambient temperatures. Inclusion of preserved gel PCR reagents in the system will bring this system one step closer to be effectively used at point of care.

The inclusion of desiccated gel PCR reagents within the chip has lead to the

following advantages, thereby further simplifying the system:

- There is no requirement for sample storage prior to testing.
- The desiccated gel PCR reagents can be rehydrated with the sample itself thereby simplifying sample delivery.
- Unprocessed samples like urine, blood and nasal swabs can be used directly thereby avoiding the need for sample preparation.
- Tests for multiple DNA targets can be performed on a single chip.
- All the reagents are already present in the chip and hence do not require any technical expertise at the user end, in the preparation of reagents.

It is important to point out that the desiccation strategy developed here had an overriding second advantage that effectively solved critical problems in sample delivery. The use of desiccated gel units enabled novel methods for sample delivery that proved to be extremely effective for later use by others in our team who were developing reaction units with geometries different from that described here. Prior to the use of desiccated gel units for PCR, sample was manually added atop fresh gel posts, allowed to soak into the gel and then placed on the peltier for thermal cycling. With this system it was not obvious how sample could be reliably delivered in an automated manner. However with desiccated gel units, methods were readily identified to automate sample delivery via capillary flow into desiccated units, thereby facilitating the development of enclosed reaction cassettes that could be packaged and stored on a shelf, with no need for refrigeration.

The geometry of the chip that is presented in this document is one of the first methods to be developed and thus still quite simple and crude. There are several issues with this geometry such as contamination of controls, the usage of oil during PCR and the glass molds are expensive and non-disposable. In an attempt to adapt this system to be used at point care there are several other geometries that are being tested. One strategy being extensively tested at present is the use of capillaries instead of molds to hold polymerized gels. An iteration of the capillary chip/cassette and the latest instrument are presented in Figures 5.1 and 5.2. The gels that are produced are cylindrical unlike the gel posts presented in this document and when desiccated form a "noodle" that is exposed to air on all sides through the open ends of the capillary tube. There are several advantages to using capillaries. There can be several capillaries that can be included on a single cassette, termed multi-parameter testing. Each capillary can have a different set of primers thereby allowing testing for several infections on a single chip. Since each capillary is a separate entity, the contamination of negative controls can also be avoided, and experimentally does not occur. However, there are some issues with desiccating the gel PCR reagents within the capillaries due to the small surface area of exposure to vacuum and more importantly the large proportion of surface exposed to air after desiccation, making rigorous packaging a necessity to prevent loss of minimal residual water. Another strategy that is being pursued is the use of gel strips, which are small cylindrical gels placed in a plastic chip. However, only preliminary

testing has been done with gel strips and is too early to determine their effectiveness in developing a point of care system.

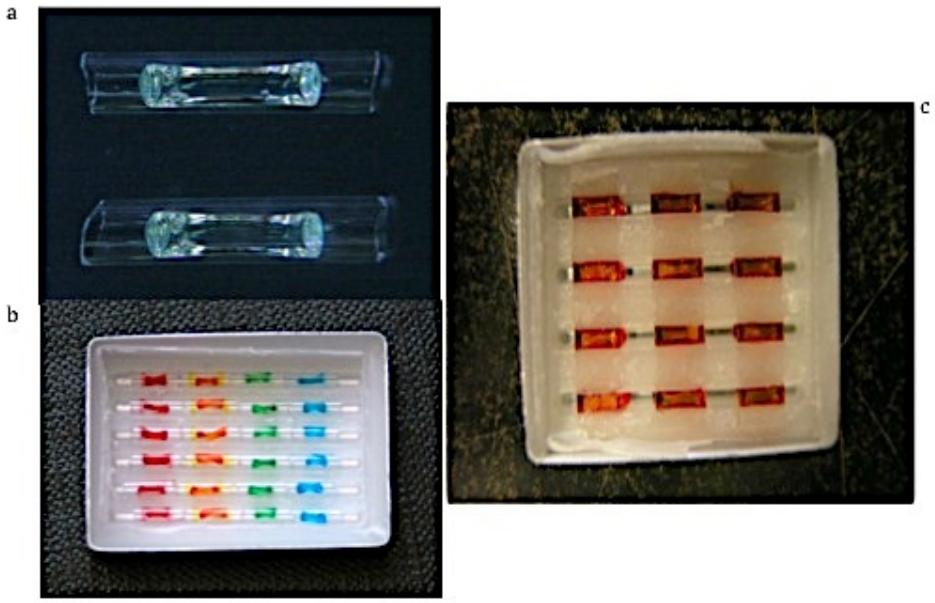

Figure 5.1: Latest iterations of In-gel PCR system. (a) Gel PCR reagents desiccated within capillaries. (b) An example of a cassette holding gel capillaries. (c) An example of a cassette holding gel strips. Each gel strip/capillary presented in the figures can have a different set of primers and can test for multiple targets at the same time. (Pictures (a) and (b) provided by Dammika Manage (MDG) and picture (c) provided by Anita Howell (Aquila Diagnostic Systems))

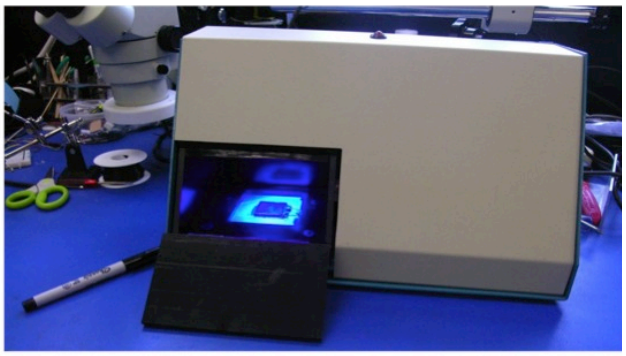

Figure 5.2: Latest iteration of the gel PCR instrument: The Gel Cycler. (Picture provided by Dammika Manage (MDG))

Novel advances during the work reported for this thesis are:

- Preliminary experiments on drying gels containing entrapped PCR reagents in air have been reported before. However, detailed studies on the preservation of PCR reagents entrapped within polyacrylamide gels are being reported for the first time in this document.
- Previous literature on the preservation of PCR reagents, involve lyophilizing the reagents. The use of vacuum desiccation, determination of excipients and the characterization of the drying process for the preservation of the PCR reagents entrapped within polyacrylamide gels have been pursued for the first time.
- The inclusion of desiccated gel PCR reagent module in our chips has simplified sample delivery to a great extent in the context of point of care systems.

In conclusion, with further improvement in instrumentation and chip design along with the inclusion of desiccated gel PCR formulations can lead to the development of a simplified system that requires minimum user interference and can be used at the point of care.