UNSW AUSTRALIA

SCHOOL OF MECHANICAL AND
MANUFACTURING ENGINEERING

Development of an Automated Magnetic Silica Bead Based DNA/RNA Extractor

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November 2016

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Abstract

Most of these first few pages are generated by the file header/frontpage.tex

However, this file contains a lot of important (and messy) commands for setting up the document which should rarely need to be changed, so I've moved the abstract to a separate file (header/abstract.tex) using an \include{} command.

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I, Jarrod Herbert Stilp, hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the projects design and conception in style, presentation and linguistic expression is acknowledged.

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Acknowledgements

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Chapter 1

Introduction

1.1 Background

Needed: -Place in industry -Background of diagnosis -Requirement and fit in
company -Existing equiptment

Within the healthcare industry, there is a continuous need for fast and
reliable diagnostics of pathogens within patients. Identifying the presence of
pathogens responsible for disease in a patient allows the appropriate preven-
tive or corrective action to be taken and represents a crucial step in treating
or preventing illness. This thesis was conducted for the benefit of and in col-
laboration with AusDiagnostics Pty Ltd.

Successful diagnostics, within the context of this thesis, can be summarised
in three overall stages. Namely these are extraction, amplification and finally
analysis. This thesis concerns itself only with extraction. It should be noted
that not all pathogen analysis and/or commercial diagnostic processes fol-
low these steps strictly, however the processes and technologies applied by
AusDiagnostics follow this procedure. The stages of this procedure may be

summarised as follows:

1. Extraction To begin the diagnosis, a clinical sample is obtained from the patient. This sample may consist of cerebrospinal fluid, faecal matter, urine or others, depending on the disease to be diagnosed. These samples contain the target DNA or RNA which will later be analysed to determine the presence of the pathogen and hence disease. They also contain however a number of inhibitors to the process of amplification and analysis. Extraction is the process of removing said inhibitors and retaining only the target DNA or RNA. The result is referred to as a clean sample.

2. Amplifcation Amplification takes the clean sample and by one of many methods increases the overall count of the DNA. This may be with the intention of allowing multiple targets to be detected or to increase the sensitivity of the analysis.

3. Analysis Analysis uses one of many available methods to search for the presence of biomarkers within the amplified clean sample. The presence of the biomarker indicates the result of the diagnosis.

AusDiagnostics currently supplies customers with the instruments and chemical products required to complete stages two and three (amplification and analysis) of the diagnosis. This requires customers to purchase extraction equipment from alternative suppliers and represents a significant weakness and loss of profit. Research and development conducted by AusDiagnostics has determined that the optimal approach, when considering speed and efficiency, is super-paramagnetic bead based extraction. The beads utilised are of the shell-core variety. The core is composed of an iron oxide, which provides the super paramagnetic properties required for physical manipulation of the

beads via a magnetic field. The shell is comprised of silica, which via chemical modification has the propensity to bond DNA and RNA to the bead surface. The techniques developed by researchers at AusDiagnostics utilising the magnetic silica beads have been validated and verified via manual operation. This thesis concerns itself with the automation of the developed extraction process, to produce a commercially viable robotic instrument. This instrument will be referred to as the Gene-Plex Extractor.

The extraction process to be automated has a number of notable requirements. These include liquid handling via precise pipetting, including mixing and liquid transfer. Also required is manipulation of the magnetic silica beads to separate the bonded and hence captured target DNA or RNA, along with heating to a specified, constant temperature to act to increase the rate of the chemical processes. The automation of the extraction process will be achieved by integrating the required capabilities into the robot produced by AusDiagnostics to conduct the amplification stage of diagnosis. The instrument, based of the Gene-Plex platform and sold as the High-Plex Processor, is pictured in it's current application in Figure 1.1. The Gene-Plex platform is essentially a liquid handling robot. The robot carries out the amplification stage by precisely pipetting and transferring liquid mixtures between the tubes and instruments on the deck, using disposable tips. Each individual assay (an analysis conducted to determine the presence and amount of a substance within a volume) utilises an individual layout of components on the robots deck. This makes the platform highly configurable for differing setups.

Fig. 1.1: The Gene-Plex liquid handling robot platform, as implemented as the High-Plex Processor.

1.1.1 The Extraction Process

In order to allow the aim and scope of the work to be clearly defined, a condensed overview of the extraction process developed by AusDiagnostics is presented. It should be noted that unless explicitly stated, all operations are to be automated.

The extraction process requires that the clinical sample undergoes a number of chemical steps across different locations on the robot. To aid in understanding the liquid handling involved, Figure 1.2 displays the important sites on the deck. The process will begin with the operator manually loading 24 individual clinical samples into the location labelled "Clinical Samples". In

order to conform to existing products used by customers, it is then required that the chemical processing takes place in the locations labelled "Samples". In order to transfer the liquid between locations, the robot will pick up 1000μL tips from the locations marked "Tips".
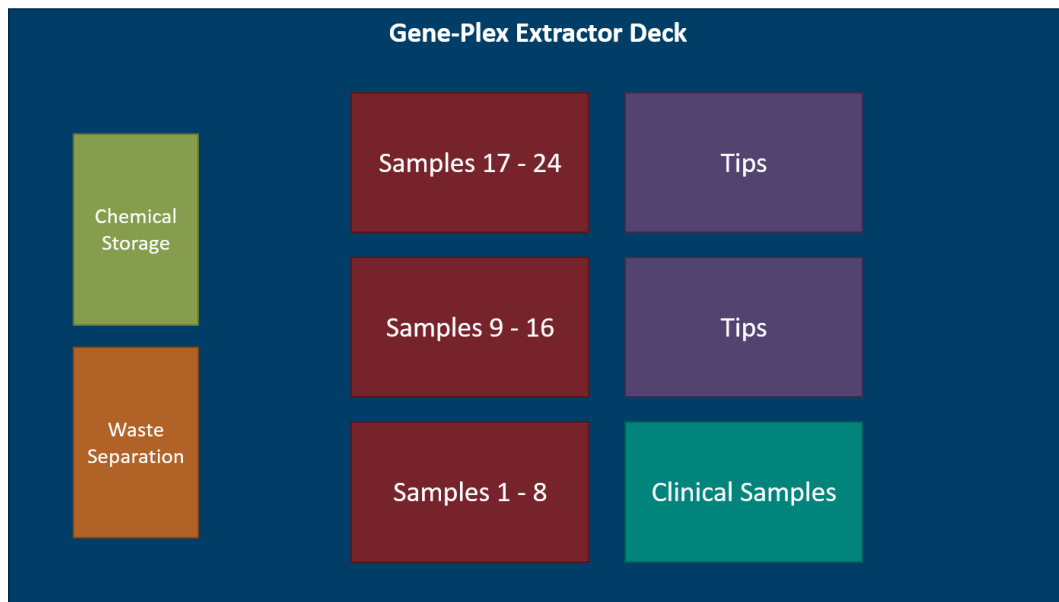
Fig. 1.2: The layout to be used for the Gene-Plex Extractor.

The sample processing locations must accept modules that fit within the standard block size (SBS) format. These blocks are required to each accept 8 cassettes, one for each sample (pictured in Figure 1.3). Each cassette includes 6 tubes, which will be the site of a particular chemical reaction in order to extract the target DNA and RNA:

1. 500μL of clinical sample is pipetted into tube location 2, as marked in Figure 1.3. Within this tube as supplied by AusDiagnostics, there will already be 10μL of magnetic bead mixture along with 440μL of lysis buffer. The lysis buffer will destroy the cell walls, releasing the target

DNA or RNA to be bound to the magnetic silica beads. This process is required to take place at 60°C in order to increase the rate of reaction Due to the low volume of liquid in this step, it is to be completed in one of the 1mL low profile tubes. This aids with liquid handling precision.

2. The target DNA or RNA is now bound to the beads, which are suspended in the waste liquid (supernatant). In order to capture only the bead suspension, the entire liquid mixture is aspirated via pipette tip and subsequently move to the location marked "Waste Separation". In this location, a magnetic field is to be applied to capture the magnetic silica beads within the pipette tip. While captured, the supernatant must then be expelled into a waste container in this location. The tip now contains only the magnetic beads with bound targets.

3. Despite the supernatant having been expelled, there will still be a significant amount of waste retained on the bead surface. To remove this and clean the beads, a sequence of 3 wash steps must then occur in tube locations 1, 5 and 6. To achieve this, the beads are first re-suspended in the tube, which contains 800μL of a particular wash buffer. The suspension must then be mixed via pipetting in order to to ensure the bead surface is properly exposed. The entire liquid mixture is then aspirated once again and transferred to the waste location, where via the same means as step 2, the waste liquid is disposed of while the beads are retained. This process is repeated 3 times in the tubes noted above to ensure no waste matter clings to the bead surface.

4. With the beads now clean and still bound to the target, the mixture is transferred to location 3 of the cassette. The elution buffer is used to break the bond between the target DNA and RNA and the magnetic

silica beads. This process is also required to be completed at 60°C to reduce the time required. Following this step, the target DNA or RNA of interest is now contained within the elution buffer, along with the magnetic silica beads which are now waste.

5. The final step is to capture only the target DNA or RNA within the elution buffer, leaving behind the magnetic beads. This is required to take place in tube location 4. This is a low volume reaction, containing only 100μL of elution buffer and is therefore completed in the final small profile tube. The pipette must then aspirate this mixture, following bead removal, and contain only the elution buffer and target mixture required for amplification in stage 2 of the assay.
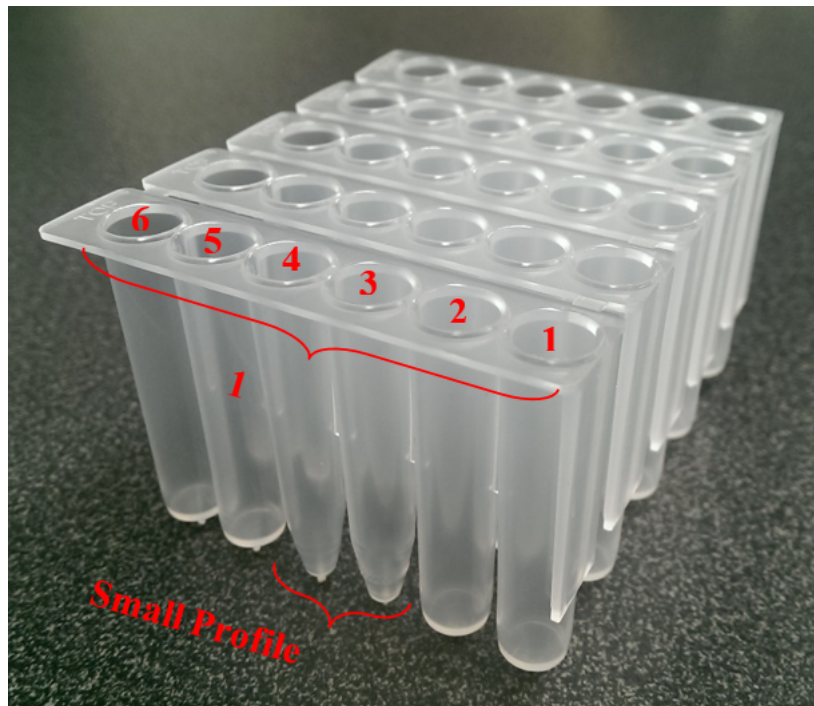

Fig. 1.3: The tube format to be used as the site of the sample processing.

1.2   Aim

This work aims to develop and integrate into the Gene-Plex Robot Platform the hardware necessary for carrying out the described chemical processes, the required magnetic manipulations and the controller necessary to maintain the stipulated constant 60°C temperature.

1.3   Scope

The features required by the Gene-Plex Extractor, over those already part of the Gene-Plex Processor, can be grouped into 3 overall categories. These are detailed below, including clear definition of their inclusion or exclusion from the scope of this work.

1.3.1   Mechanical

The summary of the extraction process to be implemented, given in Section 1.1.1, revealed a number of missing mechanical components when compared to the description of the Gene-Plex Processor given in Section 1.1.

SBS Processing Module As was noted in Section 1.1.1, the extraction process is to take place within cassettes consisting of 6 tubes. These must be located in the 3 SBS deck locations as marked in Figure 1.2, with each SBS location accepting 8 cassettes. The design of the module which will accept these cassettes is within the scope of this thesis and will form a large component of the design work undertaken.

Heating Element In order to create a commercially viable product, it has been stipulated that the tubes within each cassette where the the lysis buffers and elution buffers are held (tubes 2 and 4), a temperature of 60°C must be

maintained for the duration of processing. This is a core requirement of the extraction process and is included in the scope of this project.

Magnetic Separation The Gene-Plex Extractor can be seen to require magnetic manipulation of the silica beads in two different locations. In order to allow the extracted DNA or RNA to be captured after separation from the beads in tube 4, the beads must be retained in the tube while the liquid is aspirated by the pipette. Secondly, the magnetic beads must be captured within the pipette tip during the expulsion of the waste supernatant after all wash steps are completed. The magnetic separation required in both of these processes is defined to be within the scope of this work.

Waste Disposal Following the wash steps and the subsequent separation of the magnetic beads and the bound DNA or RNA from the wash buffer, the supernatant must be hygienically disposed of. This waste disposal method is required in order to ensure that no contamination occurs between the clinical samples being processed. It is also crucial to ensure that no contact can occur between the operator and the biological matter which is a by product of the extraction process. The development of this waste disposal hardware is included in the scope of this work.

1.3.2 Electronic

In order to account for the newly integrated capabilities in the Gene-Plex Extractor, some modifications to the Gene-Plex Processor electronics are required. Due to the temperature control requirements of the extraction process, an electronics board capable of communicating with the robot software and driving the heating elements appropriately is required. This however is not included in the scope of this work for a number of reasons. One of the com-

ponents of the Gene-Plex processor is a device called the MTX Cycler. This device controls the temperature of the already extracted sample (during stage 2, amplification) precisely between set temperatures to enable PCR (Polymerase Chain Reaction) to occur and hence amplify the sample. The MTX Cycler controls the temperature of liquid volumes significantly smaller than those involved in extraction and in a different form factor, making it unsuitable for this application. It does however provide the necessary electronics and interfaces required to drive any standard form of heating element used here. Further to this, the electronic board used to control the MTX Cycler is currently being integrated into a newly developed control board for the Gene-Plex Platform by AusDiagnostics engineers. This new board will also be capable of driving the heating elements selected for this application. Therefore, due to the redundancy of work on this component of the Gene-Plex Extractor and due to a suitable requirement existing, the development of the required electronic is not within the scope of this work.

1.3.3 Software

In order to enable the Gene-Plex Platforms controlling software to utilize the newly integrated capabilities, two main components of software must be created:

Temperature Controller In order to control the heating elements to maintain a stable and accurate 60°C for the lysis and elution stages, a temperature controller must be implemented. This temperature controller must be designed according to the temperature response of the heated hardware using appropriate methods and implemented as a controller in software. This element of the Gene-Plex Extractor's requirements will also form a core part of this

work and is included within the project scope.

Routine Addition A was briefly noted in Section 1.1, the Gene-Plex Platform is highly adaptable in its ability to perform assays requiring differing liquid handling operations. The platform achieves this by utilizing a number of library routines which may be called upon the assay requiring a particular movement, such as expelling liquid, moving to a certain robot position etc. While the vast majority of these library routines will be directly effective within the Gene-Plex Extractor, due to the newly integrated capabilities further routines will be required. These will include routines for the mixing of liquids in the cassette tubes, magnetic separation and a table of definitions which specifies the locations of each of the tubes and pieces of deck hardware. These software additions are not included within the scope of this work and will be implemented by software engineers at AusDiagnostics.

Chapter 2

Literature Review

2.1 Heating

The thermal requirements for the extractor to be developed, as outlined in Chapter 1, are very similar in nature to those of a Polymerase Chain reaction (PCR) Thermal Cycling Device, albeit with simplified operations. The requirements of a PCR thermal cycler are driven by the need to cycle the temperature of an array of tubes containing a small volume of sample liquid through temperatures of 55°C, 72°C, followed by 92°C [1]. Due to the process involving upwards of 20 cycles between these temperature set points, ramp times for these changes in steady steady state must also be minimised to ensure the total processing time is commercially viable. The thermal requirements of the Gene-Plex Extractor are highly matched, with two exceptions. In this application the temperature of the sample liquid shall be held at a constant 60°C, as determined by research conducted by AusDiagnostics, as opposed to the multiple temperatures of a PCR cycler. Secondly, the cool down ramp rates for a PCR cycler are of equal importance to it's ramp rates during heating, in order to achieve temperatures lower than the current steady state in

adequate times [2] [3]. In the application of the Gene-Plex Extractor, there is no need to pay attention to cooling ramp rates as the system will only cool at the completion of its operation. Due to the similarities evident between these two applications, a great deal of information may be gathered from PCR cycler design.

In order to generate the heat necessary to reach the required sample temperature, some form of thermal pump is required. A Thermoelectric Cooler (TEC), also know as a Peltier, is often used for liquid sample thermal cycling [4] among its many other applications. A TEC is a solid state heat pump, controlled by the directional application of electric current across it's two terminals [5]. The direction of the applied current determines the direction of heat pumping across the module [5]. TEC's are constructed of pellets of n-type and p-type bismuth telluride semiconductors, connected in an alternating series [6]. The connections, made of copper, are bound to a substrate of ceramic alumina. This substrate forms the surface by which the heat generated is transferred. Figure 2.1 displays the arrangement of a TEC in a general case application. The TEC has shown through the reviewed literature to be common in PCR thermal cycler applications due to the above mentioned ability to pump heat in either direction, allowing ramping of temperature to be precisely controlled equally in both directions.
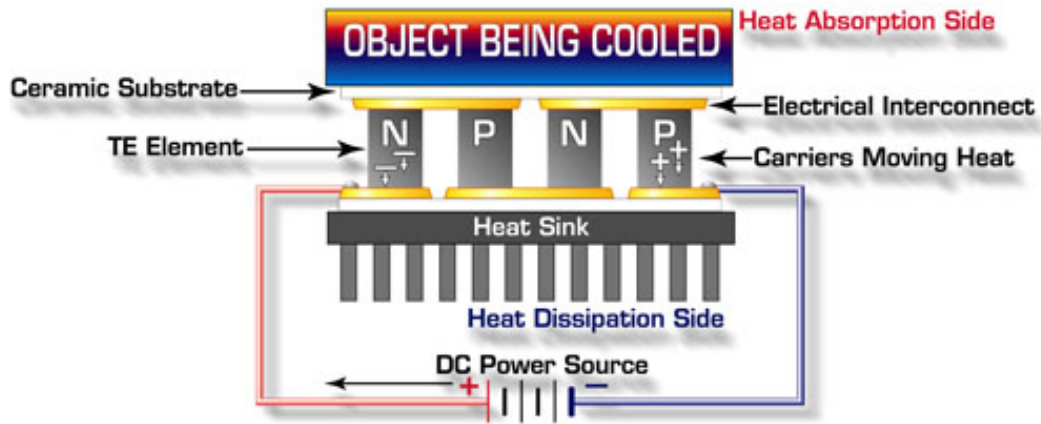
Fig. 2.1: Thermoelectric module as assembled in a typical application. [7]

When used in control applications, a TEC has the advantage of being able to not only add but to also remove heat energy as is required by the controller to achieve the desired response. Despite the TEC's ability to actively remove heat from a component, other active temperature removal methods may also be implemented. A passive approach to heat removal is that employed by a heat sink, or any simple surface area [8] [9]. This differs from an active approach, where airflow over the heat sink surface area is controlled via a fan, for example [8]. Such methods may be substituted for the directional heat pumping capabilities of the TEC, in order to achieve a similar level of temperature control. This leads to simpler possible options for heat pump selection, namely electronic resistive heaters. Despite no reviewed literature describing these devices as the main heat source in thermal control for PCR in particular, they have been demonstrated to provide a crucial supporting role by Williams et al. The thermal cycler described in the work of Williams utilizes resistive heat strips to control the temperature of a sealing lid, preventing sample evaporation at the upper operating temperatures of a PCR thermal cycler [10]. While the application noted here differs from the intended application within

the extractor, where the device will be the primary source of heat, Gene-Plex Extractor, it demonstrates the feasibility of driving component temperature to a steady state value and hence represents a design possibility.

2.2 Cooling

In order to be able to control the temperature of the liquid sample as required, a method of evacuating heat must be present. A passive method alone, such as a heat sink in isolation, will not allow the process controller to drive a reduction in heat beyond the limits of conduction to the surrounding air [9] and as such performance will not be satisfactory. Along with heat dissipation being essential for control, the TEC elements have a maximum temperature differential of which they are capable of generating across their surfaces. This imposes the following constraint on the thermal system:

$$T_{hot} - T_{cold} \leq dT_{max} \tag{2.1}$$

This has been noted as highly significant in PCR cycler design [5], where one end of the TEC must be kept at ambient temperature to enable the maximum steady state value of 92°C to be reached safely. It should be noted that due to the maximum steady state temperature in this application being limited to 60°C and assuming a reasonable TEC differential limit, dT_{max}, this constraint is not critical. However, a TEC becomes less efficient as it's temperature differential is increased [11]. As such, a method of heat evacuation must be present.

2.3 Controllability

In order to create a thermal system which is controllable within the context of stability and speed of response, attention must be paid to the thermodynamics of the device. Due to manufacturing constraints and standards, the tube which contains the sample itself is not optimal. A wall thickness of 1mm results in poor heat transfer characteristics from the heated block to the sample [12]. Another source of poor dynamics is any regions of mechanical connection between the heated block and external components [10]. Recommendations from the reviewed literature included the use of gasket material to limit thermal material between any surface contacts (Williams et al specifically recommends the use of ethylene propylene), along with the use of a groove extending through the majority of the thickness and hence limiting heat transfer to the mechanical connections [13] [11]. Mechanical connections are however only one cause of significant thermal gradient within the device. Care must be taken to reduce the severity and frequency of the occurrence of these gradients to maintain stability and performance in temperature response. After heat pumping has ceased, a temperature gradient will persist for a period of time that is proportional to the square of the distance between the hot and cold locations [10]. This has important implications for the number of sample tubes that may be heated within a single block. If the size of the sample array to be heated is too large, difficulty will be encountered achieving a uniform and stable steady state temperature [10]. Widomski et al note that despite efforts to reduce heat loss and hence gradients around the system boundaries via methods such as insulation, the region of location of the heat source will continue to maintain a higher temperature. To counter this, it is suggested to use a rod of material with lower thermal conductivity (such as stainless steel within an aluminium

block) to reduce the heat retained in this volume [11].

A number of measures may also be taken directly related to the heated block itself to ensure optimal thermal response. The concepts of static local balance and static local symmetry may be applied to obtain desirable heat transfer within the medium [10]. A state of static local balance refers to a state of constant temperature where all heat sinks are equalled by heat sources in a local area [10]. This essentially describes a state of thermal equilibrium, where the following condition is satisfied:

$$Q_{in} = Q_{out} \tag{2.2}$$

To ensure this balance is obtained, a device design should not incorporate overly effective heat sinks, without a proportional increase in the heat source capacity, and visa versa. The condition of static local symmetry requires that for a constant temperature and within a local region, the center of mass of the heat source is coincident to the center of mass of the heat sink [10]. Satisfying this condition will ensure that a constant, steady state temperature is achievable, without the presence of a permanent temperature gradient between the two centers of mass. The selection of heated block material along with manufacturing methods may also act to improve transfer properties. Aluminium 6061 is recommended due to its purity [10], which aids in even heat transfer [14]. It is further recommended that the heated block be manufactured via machining, due to the homogeneous structure that results. This is beneficial in reducing any temperature gradients that may occur within the heated element due to mechanically or otherwise joined components or impurities or inconstancies that exist as a result of other manufacturing methods such as

casting.

2.4   Temperature Sensors

The literature reviewed has presented various differing selections of temperature sensing technologies, the most prominent of which have been thermocouples and thermistors. Despite the overlapping application, the specifications of the two technologies are significantly different. The thermocouple has a large temperature range, with measurements capable of ranging from -270°C to 1800°C compared to the limited range of thermistors of usually around -90°C to 130°C [15] [3] [16]. The thermocouple and thermistor possess similar response rates along with similar accuracy, however due to the wider resistance range available, thermistors are able to achieve a high measurement resolution [15] [3] [16]. The two significant differences between the technologies are power supply requirements and linearity. Due to the welding of differing metal conductors at the measuring point, the thermoelectric effect results in a temperature dependant voltage being generated across the thermocouple terminals [15] [16] [3]. While this means the sensor does not require a power supply, it can introduce complications requiring a reference/compensating wire. Furthermore, changes to the conductor materials such as corrosion require that the sensor be calibrated on a regular basis, often as frequently as 3 months [15] [3] [16]. Thermistors use semi-conductive material that changes resistance with temperature. As such, measuring the temperature requires a current be passed through the device [15] [3]. While the output voltage of thermocouples changes linearly with temperature, this is not true of thermistors. Hence, the output signal requires linearisation before it may be of use [3]. In commercial applications, thermistor technology is often also preferred due

to its cost effectiveness [16]. While Resistive Thermal Detectors (RTD's) and Integrated Temperature Sensors appeared in a number of reviewed sources, they are not considered in depth due to the high level of signal conditioning required [16] [15] and the lack of observed uses in relevant thermal control applications.

2.5   Temperature Sensing

To utilize the thermal system to control the liquid sample temperature accurately, a method of obtaining a feedback signal is required. Complications exist however due to the constraint that the sample temperature itself cannot be directly measured. Due to the thermal transfer characteristics discussed above, the placement of the sensing device is critical to the stability of the controlled substance [13]. Vilchiz et al studied the issues of sensor hardware placement and provide numerous recommendations to increase baseline stability. Using experimental methods, it was verified that the following one-dimensional heat equation holds:

$$s = \frac{L}{a} \tag{2.3}$$

Where s is the time taken for energy to flow from location A to location B, L is the distance between the two locations, and a is the thermal diffusivity of the material. This is relevant as it allows the time lag between a controlled heating action at the heat source to the arrival of the generated heat at the location of interest to be determined. The experiment conducted tested two temperature sensing cases. One sensor was placed within the center of mass of the heated block, while the other was located as close as was possible to the heat source. The results obtained found that "there is a general advantage of control via

sensing close to the heater, whether by temperature measurement, or by heat-flow control sensing." [13]. Equation 2.3 is accountable for this result. When the sensor is placed at a greater distance to the heat source, the control effort is determined using a measurement that is not yet equalised due to the more severe temperature gradient. This error in feedback signal is reduced by the recommended placement strategy, such that "the reduction in the magnitude of the heater-induced oscillations achieved by this control strategy is at least one order of magnitude better than that of the temperature control at the core" [13].

A number of aspects of the experiments and findings the work of Wilchiz et al should however be considered. The sensing device used to make the presented conclusions were thermocouples, one of many sensing devices available. Despite the variances in accuracy and stability of measurements that are expected between differing sensing technologies, the results can be assumed independent of any associated error. Any error as a result of sensor output stability is of an insignificant order to magnitude of time when compared to the 1.07 min thermal lag situation generated by the experiment. Furthermore, one would expect a sensor technology of differing accuracy to produce a controlled output different to the desired reference input. This error is however not influential to the results given, which are concerned exclusively with the stability of the controlled response. The focus of the experiment was the application of thermocouple technology in Tian-Calvet microcalorimeter devices. This does not detract from the relevance of the reviewed work for a number of reasons. The motivation for the study was to improve baseline stability in temperature control, via the application of improved temperature measurement strategies; a general outcome that is relevant to the work of this thesis. Further to this,

the scale of the experiment conducted closely resembles the device to be developed, with the distance between sensor locations set equal to 3.5cm. Therefore, despite the differing applications of the work conducted, the recommendations and strategies found may be concluded as directly applicable.

2.6 Controller Design

With the necessary hardware in place and optimally set up, as has been discussed above, controller design and theory may be considered. The large majority of the reviewed literature, along with the instruments currently employed by AusDiagnostics, recommend or employ the Proportional Integral Derivative (PID) controller. As summarised by Vilchiz et al, "Although other types of controllers are also feasible, e.g., those based on fuzzy logic or artificial neural networks, the simplicity, good response, and low cost of PID-based control loops have motivated their popularity in industrial applications" [13] [5]. Despite the common application of the PID controller in thermal control applications, if a TEC is the selected source of heat, complications are presented. Due to the non-linearity of the device, PID coefficient selection involves the process of trial and error [5]. The strategies for PID design given were however applied to the thermal cycling requirements of PCR. This requires that 3 separate temperature controllers be implemented for each of the temperature set points, to ensure the required performance is met equally at each [5]. While the characteristics of the PID controller are not effected by this difference, it does significantly increase the complications of coefficient selection in comparision to the application in the Gene-Plex Extractor. Further to the controller to be implemented itself, a number of sources give recommendations regarding relevant hardware. Cellatoglu et al investigated the dynamic per-

formance of a process control system, with a focus on temperature controllers. The findings reveal that the word length of the ADC utilised is an important factor in reducing output error. A case study was conducted to assess the impact of increased word length on error, with results suggesting that 8 bits is the optimal value [17]. It was further found that an increase in word length beyond this point yields no reduction in error. The analysis conducted was however completed as a simulation, with no experimental verification evident. The electronic hardware implemented in other sources, such as the work of Shirafkan et al, differs from this recommendation. While not providing evidence to suggest the 8-bit recommendation is problematic, the successfully implemented controller utilized a 24-bit ADC.

2.7   Magnetic Separation

To ensure the target DNA is extracted as required, the device must include a robust means of capturing the magnetic beads. Despite offering an alternative means of magnetic bead manipulation, electromagnets are not investigated for application in the thesis due to a high level of complexity that is not required for this static, constant force implementation. Therefore, the review of literature focuses on permanent magnets. While there are numerous compositions of magnets available, the rare earth variety and in particular the Neodymium composition (ND-Fe-B) are most suitable. Neodymium magnets offer the strongest solution, with an energy product of up to 56 MGOe [18]. Good mechanical characteristics and energy to size ratio make them suitable for fastening within a manufactured product, without undue difficulty. Importantly, given the requirement to capture beads within the heated block element,

Neodymium magnets are stable in temperatures near ambient. Progressive loss of magnetism occurs at temperatures greater than 80°C [18], providing a 20°C margin of safety between the operating temperature of 60°C. This composition also holds a very high coercive force [18], allowing it to resist demagnetisation in the presence of external magnetic fields that will be present within the Gene-Plex Extractor, as a result of other hardware. Despite the specification of the superparamagnetic silica beads being a constraint determined by completed research at AusDiagnostics, a number of characteristics should be noted in order to obtain reliable bead capture capabilities. Shevkoplyas et al describes the force acting an a superparamagnetic bead due to an applied force. The context of this paper is the motion prescribed by the beads within a microfuidic chamber, however the general characteristics of the interaction between the beads and magnetic field are applicable to the capture required here. In order to saturate a suspension of superparamagnetic beads, an external magnetic field greater than 0.5T must be applied [19]. Saturation is the state where all beads magnetic poles are aligned. In a state of saturation, the magnetic beads behave simply as permanent magnets [19] [20]. In order to ensure the complete separation of the magnetic bead dispersion within the sample liquid, this condition must be achieved.

The literature reviewed has allowed a number of critical elements of thermal control and magnetic separation, being the main requirements of the extraction system, to be recognised and evaluated within the context of this thesis. The similarity between the processes demanded by the extraction technique and the works discussed allow the information gathered to be applied directly to the benefit of the developed instrument. Those works which were an exception to this allowed general strategies and design characteristics to be collected, which

may be applied with consideration in future development.

Chapter 3

Methodology

With the scope of the work now defined, the manner in which the required results will be obtained can now be detailed. The necessary additions as defined in Section 1.3, Scope, may will be implemented in the form of two independent assemblies in order to meet the requirements of the work. These two assemblies will be refereed to as the Processor Module and the Magnetic Separation Station. The former will be the site of the chemical reactions as described in Section 1.1.1, The Extraction Process, and will be the hardware element that forms the SBS block on the Gene-Plex Extractor deck. Therefore, given it will house the full set of reactions, it will also encompass the software and hardware requirements of the liquid temperature control (including the associated controller), along with the magnetic separation required in tube number 4 of the cassette. The Magnetic Separation Station will be the location of disposal of the supernatant and therefore shall include the magnetic hardware required to physically manipulate the magnetic silica beads in order to retain them in the pipette tip while the waste is expelled. Given the biological nature of the liquid waste involved, this station will also include provisions for hygienically disposing of and storing this waste.

3.1 Processor Module

3.1.1 Hardware

The hardware component of the Processor Module design is concerned with ensuring the module accepts the required cassette tubes, fits within the stipulated SBS space and provides a stable platform for the required temperature control using the knowledge gained in Section 2.3, Controllability. The work in this sections will also include the selection of an appropriate heat pumping device. In order to ensure the requirements of the hardware elements are met, the design process is applied as follows:

1. Generation of concepts related to thermally isolating the heated and non-heated regions of the block. This includes a focus on the manufacturability of the resulting components.

2. Investigation into and selection of an appropriate heating method.

3. Full CAD design of hardware including provisions to accommodate the required tubes.

4. Utilization of 3D printing to test the fit of the cassette tubes in the block design.

5. CFD simulation to verify the performance of the selected heating element and the thermal stability of the final design.

3.1.2 Temperature Controller

This work will result in the finalisation of a software temperature controller capable meeting the liquid heating requirements of the chemical reaction as detailed in Section 1.1.1, The Extraction Process.

In order to enable controller design to be commenced, the first step will be to assemble the completed Processor Module. Due to the complex dynamics of the heat transfer between the heating device and the liquid, a mathematical modelling method is not used. Instead, the system will be identified via the step input response plant identification method. To obtain the required response data, sensors will be placed strategically on the Processor Module to monitor and record key temperatures:

- The temperature at the centre of the block will be taken as this sensor data will be utilized as feedback for the controller to be implemented. This follows the recommendations given in Section 2 by Vilchiz et. al.

- The temperature of the heatsink will be plotted to monitor the temperature differential created across the module.

- The temperature of the heated region of the module will be taken on its outer surface to determine the success of the design in creating a component that responds to temperature control in responsive and stable manner.

- The temperature of the liquid within the cassette tubes will be measured as the final output of the system. The ability of the controller to drive this temperature to the desired set point will determine the success of the design.

To utilize this data for experimental plant identification, an Arduino will be used as an interface between the sensor electronics and MATLAB. This will enable the real-time monitoring and plotting of the data along with storage and subsequent analysis through the Arduino's Analogue input ports.

With the hardware now setup, the Processor Module will then be excited by a step input. The input will be provided by the same controlling electronics used in the final Processor Module. As was detailed in Section 1.3, Scope, the companies MTX Cycler electronics driver board will be utilized. The software controlling this board will therefore be modified to provide a constant step input to the Processor Module. The step input will be applied and the response data logged until a steady state has been achieved.

This data will have been logged in the MATLAB workspace in real-time, during the experiment. Prior to analysis however, the data will be pre-processed to remove any unnecessary features. For example, the data will be seroed so that the response data begins at time $t = 0$ and so that the response begins at 0°C. This will remove any effects of the varying room temperature at which the experiment may occur and ease the fitting of curves to the collected data.

To complete the plant analysis process using the pre-processed data, the MATLAB PID Tuner tool will be used. This tool provides a GUI for specifying a set of response data, the step input provided. This information is then used to interactively fit an appropriate function to the plants response data. The tool offers a number of plant structures to allow the correct plant to be identified. These range from a simple "One Pole" plant to a discrete time system.

As a result of this process, the transfer function which represents the plant of the Processor Module will be known. This plant will be in the continuous time domain and is represented by:

$$G_p(S)$$

Before utilizing the identified plant for controller design, it will be verified via simulation in Simulink. The validation will be conducted by providing the plant an identical step input as was done experimentally and verifying that the responses match. The Simulink layout used to conduct this simulated verification is shown below in Figure 3.1.
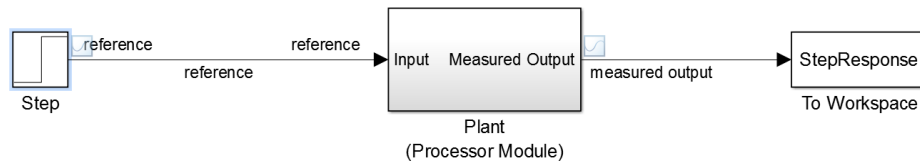

Fig. 3.1: The Simulink model layout used to validate via simulation the identified plant for the Processor Module.

With the transfer function representing the Processor Module now known, the process of controller design may be commenced. The method chosen for this design process is the Direct Analytical Method (also known as Ragazzini's Method), in the discrete time domain. As was noted in Chapter 2, Literature Review, the most common method of control for thermal cycling devices in similar applications is PID control. This method has not been selected however due the benefits of competing controller design in the z-domain with discrete time. By completing the design entirely in the z-domain, there is no need to convert the resulting controller from the s domain, allowing the controller to be directly implemented.

The direct analytical method uses the principle that if the output that is required along with the input applied is known the intermediate transfer function may be determined. This is represented by Equation 3.1, where $C(z)$ is the required output, $U(z)$ is the applied input and $F(z)$ is the intermediate transfer function.

$$C(z) = F(z)U(z) \tag{3.1}$$

The method of feedback used for the controller is a unity feedback signal. Data provided by the utilized sensor will be processed to give a feedback signal with unity gain, to ensure any features of the raw signal are removed. For the unity feedback system picture in Figure 3.2, $F(z)$ is given as [21]:

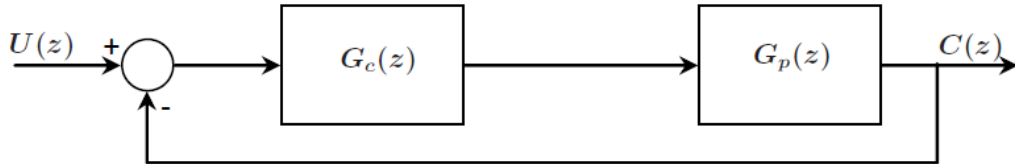$$F(z) = \frac{G_c(z)G_p(z)}{1 + G_c(z)G_p(z)} \tag{3.2}$$

Fig. 3.2: The block diagram for a unity feedback system. [21]

Due to the plant identification completed earlier, the continuous time form of the system plant is known. Using MATLAB, this may be converted to a discrete time transfer function, with the appropriate sampling time selected. Therefore, $G_p(z)$ is known.

Rearranging for the controller of interest, $G_c(z)$ gives:

$$G_c(z) = \frac{F(z)}{G_p(z)[1 - F(z)]} \qquad (3.3)$$

By equation 3.3, the direct method requires only that $F(z)$ be determined to realise the controller. To complete the formation of $F(z)$ and hence $G_c(z)$, the method below will be utilized. This method, as detailed by J. Katupitiya [21], provides a number of steps to ensure the resulting controller performs as required.

Controller Performance

To define the response time of the controller, the pole locations are set. To do so, the desired time constant of the system, τ is set and the s domain pole is found by:

$$s = -\frac{1}{\tau} \qquad (3.4)$$

This s domain pole is then transferred to the z domain via:

$$z = e^{sT} \qquad (3.5)$$

Where T is the sample time of the controller. The resulting pole is then placed as a pole of $F(z)$.

It is also important to control the steady state error of the controller. To do this, the DC gain of the system is set to 1, resulting in the output equalling the input at steady state. By setting the following, this can be achieved:

$$F(z)|_{z=1} = F(1) = 1 \tag{3.6}$$

Pole Zero Cancellation

It must be ensured that a pole or zero cancellation does not occur in the unstable region of the Z-Plane, in order to ensure that this cancelled pole does not become a component of the formed characteristic equation. If a controller component cancels an unstable plant pole or zero in this scenario, it will influence the system so that it becomes unstable. These constraints will be dealt with via two stability constraints:

Stability Constraint 1

Stability constraint 1 addresses the instability that may occur if a pole of the plant $G_p(z)$ is cancelled by a zero of $G_c(z)$. Firstly, all poles of $G_c(z)$ are tested for instability, where any pole $z = p_0$ is unstable if :

$$|p_0| > 1 \tag{3.7}$$

If any pole is found to satisfy Equation 3.7, it is unstable and must be added as a zero of $[1 - F(z)]$. This addition removed the unstable pole and ensures the controller is not able to create the unstable cancellation.

Stability Constraint 2

Stability Constraint 2 deals with the case that an unstable zero exists in the plant transfer function. Therefore, it prevents a pole of the controller from cancelling this zero in the unstable region. A zero of the controller $G_c(z)$, ($z = z_0$ may be tested for instability by checking if it satisfies Equation 3.8:

$$|z_0| > 1 \tag{3.8}$$

Any zero satisfying this equation can be identified as unstable and dealt with by including it as a zero of $F(z)$.

Causality Constraint

The causality constraint ensures that the designed controller is either proper or strictly proper. That is, that it only uses current or past terms in the calculation of controller effort and no futuristic terms. This is achieved by ensuring the pole-zero deficiency of the plant (i.e. its delay) is either that same as or less than that of $F(z)$. The pole-zero deficiency of he plant is the order of the denominator minus the order of the numerator, denoted as:

$$D[G_p(z)] = n \tag{3.9}$$

The pole zero deficiency of $F(z)$ is given as:

$$D[F(z)] = m \tag{3.10}$$

If $m = n$, the controller is strictly proper. If $m > n$, the controller is proper. However, if $m < n$, then the controller is improper (will require futuristic terms) and the causality constraint is not satisfied. In this situation, the order of the denominator of $F(z)$ must be increased until the controller is at least strictly proper.

Controller Difference Function

Using the $F(z)$ found using the previous steps, the controller transfer func-

tion, $G_c(z)$ can then be calculated. This transfer function is converted into a function of the variable Z^{-1}, which is commonly known as the Digital Signal Processing (DSP) format. This allows the transfer function to be directly written as a difference function involving multiple error and controller effort terms with varying time delays. This difference function is the realised discrete controller.

Simulated Validation

In order to validate the design of the controller prior to implementation, the design will be simulated via Simulink to validate its response to the desired reference input. The block diagram shown in Figure 3.3 will be used to conduct the validation and record the resulting data. The response recorded will be assessed against the controller requirements.
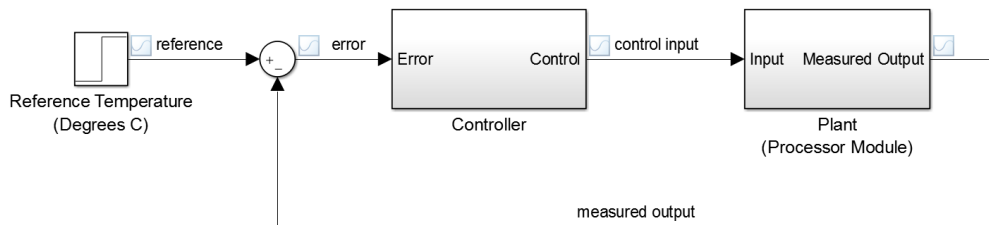

Fig. 3.3: The Simulink model layout used to validate via simulation the designed controller for the Processor Module.

Following the successful validation of the temperature controller, it will be implemented via software currently driving the MTX cycler, along with any required modifications.

Experimental Verification

In order to verify the performance of the resulting controller, the following experimental verification will be conducted. The experiment will determine the ability of the controller to meet the steady state error, disturbance rejection, response time, overshoot and stability requirements when controlling temperature in the module. The method is as follows:

1. Assemble 4 complete Processor Modules

2. Using an Arduino as an interface and MATLAB as the data logger and analyser, measure the temperature in the tubes shown in Figure 3.4 for the duration of the experiment.This distribution will allow the performance at the extremes of the device to be determined.
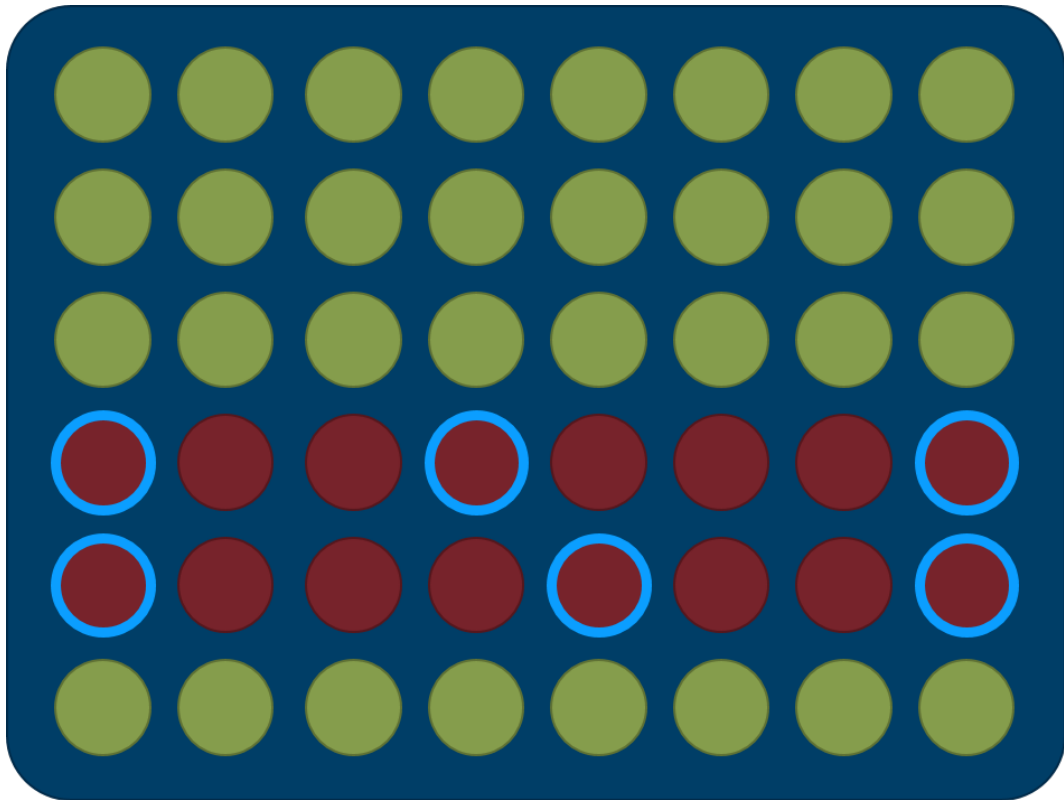

Fig. 3.4: Tube locations to be measured during temperature control verifications experiment. Non-Heated tubes are coloured green, heated tubes are coloured red, measured tubes are outlined in blue.

3. Begin data logging and live plotting.

4. Set the reference input to the controller to 60°C.

5. Collect data for a total of 30 minutes to capture the long term steady state performance of the module (to allow any slow heat transfers to be observed) along with the transient response.

6. Repeat the experiment for all four assembled modules. This allows for the controllers ability to reject disturbances caused by the differing of the plant properties to be determined as is caused by the slight differences in assembly.

7. Repeat experiment in differing environmental conditions. The experiment should be conducted in rooms at temperatures of 18°C, 22°C and 26°C. This allows for the disturbance rejection of the controller to be assessed where the disturbance is due to altered environmental conditions.

8. Use the collected data to assess the consistency of the heating profile and the temperature stabilisation across the tubes of the module. These results will be used to make conclusions regarding the designed controller along with the thermal characteristics of the hardware. It should be noted that the worst case response will be used to draw conclusions regarding performance to ensure all samples fall within the requirements.

3.1.3 Magnetic Separation

As the final stage of the extraction process is the removal of the clean sample from the waste beads, the success of extraction depends on the complete and reliable separation in tube number 4. To ensure this is achieved, the following design process and experimental verification will be conducted:

1. An investigation will be conducted into the various possible methods of magnetic separation in this application, taking into account the recommendations to use ND-Fe-B magnet compositions as found in the reviewed literature.

2. 3D Printed test jigs will be created to test the effectiveness of the most suitable configurations via manual inspection.

3. With a preferred design selected, the a test module will be 3D printed including the magnetic arrangement. This test module will be assembled on the robot deck and verified experimentaly via the method described below.

Experimental Verification

To allow a confident conclusion to be made regarding the effectiveness of the magnetic separation within the Processor Module to be made, the following experiment will be conducted. This experiment will replicate the setup used by the robot and its processes to ensure the result is representative of the true scenario.

1. Place test module with magnetic separation on Gene-Plex Extractor deck within one of the SBS spaces.

2. Place a 5mL tube on the robot deck to collect the experiment output.

3. Place a full set of 8 cassettes in the test module with 100μL of filtered water and 10μL of magnetic beads in tube 3. The water will simulate the elution buffer. Both volumes are equal to that used in the final process.

4. Instruct the robot to aspirate the mixture in tube 3 and expel it into tube 4, the location of the magnetic separation.

5. Wait for 5 seconds for separation to take place. It should be noted that this waiting period is a design variable and may be adjusted and this experiment repeated to determine the optimal wait time.

6. Aspirate the separated liquid at a distance of 1mm from the tube bottom. This distance once again may be adjusted, however is a sound starting point for experimentation.

7. Expel the liquid from the tip into the 5mL tube on the deck.

8. Repeat this separation procedure for all 8 cassettes.

9. Refill all cassettes with equal volumes of water and beads, as was defined in step 3.

10. Repeat the separation for a total of 3 modules to equal the total volume of liquid which will be processed by the Gene-Plex Extractor during the processing of all 24 samples.

11. Place the 5mL tube with extracted contents adjacent to a large ND-Fe-B magnet to draw any beads to the tube wall. Examine the tube contents for any sign of magnetic beads. The presence of a bead within the tube indicated a failed separation within tube 4. Such a result may lead to severe complications in step 2 (amplification), such as the failed diagnosis of a patient. Therefore, for a successful verification, it is required that no beads are present.

3.2 Magnetic Separation Station

The magnetic separation station is a standalone module on the robot deck which will fulfil two requirements of the extraction process. Firstly, the sta-

tion will provide the means of separating the waste liquid from the magnetic beads and captured DNA/RNA, as necessary after step 2 (refer to Section 1.1.1) of the extraction process. Following the successful separation of the supernatant, the separation station will provide a means of hygienically disposing of the associated biological waste. The work will be completed via the the methodology described below.

3.2.1 Magnetic Separation

This stage of the extraction process carries a high level of significance in relation to the final result. Poor magnetic separation may effect the reliability of the diagnosis due to a reduction in the sensitivity of the step 2 amplification, or failure of step 3 analysis due to the presence of inhibitors. The former may be caused by a lack of concentration of the target DNA or RNA in the extracted clean sample. Failed magnetic separation could cause this by not properly capturing the magnetic beads and bound target, allowing them to be expelled into waste. This loss of target will lower the sensitivity of the amplifications stage and hence may lower the accuracy of diagnosis. The second scenario of inhibitors may occur if some of the waste liquid remains after washing, contaminating the sample and lowering the effectiveness of the amplification and analysis steps. Therefore, the method used for the design of the magnetic separation capabilities of the Magnetic Separation Station is aimed at ensuring a strong and reliable separation.

1. An investigation will be carried out to determine the most suitable magnetic arrangement for providing reliable separation. Similar to the treatment of the magnetic separation capabilities within the Processor Module, the investigation will focus on those employing the ND-Fe-B com-

position.

2. The selected configuration will then be implemented as a design concept and a CAD model generated. This detailed design will also account for a number of secondary requirements. These include maintaining a minimum distance between the pipette tip and the Separation Station at all points to remove the possibility of cross contamination occurring through liquid transfer.

3. The detailed design will then be prototyped using 3D printing to allow experimental verification.

4. The prototype Separation Station will then be assembled on the Gene-Plex Extractor deck along with the necessary tubes to perform magnetic separation via the experimental procedure detailed below.

Experimental Verification

In order to verify the required performance is achieved by the designed Magnetic Separation Station, the following experiment will be performed.

1. The prototyped Magnetic Separation Station will be fully assembled and positioned on the deck of the Gene-Plex Extractor.

2. 3 cassettes and therefore 24 tubes will be positoned on the deck with 640μL of water and 10μL of magnetic silica beads in each. These volumes are equal to those used in the extraction process and this will be repeated 24 times, once for each of the 24 samples. Therefore, this setup matches the volumes to be processed by the implemented extractor.

3. The robot will then be setup to aspirate the liquid mixture from one of the tubes.

4. The pipette will then be positioned within the magnetic separation station and lowered to full depth.

5. The pipette tip will then be raised and lowered with the full liquid height passing along the magnetic arrangment a total of 2 times.

6. With the beads now separated from the liquid and captured on the pipette wall, the water will be ejected into the waste disposal system. For this experiment, the waste disposal will be replaced by a small glass container that may be used to collect and analyse the ejected liquid.

7. This procedure will then be repeated for all 24 "samples".

8. The contents of the waste container will then be subjected to a magnetic field to collect any beads which were not correctly separated by the process. The presence of any bead within the waste liquid indicated a failed separation.

3.2.2 Waste Disposal

The work on this component of the Magnetic Separation Station is concerned with ensuring the product meets key standards and requirements in order to dispose of the biological waste in a hygienic manner. The disposal of the waste generated by the process is a key factor in ensuring a successful sample extraction. Any failure to safely dispose of this waste may introduce the risk of a cross contamination occurring between two independent samples and therefore a failure to correctly diagnose the patient.

To ensure the waste disposal system negates these risks, the process used will focus on researching the related standards initially in order to determine

the best practices which must, by regulation, be adhered to. This research will be supplemented by end user feedback. To gain this, a group of key product users will be consulted on preferred methods of waste disposal and hygiene to guide the design process. Using the results of this investigation, the design process will be used to form a final design which takes into account the gathered information and requirements and therefore produce a functional and hygienic waste disposal system.

Chapter 4

Results

The problem at hand along with the need for a solution are now well defined. Paired with an understanding of important previous works in this area and the technologies available, the design of each of the components may be given consideration. This chapter will present the designs of the required additions to the Gene-Plex Extractor, as were defined in Section 1.3, Scope. This will include relevant aspects of the design process, the conducted simulations along with validations and verifications via experimental methods. Through the sections below, the reader will be familar with the design of each of the components along with the resulting performance of the realised and implemented component.

4.1 Processor Module Hardware

4.1.1 Requirements

4.1.2 Design

4.1.3 Simulation

4.2 Temperature Controller

4.2.1 Requirements

4.2.2 Design

4.2.3 Simulation

4.3 Processor Module

4.3.1 Validation

4.3.2 Verification

4.4 Magnetic Separation Station

Chapter 5

Discussion

Chapter 6

Conclusions

Chapter 7

Future Work

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Appendix A

Raw Results