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LAB-ON-A-CHIP: DESIGN AND IMPLEMENTATION OF AN ELECTRONIC CONTROL BOARD FOR A SELF-TESTING INSTRUMENT DETECTING SEXUALLY TRANSMITTED INFECTIONS

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ABSTRACT

This work describes the collaboration in a project aimed at realizing a biomedical device able to analyze biological samples and detect the appearance, if present, of pathologies. The activity had been developed during an ERASMUS exchange experience at Brunel University, West London. The host research group, CESR (Centre For Electronic System Research), offered the kind possibility to work with them on this important project they are still investing on a lot. Other research institutes and university of UK are collaborating on the same, like for example: St. George's University of London, University College of London and UK Clinical Research Collaboration.

The final system is thought to be characterized by: a fast response, small dimensions and low price, distinctive requirements for a biomedical instrument. The graphic interface is very simple, easy to use, important aspect to help the user's work; the dimensions are reduced just to create a light and easy to handle instrument. The price requirement is one of the main aims, just because we want the device to be widespread, in order to start the treatment as soon as possible, and to be sold to everyone in every pharmacy.

More in detail, it's required to realize a device able to extract the genetic information from a biological sample, inserted using a particular cartridge, to amplify it and eventually analyze it. The main goal is to detect the presence of virus, due to sexually transmitted diseases affection, as HIV or hepatitis; this can explain the reason of the requirement about the fit price for the large distribution. The project is still at the beginning and it requires the collaboration of many different specialized figures, like doctors or chemists, electronic engineers and product specialist designers as well.

The work, described here, was firstly directed towards the design of the electronic board, which has to control the amplification reaction of the DNA, extracted from the sample. In particular, the circuit has to impose the proper timing for the reagents injection into the tester; to provide a proper mixture of them and to maintain a constant temperature, which is essential for the specific reaction. After the validation of the circuit, the electronic board was realized just using a simple breadboard and an *Arduino* board; the necessary components were chosen, purchased and correctly placed on the breadboard itself. Eventually, some experimental measurements were taken. The outcome of the tests confirmed that the system does work properly.

The choice of using an open-source prototyping electronics platform, Arduino, for the implementation of the logic control reduces the space needed, the number of components and also the potential noise sources, therefore making the device more reliable. The versatility is one of the strengths of the device, as it is possible to drastically vary the performance just by acting on the firmware of the microcontroller.

The system is conceived as easily expandable, because of the large number of pins and peripheral devices that are not used by the microcontroller

SOMMARIO

L'elaborato descrive il lavoro di partecipazione a un progetto per la realizzazione di un dispositivo biomedicale in grado di analizzare campioni biologici e rilevare l'eventuale presenza di patologie. L'attività è stata svolta nel corso di un'esperienza di scambio culturale ERASMUS presso la Brunel University, West London. Il gruppo di ricerca ospitante, CESR (Centre For Electronic System Research), ha gentilmente offerto la possibilità di collaborare al progetto sul quale molto sta tuttora investendo. Oltre alla Brunel University collaborano allo stesso altri istituti di ricerca e università inglesi, quali ad esempio: St George's University of London, University College of London e UK Clinical Research Collaboration.

Il sistema finale è pensato essere caratterizzato da: una rapida risposta, piccole dimensioni e prezzo contenuto, requisiti peculiari per quel che riguarda uno strumento biomedicale. L'interfaccia grafica è molto semplice e di facile utilizzo, aspetto fondamentale per agevolare l'utente; le dimensioni sono ridotte per creare uno strumento leggero e facile da maneggiare. Mentre il requisito sul prezzo è posto tra gli obbiettivi principali proprio perché si vuole che lo strumento abbia larga diffusione, agevolando quindi anche la tempestività della cura, e che possa essere facilmente acquistabile da chiunque presso una qualunque farmacia.

Più in dettaglio quello che si vuole realizzare è un apparecchio in grado di estrarre l'informazione genetica contenuta in un campione biologico, inserito nello strumento attraverso una microcapsula, amplificarla e analizzarla. Lo scopo primario è quello di identificare la presenza di virus, dovuta all'affezione di malattie sessualmente trasmissibili, quali HIV o epatite, questo appunto spiega il vincolo sul prezzo adatto alla grande distribuzione. Il progetto è ancora agli albori e richiede la collaborazione di diverse figure specializzate, da uno staff medico e chimico all'ingegnere elettronico per poi finire con il product specialist designer.

Il lavoro compiuto, e illustrato in questo elaborato, ha riguardato dapprima la progettazione della board elettronica in grado di controllare il corretto avvenimento della reazione di amplificazione del DNA estratto dal campione. In particolare il circuito deve essere in grado di dettare le corrette temporizzazioni per l'iniezione dei reagenti nel tester, provvedere all'opportuna miscelazione degli stessi e mantenere costante la temperatura richiesta dal particolare tipo di reazione. Una volta progettato, si è passati alla temporanea realizzazione del sistema utilizzando una semplice

breadboard e una board *Arduino*; questa fase ha compreso la scelta e l'acquisto dei componenti necessari, la disposizione degli stessi sulle schede. Infine sono state compiute diverse misure sperimentali che hanno confermato il funzionamento del sistema.

L'utilizzo della piattaforma open-source di prototipazione *Arduino,* basata su microcontrollore, ha permesso di creare una logica di controllo che richiede poco spazio, riduce il numero di componenti impiegati e le possibili sorgenti di errore, il che rende lo strumento più affidabile. La flessibilità è un punto di forza del dispositivo realizzato, in quanto è possibile cambiarne anche radicalmente il funzionamento modificando il solo firmware del micro.

Il sistema è concepito per essere facilmente espandibile, grazie al grande numero di pin e periferiche non utilizzate sul microcontrollore.

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INTRODUCTION

Sexually Transmitted Infections (STIs) present a significant burden to UK and worldwide healthcare systems and have been identified as an area of priority for world health. Unfortunately, the uptake of microsystems in point of care diagnostics has been relatively slow. This is for a number of reasons, but a principal one is that not all of the elements in the analysis chain are robust or easily manufactureable. Examples of these are sample preparation and rapid DNA extraction, amplification and detection within a microsystem.

Working with commercial partners the microengineering component of the project will indentify and investigate a number of these key technological challenges that are barriers to developing rapid point of care microsystems based polymicrobial STI diagnostics.

An important aim is to produce a "front-end" STI sample processing system, usable by industry to evaluate novel bio-sensing technologies that are rapidly emerging from nanotechnology and semiconductor research and thus be an aid to translation of promising diagnostics. In addition work will also focus on the control elements and wireless communications required to enable integration into a larger system

In this scenario, the focus of the Brunel BioMEMS group is to develop a rapid, accurate microfluidic device for polymicrobial pathogen detection from complex matrices such as blood, urine or urethral swab samples.

This design would allow for prompt diagnosis and treatment of STIs for patients and their sexual partners, a factor which has been identified as a challenge to effective STI control. The compact nature of this device affords it the potential to penetration into high risk groups such as youth and immigrant sectors who are reluctant to seek prompt primary care in the traditional settings.

In addition to the individual benefits of this electronic system is highly amenable to incorporation into broadband and mobile communication systems with a wide scope of possibilities, including assisted or automated patient and partner notification, community epidemiological surveillance and telemedicine for geographically isolated individuals.

1

IMMUNO ASSAY AND MOLECULAR DETECTION:

molecular recognition technologies in comparison

Over the past several years, the development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases. Though microbial phenotypic characteristics, such as protein, bacteriophage, and chromatographic profiles, as well as bio typing and susceptibility testing, are used in most routine laboratories for identification and differentiation, nucleic acid techniques and immunological detection are making increasing inroads into clinical laboratories. Bacterial fermentation, fungal conidiogenesis, parasitic morphology, and viral cytopathic effects are a few phenotypic characteristics commonly used. Some phenotypic characteristics are sensitive enough for strain characterization; however, most phenotypic variables commonly observed in the microbiology laboratory are not sensitive enough for strain differentiation. Therefore, when methods for microbial genome analysis became available, a new frontier in microbial identification and characterization was opened.

It is difficult to draw firm conclusions as to type of bio recognition molecule to use for a given analyte. However, the detection method and reagents are generally target-driven and the user must decide on what level the detection should be performed. In general, nucleic acid-based detection is more specific and sensitive than the immunological-based detection, while the latter is faster and more robust.

1.1 Immunological detection

Enzyme immunoassay (EIA) is based on two important biological phenomena: the extraordinary power of antibodies, based on the ability of immune system of vertebrates to produce a virtually unlimited variety of proteins (antibodies), each with an affinity for a specific foreign compound (antigen or hapten); and the extremely high catalytic power of enzymes, which may quite often be detectable with great ease.

Today, fully automated instruments in medical laboratories around the world use the immunoassay principle with an enzyme as the reporter label for routine measurements of innumerable analytes in patient samples. But, before the introduction of enzyme immunoassay there were two widely accepted assays that employed labelled antibodies and antigens. They are immunofluorescence, in which a fluorescent dye is conjugated to the antibody; and radioimmunoassay, in which isotopes are attached to antibodies or antigens. In practice immunofluorescence is not easy to quantify for antibody assays, since it depends on subjective visual assessments of fluorescence and the results are usually expressed as the serial dilution of serum that gives the least fluorescence. Radioimmunoassay, on the other hand, is highly sensitive and permits precise quantification. However, the isotope labels decay rapidly, so that the conjugates have a short shelf life; complex equipment is necessary for their assessment; and, because of the medical hazards, only highly trained personnel must handle them. The enzyme immunoassays offered an attractive alternative to these labelled antibody/antigen methods. In particular, they employ antibodies or antigens conjugated to enzymes in such a way that the immunological and enzymatic activity of each moiety is maintained. These assays give objective results and are extremely sensitive; the reagents present no health hazards, they are stable, and have long shelf lives. Moreover, the estimation of results can either be visual or be made with a rather simple spectrophotometer.

EIA consists thus of a two-pronged strategy: the reaction between the immuno-reactants (antibody with the corresponding antigen) and the detection of that reaction using enzymes, labelled to the reactants, as indicators. In this way it is possible to measure the presence or concentration of a substance in solutions that frequently contain a complex mixture of substances.

Such assays are based on the unique ability of all antibodies to bind with high specificity to one or a very limited group of molecules. In particular, the specificity of the assay depends on the degree

to which the analytical reagent is able to bind to its specific binding partner to the exclusion of all other substances that might be present in the sample to be analysed. In addition, to the need of specificity, a binding partner must be selected that has a sufficiently high affinity for the analyte to permit an accurate measurement.

The other key feature of this kind of analysis is a means to produce a measurable signal in response to a specific binding. This can depend on the use of an analytical reagent that is associated with a detectable label. Such labels serve for detection and quantitation of binding events either after separating free and bound labelled reagents, or by designing the system such a way that a binding event effects a change in the signal produced by the label.

Immunological detection with antibodies is perhaps the only technology, which has been successfully employed for detection of bacterial cells, spores, viruses and toxins alike. Virtually any chemical compound can serve like an antigen if it is able to trigger an immune response.

1.2 Molecular recognition-based detection technologies

By definition, almost any self-replicating biological entity can be discriminated on the basis of nucleic acid sequences to that particular organism. This approach justifies the claim that an assay can be developed for virtually any organism given a sufficient effort to identify specific sequences unique to the target organism. The challenge is to make these assays totally inclusive and thus make it recognize all potential biological variants.

Early DNA hybridization studies were used to demonstrate relatedness amongst bacteria. This understanding of nucleic acid hybridization chemistry made possible nucleic acid probe technology. Advances in plasmid profiling and bacteriophage recovery and analysis have made possible plasmid profiling and bacteriophage typing, respectively. These technologies, however, like the determinations of phenotypic variables, are limited by microbial recovery and growth. Nucleic acid amplification technology has opened new avenues of microbial detection and characterization, such that growth is no longer required for microbial identification. In this respect, molecular methods have surpassed traditional methods of detection.

The polymerase chain reaction (PCR) and other recently developed amplification techniques have simplified and accelerated the in vitro process of nucleic acid amplification. The amplified products, known as amplicons, may be characterized by various methods, including nucleic acid probe hybridization, analysis of fragments after restriction endonuclease digestion, or direct sequence analysis.

1.3 Enzyme immunoassay: a review

Most of the enzyme immunoassays are analogous to the fluorescence or radioimmunoassay in that they involve at least one separation step in which the "bound" enzyme labelled reagent is separated from the unbound enzyme, enabling measurement of either bound or free activity. This is the basis of all "heterogeneous enzyme immunoassay", for example, the enzyme-linked immunosorbent assay (ELISA).

The development of various EIA procedures has led to confusing terminology and classifications. Here, EIA will be classified in "homogenous enzyme immunoassay" and "heterogeneous enzyme immunoassay". A diagrammatic outline of the first type of assay is given in fig.1.1.

The hapten (a small molecule that can elicit an immune response only when attached to a large carrier such as protein) is labelled with an enzyme, so that it retains its activity. However when the hapten reacts with the antibody the enzyme activity of the hapten enzyme complex is inhibited. In the test the unknown sample is mixed with the labelled hapten and with specific antibody to the hapten. If there is hapten in the sample it competes with the labelled hapten for the limited amount of antibody.

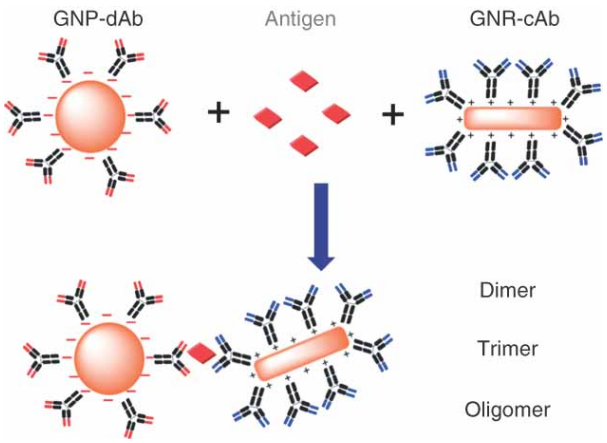
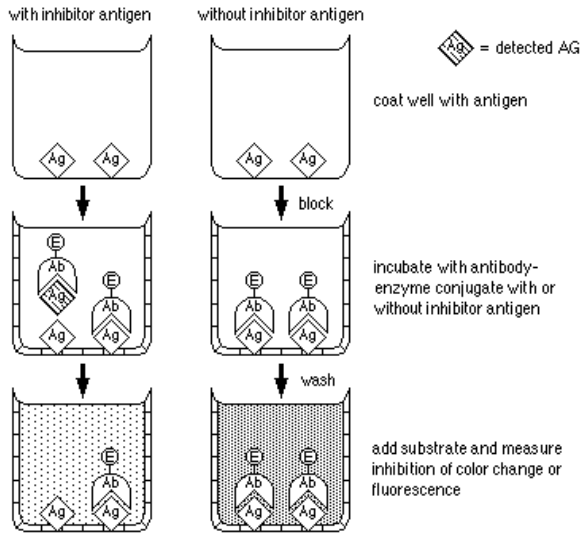


Fig. 1.1: Homogenous enzyme immunoassay.

Thus, there are fewer antibodies available to inhibit the enzyme activity of the labelled hapten. This method is the basis of the EMIT system. It is especially useful for the rapid assay of low molecular weight compounds.

The heterogeneous enzyme immunoassays are known under a variety of names, the most recognized is ELISA (Enzyme-Linked Immunosorbent Assay). ELISA tests can be competitive (fig.1.2) for the assay of antigen.



Immunoglobulin containing specific antibody is attached to the solid carrier surface. A mixture of solution thought to contain antigen and enzyme-labelled antigen are incubated in various proportions on the carrier. The amount of enzyme-labelled antigen attached is again measured by the rate of hydrolysis of its substrate. The more antigen there is in the unknown solution, the less labelled antigen will be attached.

Fig. 1.2: Competitive ELISA for assay of antigen.

An alternative method for antigen measurement is the double antibody sandwich technique (fig.1.3).

In this variation immunoglobulin containing specific antibody is used to sensitize the carrier surface. The solution containing the antigen is then incubated with the sensitized surface and the excess solution is washed away. A conjugate consisting of enzyme-labelled specific antibody is then added and this becomes attached to the antigen already captured by the sensitized surface. After incubation, the excess conjugate is washed away and the amount attached is measured by the rate at which it degrades added substrate.

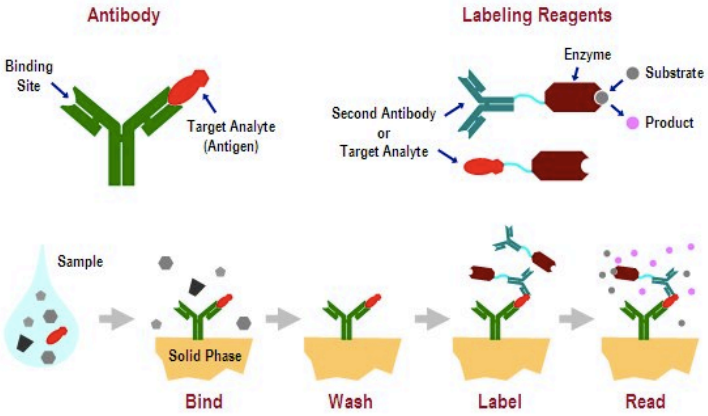


Fig. 1.3: Double antibody sandwich method of ELISA for assay of antigen.

In the indirect method (fig.1.4) the antigen is coupled to a solid-phase support and the sera thought to contain antibody are incubated in this sensitized carrier. Excess serum components are washed away and then the enzyme-labelled antiglobulin (conjugate) is added. The conjugate will become attached to the antigen-antibody complexes on the carrier surface, and the amount of conjugate attached is measured by the amount of substrate that it degrades.

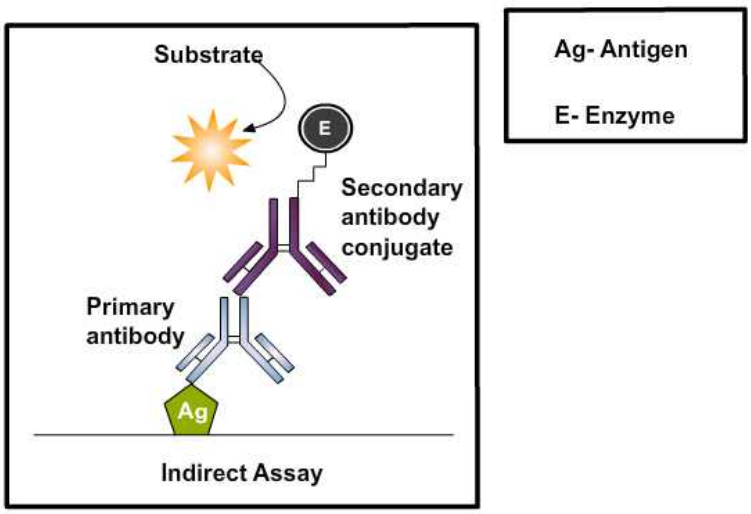


Fig. 1.4: The indirect method of ELISA for assay of antibody.

This a very useful method, since a single enzyme-labelled antihuman globulin can be used to detect any human antibodies regardless of the disease state that is being investigated.

In all these methods the end result is a change in colour of the enzyme substrate. This can be measured accurately in a spectrophotometer. As far as the carrier surface is concerned, it may be beads, tubes or plates, which permit covalent linking of antigen or antibody to the surface. In addition it is important to ensure that all wells or tubes are treated in exactly the same way.

The results can be expressed in a variety of ways, as the following points indicate.

(a) Under carefully controlled conditions, the results may be expressed in absorbance values and this is the easier method available. A value indicative of infection is then chosen above the control level. Sera are classified as positive if above and negative if below that value.
(b) In terms of reference samples using a standard curve.
(c) All sera can be titrated by serial dilution. An absorbance value is then chosen and the dilution of serum yielding such as absorbance value is the “titre”.

Endocrinology is the area where much of the pioneering ELISA research was done. In particular insulin is a large hormone, which has been measured successfully, but also smaller molecular weight hormones have been evaluated with high sensitivity. Another area where ELISA appears to offer great practical possibilities is in the measurement of onco-fetal proteins, especially in the large-scale screening. The published literature indicates that the main impacts of ELISA have been in the measurement of serum proteins, like immunoglobulin B, antigens of infectious agents, but overall of antibodies.

1.4 Nucleic acid-based detection: a review

Nucleic acid-based typing systems such as plasmid analysis, restriction endonuclease analysis or ribotyping, present several disadvantages: lack of reproducibility, poor discriminatory power, and difficulties in typing. Nucleic acid probes, on the contrary, are capable of identifying organism at, above, and below the species level. They are more specific, more easily quantified and standardized among the different organisms. The quantity of target detectable by the method depends on the size and homology of the probe chosen and the nature of the original specimen; identification and organism in pure cultures or from isolated colonies is usually easier than detection of organism in direct specimen. DNA probes facilitate the identification of infectious agents that do not grow rapidly. Additionally, this technique allows for the diagnosis of infections in which the organisms are not easily cultured or cannot be cultured at all.

Nucleic acid-based assays can be broadly classified into two categories: (1) direct target probing with signal amplification; and (2) target amplification.

Direct target:

The basis of virtually all nucleic acid target probe system is the ability of complementary nucleic acid strands to form stable hybrid complexes, and the stability of the hybrid complexes is correlated with the temperature at which the hybrids dissociate (melting temperature). Most hybridization assays require post-hybridization separation of the hybrid complex from free probe. The labelled probe in most of these systems does not have any inherent property that makes it significantly different when bound to the probe as opposed to when free in solution. The separation step can be accomplished in a number of ways but usually involves either immobilization of probe and target.

One widely used format is sandwich hybridization involving use of two probes. The general format of this assay is shown in fig. 1.5. One probe is immobilized to a solid support and serves to capture target. A second probe labelled with a reporter binds to a spatially distinct portion of the target and detects bound target.

For signal generation, the probe can be labelled with a variety of reporter molecules including radioisotopes, fluorophores, enzymes or haptens. While radioisotopes were the first reporters utilized, they have fallen out of favour due to limit half-life and handling concerns. Direct labelling with an enzyme or a fluorophore eliminates post-hybridization steps but may limit the performance of the probe. Hapten labelling as well as labelling with low molecular weight molecules for which high affinity capture system exist, is frequently used, especially during assay development.

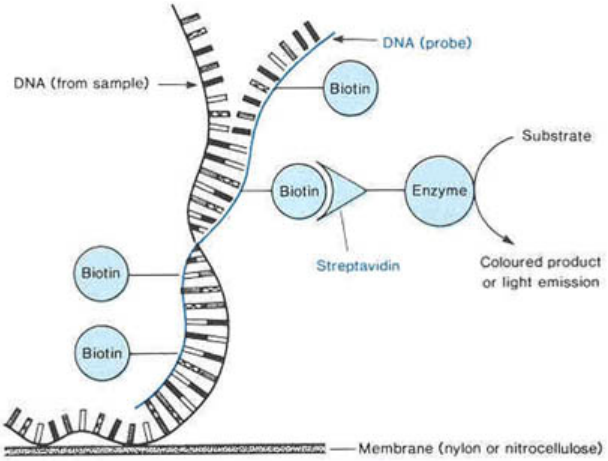


Fig. 1.5: Double probe sandwich hybridization.

These assays are popular for field usage where use of instrumentation is not viable option. Small hand-held spectrophotometers are currently available with a digital readout and programmable threshold setting to score assays either positive or negative. These assays cannot be scored visually and require instrumentation.

Although direct hybridization is better than PCR for quantifying target, it is not as sensitive. To increase sensitivity and specificity of direct hybridization assays, branched DNA (bDNA) was developed for detection and quantization of viral load for HIV and Hepatitis. In the contrast to target amplification methods, e.g. PCR, the bDNA signal amplification method quantitates target nucleic acid at physiological levels, involving a series of hybridization reactions without thermal cycling. bDNA is also more specific and highly reproducible, and thus represents an excellent technological platform for monitoring therapeutic response and quantifying nucleic acids.

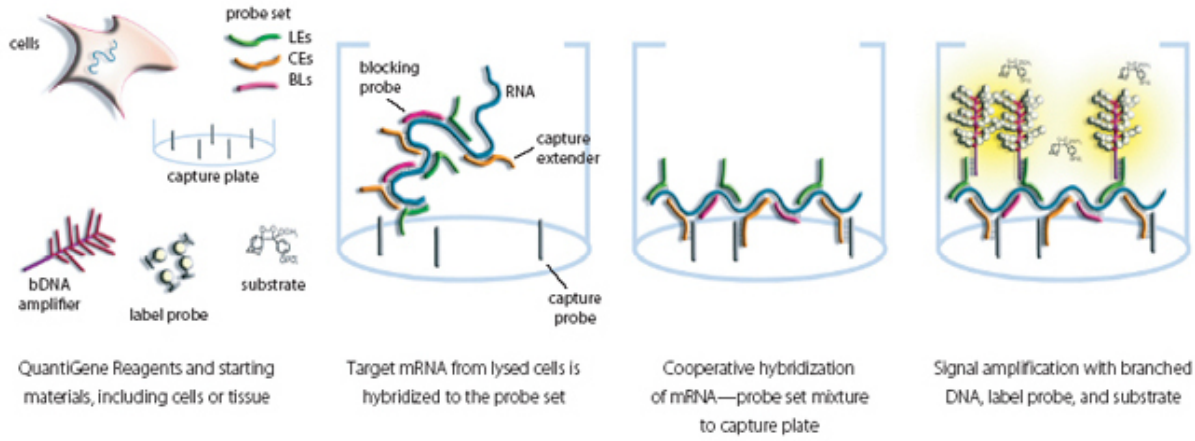


Fig. 1.6: Branched DNA signal amplification.

Visualization of captured target DNA is achieved by fluorescence imagers and CCD cameras. Sensitive detection of DNA/RNA by direct hybridization methods have also been reported using a CCD, light addressable potentiometric sensor and an evanescent wave sensor. These methods though seemingly straightforward and easy to perform, are time-consuming; they involve pre-hybridization sample preparation and are generally less sensitive than enzymatic amplification techniques.

Target amplification

As mentioned above, for direct application to the diagnosis of infections, nucleic acid without amplification often has the disadvantage of low sensitivity. Nucleic acid amplification techniques increase sensitivity dramatically while still retaining a high specificity.

PCR is the best developed and most widely used method of nucleic acid amplification. It is based on the ability of the DNA polymerase to copy a strand of DNA by elongation of complementary strands initiated from a pair of closely spaced chemically synthesized oligonucleotide primers. The basic technique of PCR includes repeated cycles of amplifying selected nucleic acid sequences. Each cycle consists of three steps: (a) a DNA denaturation step, in which the double strands of the target DNA are separated; (b) a primer annealing step, performed at a lower temperature, in which primers anneal to their complementary target sequences; and (c) an extension reaction step, in which DNA polymerase extends the sequences between the primers. At the end of each cycle, the quantities of PCR products are theoretically double. The whole procedure is carried out in a programmable thermal cycler.

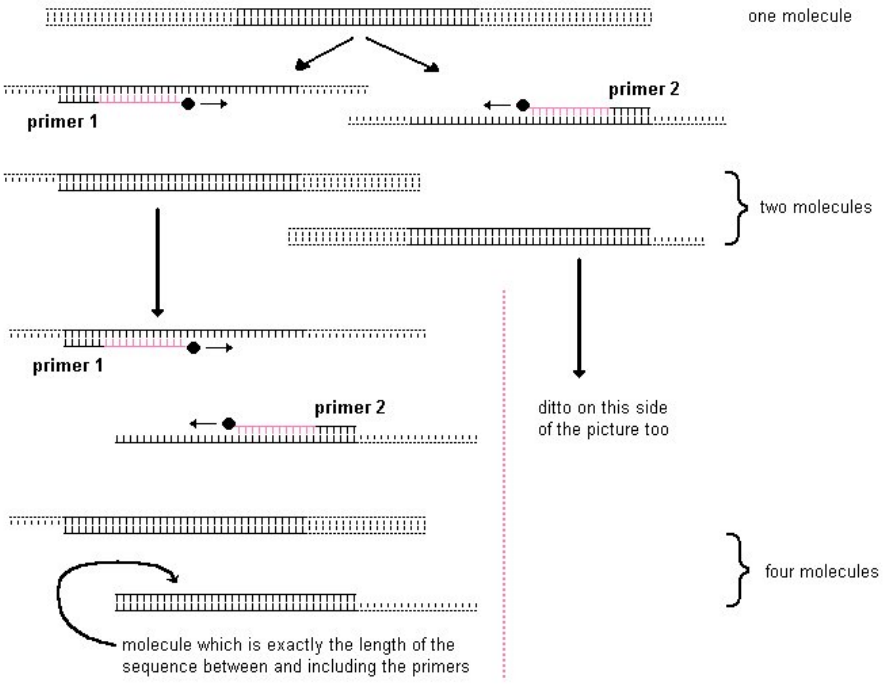


Fig. 1.7: PCR cycles.

Polymerase chain reaction techniques have led the way into this new era by allowing rapid detection of microorganisms that were previously difficult or impossible to detect by traditional microbiological methods. Early PCR methodologies involved use of Escherichia Coli DNA polymerase and manual rounds of heating to denature the template strand and cooling to allow the primers to anneal to the target. However, the Escherichia Coli DNA polymerase was also denatured at the temperatures required to denature the nucleic acid template and as a consequence additional polymerase was required to be added after each cycle. Numerous modifications of the standard PCR procedure have been developed since its inception. Some of these modifications effectively expand the diagnostic capabilities of PCR and have increased its utility in the clinical laboratories.

RT-PCR (Real Time PCR) was developed to amplify RNA targets. In this process, RNA targets are first converted to complementary DNA by RT, and then amplified by PCR. RT-PCR has played an important role in diagnosing RNA-containing virus infections, detecting micro bacteria species, and monitoring the effectiveness of antimicrobial therapy. In particular real time assays are characterized by: rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity, easy standardization and they are easy to transfer research laboratory protocols to diagnostic laboratories. All RT-PCR systems rely upon the detection and quantization of fluorescent reporter, the signal of which increases in direct proportion of the amount of PCR product in a reaction. The most famous one is Sybr Green that binds to double stranded DNA and upon excitation emits light. It is inexpensive, easy to use and sensitive, but sometimes the result can be an overestimation of the target concentration.

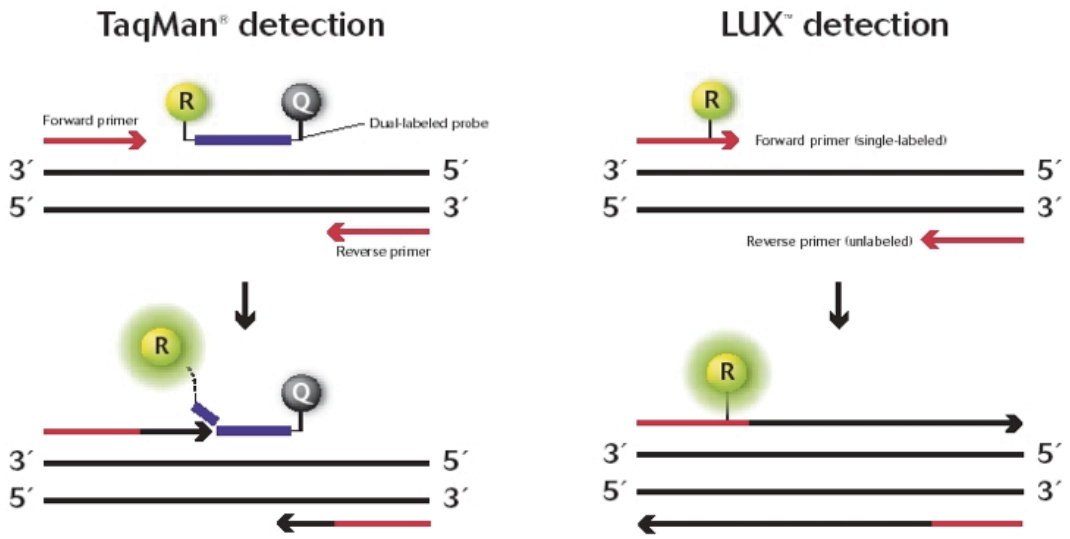


Fig. 1.8: SYBR Green.

An early deficiency of PCR was the inability to discriminate between targets that differed in only a single base pair. An alternative amplification scheme, the LCR, has been developed from the ligation detection reaction (LDR). LCR is a method of DNA amplification similar to PCR, but it differs from PCR because it amplifies the probe molecule rather than producing amplicons through polymerization of nucleotides. Two probes are used per each DNA strand and are legated together to form a single probe. LCR uses both a DNA polymerase enzyme and a DNA ligase enzyme to drive the reaction. Like PCR, LCR requires a thermal cycler to drive the reaction and each cycle results in a doubling of the target nucleic acid molecule. LCR can have greater specificity than PCR.

1.5 Current applications of molecular diagnostics and conclusions

Traditionally, the clinical medical microbiology laboratory has functioned to identify the etiologic agents of infectious diseases through the direct examination and culture of clinical specimens. Direct examination is limited by the number of organism present and by the ability of the laboratorian to successfully recognize the pathogen. Similarly, the culture of the etiologic agent depends on the ability of the microbe to propagate on artificial media and the laboratorian's choice of appropriate media for the culture. When a sample of limited volume is submitted, it is often not possible to culture for all pathogens. In such instances, close clinical correlation is essential for the judicious use of the specimen available. Some organisms are either uncultivable at present, extremely fastidious, or hazardous to laboratory personnel; and sometimes the laboratories are not well set up, or economically independent.

The addition of molecular detection methods to the microbiology laboratory has resolved many of these problems. The exquisite sensitivity and specificity of many molecular methods allow the accurate detection of very small numbers of organisms. The technology allows for the rapid and accurate identification of the etiologic agent in a time substantially shorter than traditional methods. This allows for earlier initiation of a focused antimicrobial regimen and decreases the likelihood of disease progression. In selected situations, the limitations imposed by the ability of an organism to be cultured and the selection of appropriate media and culture conditions may be replaced by the use of molecular microbiology. Microbial DNA/RNA extracted from a clinical specimen may be analysed for the presence of various organism-specific nucleic acid sequences regardless of the physiological requirements or variability of the organism.

As alluded to earlier, molecular methods may also be useful in instances of limited specimen volume. Even in low-volume specimens, enough DNA/RNA can often be extracted to allow performance of numerous molecular assays. However, though molecular methods are very sensitive, like culture and direct examination, clinically relevant results are ultimately reliant in the submission of quality specimen.

2

LAB-ON-A-CHIP: idea and applications

Semiconductor micro fabrication is a well-established technology that has been developed and optimized by the microprocessor industry. Due to excellent benefit of this technology, research is being pursed with a view to implement laboratory works into miniature integrated analysis systems on a micro/nano scale. Over the past, decade, miniaturization of analytical techniques has become a dominant trend in research, such as miniaturization of laboratories processes and diagnostics. This miniaturization is primarily driven by the need to reduce costs, by reducing the consumption of expensive reagents, by increasing throughput and automation and shortening analysis time. The standard method is collecting the samples on site and sending them to specialized laboratories for analysis, which is cost intensive and time consuming due to a large and expensive instrumentation and also due to needs for qualified personnel to carry out the analysis.

DNA molecule, which contains vital and identical codes, can be used for diagnostic purpose. To date about 400 diseases are diagnosable by the molecular analysis of DNA. For example, lung cancer, which is a genetic disorder, can be found thorough DNA codes. Therefore, combination of integration and automation of diagnostic processes on DNA molecule in low-cost fast and simple integrated chip may have the greatest impact on modern society's health and life. These easy-to-use portable diagnostic devices are called LOAC systems that in general would effectively simplify standard tasks in the area of medical, biological and biochemical treatments. Recently, progress in the field of microfluidics such as integrated multiplexor, micro fabricated reaction and separation systems, integrated microfluidic DNA amplification or miniature integrated LOAC system propose

molecular detection in an integrated microfluidic device that will be commercially available within a few years.

Figure 2.1 shows a system-level block diagram of a specific LOAC as a DNA diagnostic chip. As it is shown in this figure, this system consists of three main parts: extraction, amplification and detection, which are connected to each other through microfluidic networks. Extraction is the first part of same diagnostic chips where the sample is delivered. In this part a biological entity of interest is extracted from the sample. This is achieved by mixing the test sample with an appropriate buffer to lyse the cell. This is done using a micro mixer. Then the biomolecule of interest is extracted using an appropriate separation technique. In such a system, the quantity of the extracted biomolecule (e.g. DNA) is often insufficient for detection. Therefore, in the next stage a PCR process is incorporated to amplify the DNA molecule to obtain sufficient quantity for detection. In the final stage of the system, a specific type of electro-chemical sensor is implemented to detect the desired DNA molecules. This work is concerned about the last part of the process, which may be the key point of the whole process.

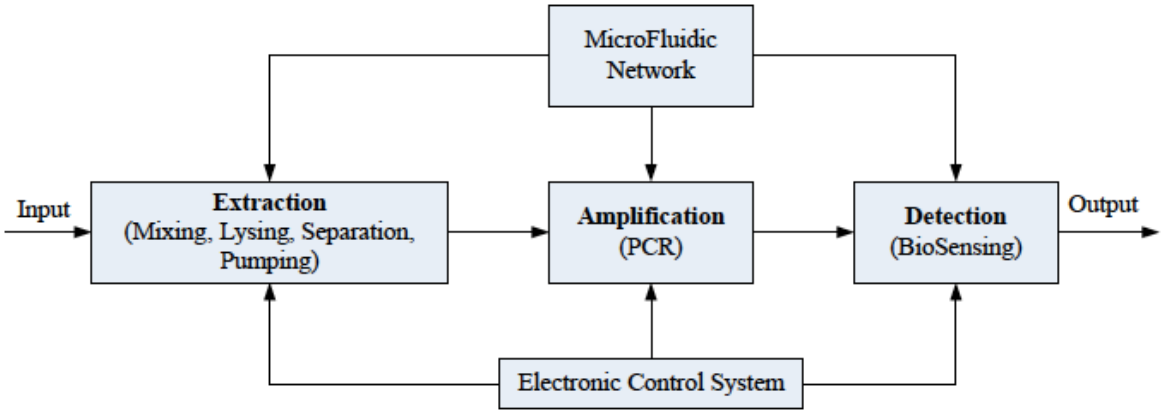


Fig. 2.1: Block diagram of a typical LOAC for DNA diagnostic chip.

2.1 DNA sensors classification

Significant effort has been directed so far to make an accurate sensor for detection of DNA molecules. Different groups have proposed different methods but none of them is commercially available yet and research is still in progress. Almost all of the methods for specific detection of DNA molecules are based on hybridization process and for this purpose, usually a short sequence of DNA, namely probe DNA, is immobilized on transducer's surface that is a sensitive surface to the occurrence of the hybridization of the probe DNA to the complementary sequence, namely target DNA. This sensitive area and both probe and target are in an aqueous medium and usually this procedure is applied to a DNA microarray or DNA chip that consist of a large number of individual spots with different DNA probe for sensing different sequences. Since the sequence and position of every probe DNA spot is known, the composition of the sample is mapped if the amount of hybridized analyte DNA is detected at each spot. Hybridization of single strand or probe DNA with complementary strand or target DNA results in a change in the transducer property, which could be converted to an appropriate electrical signal.

Widely established and accepted method in DNA detection is fluorescent-based method with fluorescent dyes of different colours, e.g. rhodamine (red) and fluorescein (green). Due to the intensive cost of reagents and associated equipment, such as lasers and suitable scanners as well as the time consumption and complicated computer analysis, photo-based detections are limited to the laboratories and are impossible to fabricate in a microchip. Recently progress in the field of functionalized fluorescent semiconductor nanocrystals has made them a very good option for traditional dyes but the principle and complexity remain the same. Nevertheless, introduction of DNA microarrays has made it possible to analyse most clinical diagnostics and genetic research in parallel, which decrease the total process time. DNA microarrays are now available from a large number of vendors, both prefabricated for specific common tasks and custom made.

While the principle of sequence analysis by hybridization is common to all detection methods, there is a wide range of possibilities about how to identify the hybridized molecular pairs within a specific probe DNA spot. They can be classified into two major categories: the ones, which add additional markers and the ones that try to detect the hybridization events directly. The former method has the advantage of large signals and low noise levels since the markers generally possess properties that can easily be distinguished from the other materials involved in the hybridization

procedure (e.g. they emit light, transfer charge in an electrochemical redox process, or produce a magnetic stray field). However, the use of markers requires an additional molecular recognition step, and because these are always dynamic equilibrium process between binding and unbinding events, they can never reach 100 % efficiency. Thus, there are always a percentage of hybridized pairs, which do not contribute to the signal because no markers are bound to them. From this point of view, a direct detection of hybridized molecular pairs would be advantageous, but sensing these events is rather challenging because there is no inherent difference in the kind of signal between single strands and hybridized pairs. Possible measures are the mass or the charge of the molecules, but these signals only change incrementally when small amounts of analyte DNA are added to an already existing large number of probes DNA strands. Furthermore, other ingredients of the surrounding solution like trapped ions can falsify the outcome of the measurements, so that great care has to be taken in interpreting the results. Following, different current approaches for direct and indirect hybridization detection are presented briefly.

- *The main class of DNA-based detections is based on detection with markers that can be subdivided to three groups, namely redox-active markers, enzyme labels and metal nanoparticles.*

Employing redox active markers, that is involved with a modification step of DNA with a molecule that has reduction or oxidation property with redox active molecules contained in solution and after interaction between the DNA and binding molecule, an electrochemical technique is applied to the electrode to measure the surface species. Some redox-active molecules are intercalator such as daunomycin that binds to DNA and inserts itself into the DNA double-helix structure and an enhancement in the redox signal would be observed. Some other redox-active molecules have an affinity towards ssDNA, such as methylene blue, and then a high signal would be observed from the probe-modified electrode. These changes in the peak potential current of the labels for the probe and hybrid molecules provide the basis for detection of the label-based hybridization. Several metal complexes such as cobalt phenanthroline, cobalt bipyridine and ruthenium bipyridine, anticancer agents such as echinomycin and epirubicin, and organic dyes such as methylene blue were used as labels for the detection of hybridization.

Another technique for labelling of probe DNA is enzyme labelling of probes. The same as metal tags, enzyme-modified electrode surface, has electrochemical activity with appropriate substrates in solution. And this electrochemical activity can be used for detection of hybridization of DNA.

Horseradish peroxidase (HRP) has been used extensively as a label for colorimetric detection in biological studies.

Metal nanoparticles offer excellent prospects for chemical and biological sensing because of their unique optical and electrical properties. The application of gold nanoparticles as labels in DNA hybridization detection assays has become common. Also silver nanoparticles have desirable compositions as labels in electrochemical detection assays for example, silver particles exhibit better electrochemical activity than gold particles. Particularly metal nanoparticles can be treated with or without acid for producing electrochemical signal on electrode surface. Also gold nanoparticle can be used with a silver coating to enhance the electrochemical signal of silver.

The labelling of probes with different metal nanoparticles or enzymes enables the simultaneous detection of more than one target in a sample by encoding technology. Three encoding nanoparticles (zinc sulfide, cadmium sulfide and lead sulfide) were used to differentiate the signals of three DNA targets in connection with stripping-voltammetric measurements of the heavy metal dissolution products.

In summery, metal nanoparticles provide a very sensitive and versatile method for analysing biomolecules, and several companies are already offering or developing kits based on this approach. Thus, nanoparticle based DNA assays can be regarded as a serious competitor to the established fluorescent detection scheme.

The use of carbon nanotubes will improves the sensitivity and selectivity of mentioned sensors due to large charge-transport characteristics of carbon nanotubes.

- *The second main group of DNA detection method is label-free detection of hybridization based on changes in mass density by using micro-cantilevers and quartz crystal microbalance, and changes in ionic strength of solution by using semiconductor devises such as field effect transistors and field effect capacitances.*

Label-free electrical detection of the DNA-hybridization by using semiconductor field-effect devices offers a new approach for DNA chips with direct electrical readout for a fast, simple and in-expensive analysis of nucleic acid samples. The inherent miniaturization of such devices and their compatibility with advanced micro fabrication technology can make them very attractive for DNA diagnostics. Therefore, in recent years, several attempts have been made to detect DNA by its

intrinsic molecular charge using field-effect devices, like capacitive electrolyte-insulator-semiconductor (EIS) and field-effect transistor (FET) structures.

Guanine and adenine are the most electro active bases of DNA, because they can easily be adsorbed and oxidized on carbon electrodes. Monitoring the changes in these signals upon duplex formation enabled the label-free detection of hybridization of DNA by its intrinsic property.

Recently due to progress in the field of magnetic resistors, they have found their way in the field of biomedical research and computer technology. Change in resistance of Giant magnetic resistance is a physical effect of ferromagnetic thin layers sandwiched with non-ferromagnetic layers in the presence of external magnetic field and magnetic particles. After instant magnetization of magnetic micro particles, stray magnetic field in micro particles will change the resistance of GMR sensor that can be measured by measuring the electrical current. In the field of DNA sensor, magnetic particle can be used, as marker for duplex DNA and it will signal the formation of duplex DNA.

3

DNA DETECTION: optical methods

The detection of pathogenic bacteria is key to prevention and identification of problems related to health and safety. Recent advances in ultrasensitive instrumentation have allowed for the detection, identification and dynamic studies of single molecules in the condensed phase. Molecular diagnostic that report on DNA sequence information are making increasingly important contribution to medicine and research. Pathogen identification based on a DNA sequence is more accurate, less subjective and often much faster than culture-based methods. In addition to improving existing services, DNA diagnostics allow access to genomic information previously unavailable to clinicians.

Methods for routine identification of microorganisms take at least several hours, a day or even more. Typically they take between 24~48 hours to yield results. They rely on time-consuming growth in culture media, followed by isolation, biochemical identification and sometimes serological determination. The culture-based tests have relatively low sensitivity, about 30~50%. That means, for example, that the probability of identifying a particular bacterium from the sepsis patient is less than 50%. That is where new analytical techniques offer significant benefits. Development of nucleic acid-based detection systems is the main focus of many research groups and high technology companies. Thus, new challenges and requirements for an detector suitable for nucleic acid analysis, include high sensitivity and high specificity protocol that can be completed in a relatively short time offering at the same time low detection limit. Moreover, systems that can be miniaturized and automated present a significant advantage over conventional technology, especially if detection is needed in the field.

For these reasons, using micro fluidics technologies can be an obvious advantage, just because of the ability to handle small sample sizes and also as a result saving valuable reagents used in the assays. Many micro fluidics systems are integrated with sensing modules or sample-pre-treatment modules, which increase the efficiency of the assays and reduces cross contamination. They are commonly called *Biosensors* and they have the potential to shorten the time span between sample uptake and results, but their future lies in reaching selectivity and sensitivity comparable to established methods at a fraction of the cost.

The following review offers an overview of the various approaches most commonly taken to detect and identify pathogenic bacteria using optical methods, describing also the main techniques compared with traditional methods, and recent developments in the field of pathogen bacteria optical biosensors.

3.1 About DNA diagnostic

Like all diagnostic technology, DNA diagnostics require both a *detection method* and a *signal transducer*. Most current detection methods for the sequence-specific recognition of DNA make use of the special property of single-stranded DNA (ssDNA) to base pair with high specificity to a complementary molecule. The other molecule may be another ssDNA, ssRNA, peptide-nucleic acid or other base-pairing molecular analog. Such specific annealing or hybridization forms the basis for such common technologies as PCR amplification with specific primer sets, DNA microarray and fluorescent in situ hybridization. The second component required is a signal transducer, which converts the sequence-specific recognition event into a signal that can be quantitatively measured. Typically the signal is optical or electrical.

While the specificity of a DNA diagnostic will depend on the fidelity of the detection method, the sensitivity will largely be a function of a signal transducer. However, detection of unique genomic sequences is limited by the difficulty in detecting the weak signal of one fluorescent molecule over background. To improve sensitivity, several ingenious methods have recently been developed to sensitively detect the recognition event, or amplify the transduced signal.

The most popular methods are, by far, those based on culture and colony counting methods and the polymerase chain reaction, PCR. This can be explained on the grounds of selectivity and

reliability of both techniques. Culture and colony counting methods are much more time consuming than PCR methods but both provide conclusive and unambiguous results. On the other hand, recent advances in PCR technology, namely real time PCR, now enable obtaining results in few hours.

Biosensor technology comes with promises of equally reliable results in much shorter times, which is perhaps why they are currently drawing a lot of interest. In fact the miniaturization of nucleic acid analytical platforms has many advantages over the conventional bench-top counterparts. These include low samples/reagents consumption as well as short assay time. Most importantly, they permit the integration of a number of functions including sample preparations, target amplification, and product detection, thus enabling a fully automated operation that can be used by untrained individuals.

3.2 Biosensors in pathogen detection: optical systems

Biosensors have recently been defined as analytical devices incorporating a biological material, a biologically derived material or a bio mimic intimately associated with or integrated within a physicochemical transducer or transducing micro system, which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical. In particular, in this review, the attention will be focused on optical detection schemes integrated on the micro fluidic chips, because they can offer many benefits.

Optical biosensors are probably the most popular in bio analysis, due to their selectivity and sensitivity. They have been developed for rapid detection of contaminants, toxins or drugs and even pathogen bacteria. Labelling techniques are relatively sensitive for detecting bio molecules in traditional bioassays. Shrinking the sensing scheme to on-chip operation can potentially save reagents due to the small volumes required for micro fluidics, offer integration and therefore fewer steps in assays as well as the possibility for automation.

Fluorescence detection

Fluorescence occurs when a valance electron is excited from its ground state to an excited singlet state. The excitation is produced by the absorption of light of sufficient energy. When the electron returns to its original ground state it emits a photon at lower energy. Another important feature of fluorescence is the little thermal loss and rapid light emission taking place after absorption. The emitted light is at longer wavelength than the absorbed light since some of the energy is lost due to vibrations, this energy gap is termed Stoke's shift, and it should be large enough to avoid cross talk between excitation and emission signals.

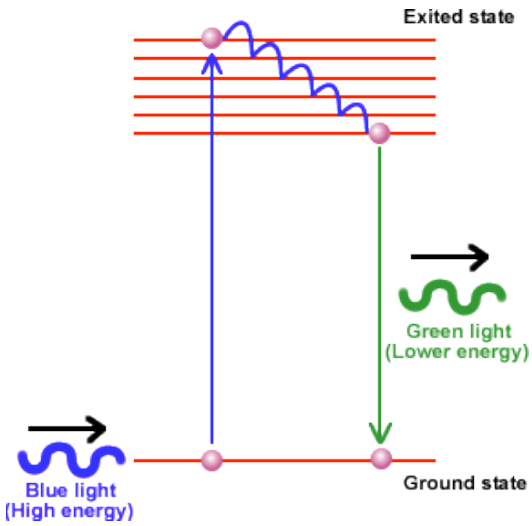


Fig. 3.1: Fluorescence process.

One of the most common applications of fluorescence is the fluorescent labelling, which typically uses small molecules of proteins that can be excited at a particular wavelength and emit at a longer wavelength. Their use is common is biology and chemistry due to the simplicity of the method. The advantage in integration fluorescent labels with micro fluidics is the reduction in the sample volume, which reduces background signal noise, hence increasing the sensitivity, signal-to-noise ratio. However, using fluorescent labels with micro fluidics can also present some challenges. The chip material needs to be microscopy-compatible, e.g. low auto fluorescence and non-adsorbent to the molecules.

Typically, samples are labelled with a specific fluorophore, the most common is *Fluorescein Isothiocyanate* (FITC), and then the fluorescence signal induced by a laser source is detected as sample flow through a channel.

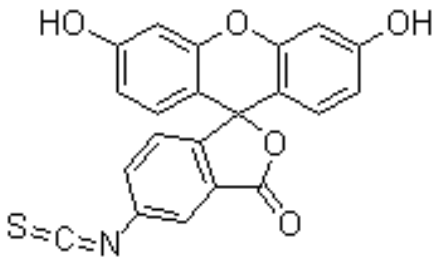


Fig. 3.2: Fluorescein Isothiocyanate structure.

A more sensitive technique, *Fluorescence Cross-Correlation Spectroscopy* (FCCS), uses cross-correlation of the temporal fluorescence fluctuations from dual-labelled molecules, which allows detection of very low concentration of proteins in microliter volumes of samples. It extends the *Fluorescence Correlation Spectroscopy* (FCS) procedure, by introducing high sensitivity for distinguishing fluorescent particles, which have a similar diffusion coefficient. Fluorescence correlation spectroscopy (FCS) is a correlation analysis of fluctuation of the fluorescence intensity. The analysis provides parameters of the physics under the fluctuations. One of the interesting applications of this is an analysis of the concentration fluctuations of fluorescent particles in solution. In this application, the fluorescence emitted from a very tiny space in solution containing a small number of fluorescent particles is observed. The fluorescence intensity is fluctuating due to Brownian motion of the particles. In other words, the number of the particles in the sub-space defined by the optical system is randomly changing around the average number. The analysis gives the average number of fluorescent particles and average diffusion time, when the particle is passing through the space. Eventually, both the concentration and size of the particle are determined. In these techniques light is focused on a sample and the measured fluorescence intensity fluctuations are analysed using the temporal autocorrelation. FCCS, on the other hand, uses two species, which are independently labelled with two spectrally separated fluorescent probes. These fluorescent probes are excited and detected by two different laser light sources and detectors commonly known as green and red respectively. Both laser light beams are focused into the sample and tuned so that they overlap to form a superimposed confocal observation volume.

Another technique, *Fluorescence Resonance Energy Transfer* (FRET) is used to measure molecular interactions. Two different fluorophores are tethered to two molecules of interest. When the molecules interact and bring the fluorophores close together, the emitted photon from the donor is transferred to the acceptor, which emits at a different wavelength from the donor. Changes in ratio metric measurements of the two colours suggest molecular interactions. This technique enables the distinction between hybridization and non-hybridization DNA oligomers, with an improved spatial and temporal resolution compared to simple fluorescently labelled molecules. The study of intermolecular interactions is improved in microfluidics devices, due to enhanced mixing that overcomes slow hybridization caused by limited diffusion in regular microarray chips.

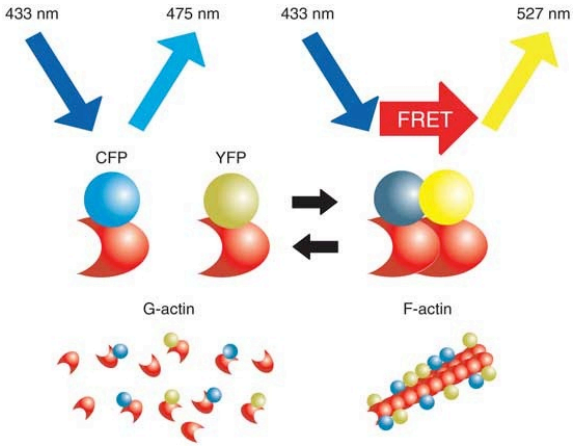


Fig. 3.3: Fluorescence Resonance Energy Transfer process.

To overcome photo bleaching and pH sensitivity issues of traditional labels, *Quantum Dots* (QD), are used to provide better stability and a higher signal. A quantum dot is a semiconductor whose excitons are confined in all three spatial dimensions. Consequently, such materials have electronic properties intermediate between those of bulk semiconductors and those of discrete molecules. They are bright, and their emission wavelength can be easily tuned by varying size and composition, therefore allowing multiplexing with labels across a wide range of accessible wavelength.

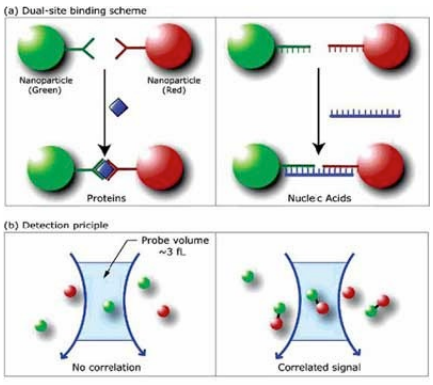


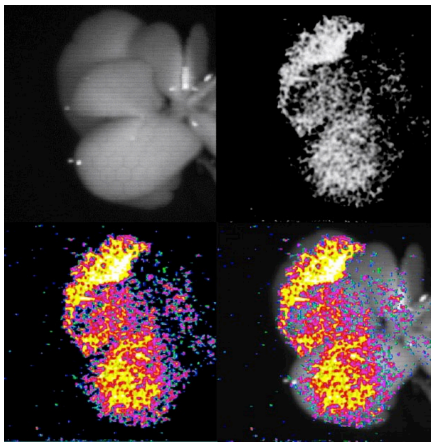
Fig. 3.4: Quantum Dots.

QDs are largely used in microfluidic assays for microbe and virus detection, sensing of single molecules, or in combination with FRET for applications such as signal amplification during DNA sensing and detection of molecular orientation, size and binding. This is because it has been estimated that quantum dots are 20 times brighter and 100 times more stable than traditional fluorescent reporters and they are particularly significant for optical applications due to their high extinction co-efficient. Being zero dimensional, quantum dots have a sharper density of states than higher-dimensional structures. As a result, they have superior transport and optical properties, and are being researched for use in diode lasers, amplifiers, and biological sensors.

Fluorescence detection, in contrast to SPR (Surface Plasmon Resonance), which will be explained below, is also used in combination with established techniques such as PCR and ELISA.

Chemiluminescence

Chemiluminescence is the emission of energy in the form of light as the result of a chemical reaction. Specifically, the analyte binding to a substrate or with an enzyme causes light emission. The advantage of this technique is that no excitation instrumentation is required, and therefore background interference is virtually eliminated. However, emission is generally low which requires a more sensitive detection mechanism.



Chemiluminescence differs from fluorescence in that the electronic excited state is derived from the product of a chemical reaction rather than the more typical way of creating electronic excited states, namely absorption. It is the antithesis of a photochemical reaction, in which light is used to drive an endothermic chemical reaction. Here, light is generated from a chemically exothermic reaction.

Fig. 3.5: Chemiluminescence imaging of free oxygen radicals formation in rat liver subjected to oxidative stress. Free oxygen radicals are detected by means of the chemiluminescent reagent lucigenin. Live image (top left), chemiluminescent image (top right), pseudocolored chemiluminescent image (bottom left), and pseudocolored chemiluminescent image overlapped to live image (bottom right).

Nanoparticles

Nanoparticles, including magnetic and gold particles coated with antibodies or aptamers can be used as labels for the separation and detection of cells and specific biomolecules. In standard bench top protocols, magnetic particles have been used extensively for molecular and cell separation purposes due to their simplicity of use and relatively high efficiency.

Fluorescence is the standard method for detection of binding events in molecular biology. Fluorescence markers are highly sensitive, and a variety of fluorescence-conjugated biomolecules are available. However, this technique suffers from photochemical instability and environment-dependent quantum yield, and the needed readers are expensive. Metal nanoparticles promise a high stability combined with a simpler optical detection, and were therefore proposed as alternative for the detection of binding events. So alternative optical readout devices can be applied for the detection of specific DNA-binding on micro structured spots of complementary, surface-immobilized capture DNA.

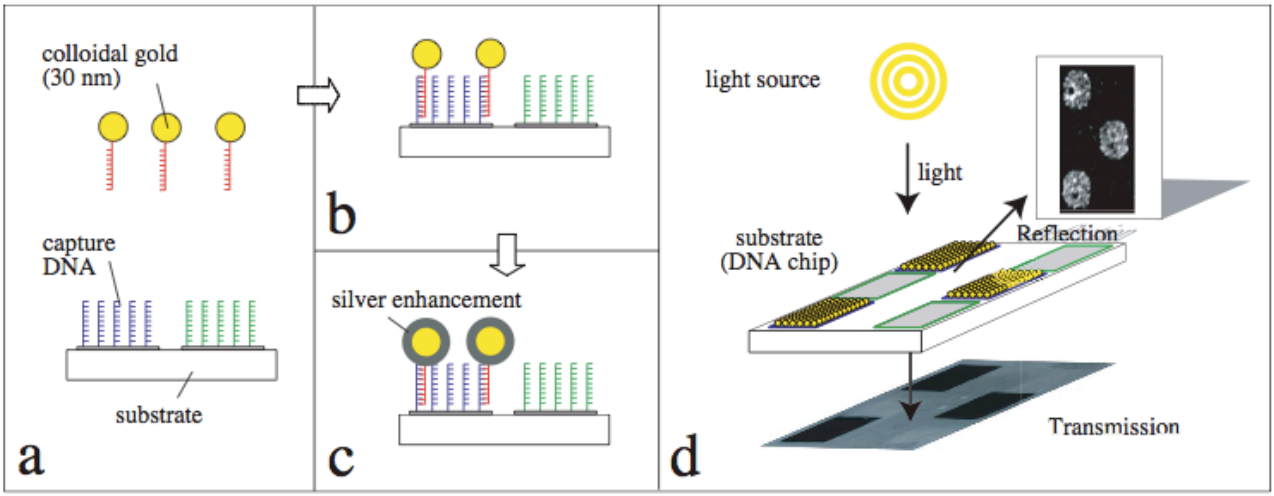


Fig. 3.6: Scheme of nanoparticle-based labelling for the detection of molecular interactions. a) Molecules of interest are labelled with colloidal gold particles. Arrays of capture molecules, which are complementary to the molecule of interest, are prepared on solid substrates. b) After incubation, the molecules of interest bind to the complementary capture molecules, resulting in surface-bound colloidal particles. c) These particles can be enhanced by selective growth of silver on the nanoparticles. d) A perspective scheme shows a substrate with immobilized nanoparticles. Optical detection in reflection or transmission mode is applied for localization of the areas of nanoparticle binding.

The high stability of DNA-nanoparticle conjugates in solution and on solid substrates were demonstrated in various studies. There are several possibilities for the detection of the bound nanoparticles; the simplest one is the detection of colour change in solution or on a substrate with the naked eye. This technique is only applicable for a greater ensemble of DNA-nanoparticle complexes. For single-molecule detection, the Surface Plasmon Resonance of nanoparticles can be used, which can be tailor-made to enable multicolour tests. To meet the demand of today's molecular diagnostic, highly paralleled tests with a simple detection scheme are needed. The parallelization of nanoparticle-based tests was already demonstrated by micro structuring DNA-spots on substrates down to 50 μm or even 4 μm squares. However, these spots were still examined by an optical microscope.

Associated with nanoparticles magnetic beads can be used to detect and quantify biomolecules. External permanent magnets are frequently used, but several groups have integrated sources of magnetic field on-chip for better control. Gold particles too, are commonly used nanoscale materials in molecular diagnostics, because of their low toxicity, easy conjugation to biomolecules and their versatility in detection methods employed for their analysis. Readouts based on optical absorption, fluorescence and electrical conductivity have all been implemented in microfluidic devices.

Barcodes

One of the main advantages of microfluidics is that it enables high throughput, parallel and multiplexed experiments. One of the major challenges in multiplexed analysis is to identify each specific reaction with a distinct label or code. Two encoding strategies are currently used: positional encoding, in which every potential reaction is preassigned a particular position on a solid-phase support such as a DNA microarray, and reaction encoding, where every possible reaction is uniquely tagged with a code that is most often optical or particle based.

In order to simplify the readout of these assays and reduce the total time, several groups have developed methods to create barcodes on-chip. Recently, dendrimer-like DNA (DL-DNA) nanostructures have been synthesized. The multivalent and anisotropic properties of DL-DNA were used as fluorescent dye carriers to construct fluorescence-intensity-encoded nanobarcodes. First the fluorescence-labelled Y-shaped DNA (Y-DNA) was synthesized, where each Y-DNA consisted of three oligonucleotide components that were complementary to each other. One of the oligonucleotides had a sticky end, and the other two were labelled with either fluorophore or a molecular probe. After hybridization, these oligonucleotides formed a fluorescence-labelled Y-DNA

(Fig. 3.7a) that was used as a peripheral outermost layer of DL-DNA to construct fluorescence-labelled DNA nanostructures. Since both dye type and dye number can be precisely controlled, multicolour fluorescence-intensity-encoded nanobarcodes could be fabricated (Fig. 3.7b). The decoding is based on the different ratios of different fluorescent dyes, independent of the dye positions (Fig. 3.7c). During the construction of DNA nanobarcodes, molecular probes were linked to the free reactive ends of DL-DNA. A myriad of DNA-manipulation enzyme tools makes it very easy to attach molecular probes (e.g. DNA or RNA probes, or even antibodies) to DNA nanobarcodes. Consequently, the resultant DNA nanobarcodes not only had coding capacity, but also contained molecular recognition elements that could be used for molecular detection. The resultant nanobarcodes were evaluated using agarose gel electrophoresis (Fig. 3.7d). The diameter of DNA nanobarcodes could be 30 nm, which is far below the detection limit of optical microscopy.

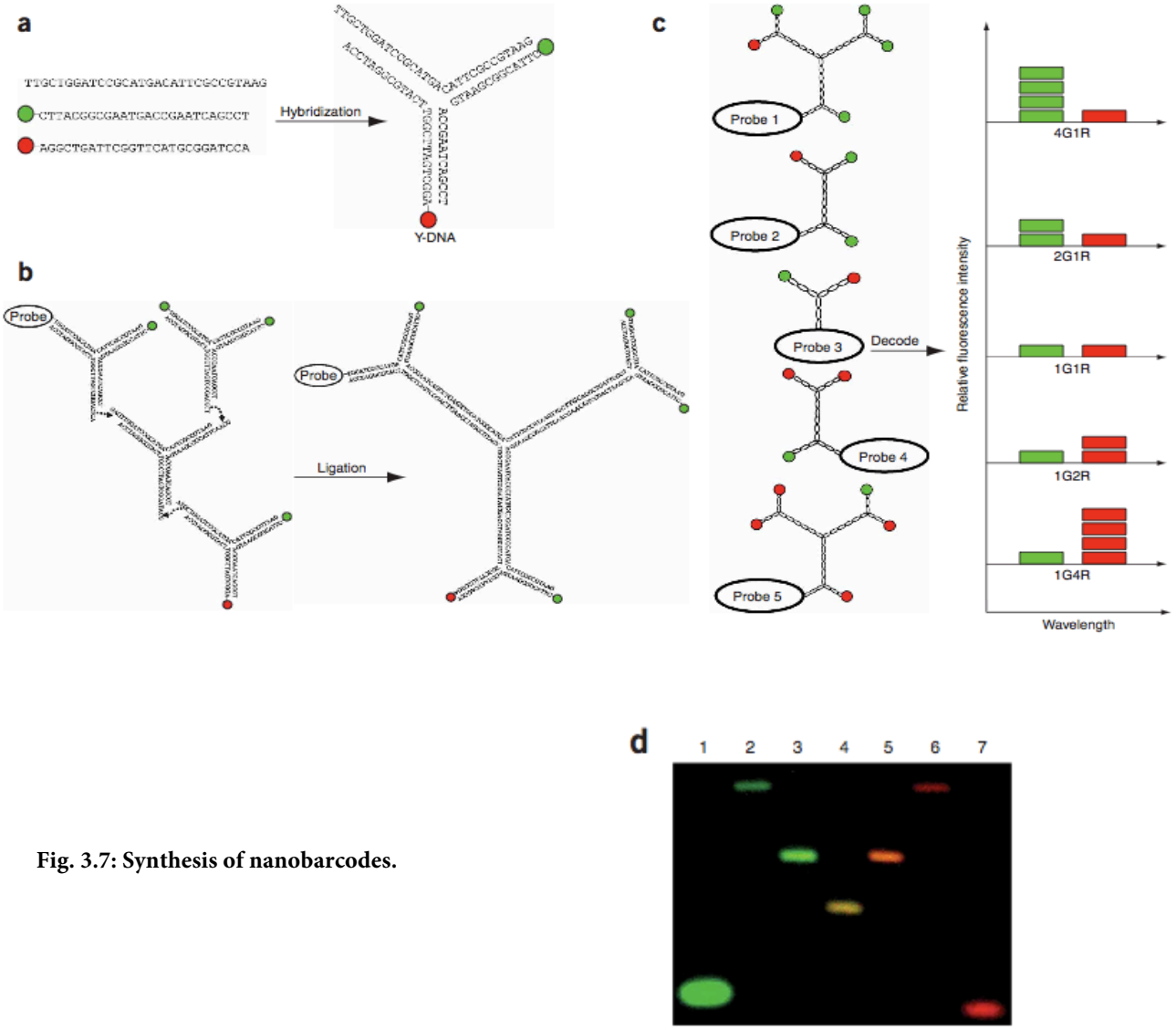


Fig. 3.7: Synthesis of nanobarcodes.

Encoded barcodes, as advantage, overcome the intrinsic limitation of the number of available colours offered by fluorescent labels. They enable on-chip blood separation and rapid measurement of a panel of plasma proteins from quantities of whole blood as small as those obtained by a finger prick.

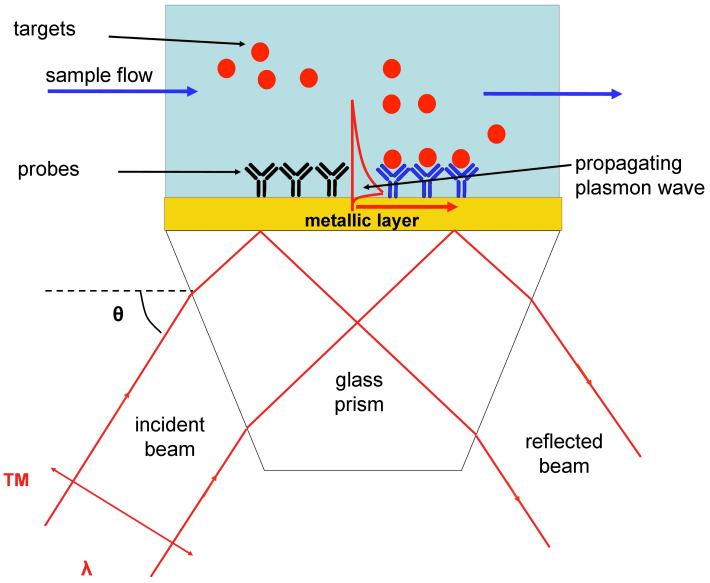
3.3 Label-free detection

While label-based methods provide high sensitivity, the additional steps required for the labelling process increase the probability of error, and may also induce conformational changes, resulting in variations in the binding affinities or functions of the biomolecules to be detected. Label-free methods therefore offer the alternative of a simplified assay, with good sensitivity, and integrated detection systems. The assay simplification as well as its high throughput capabilities could potentially facilitate point of care diagnoses as well as sensor applications in other microfluidic systems.

Surface Plasmon Resonance (SPR)

SPR is an optical detection method that detects subtle changes in the refractive index at the surface of a gold substrate, by monitoring the deflection angle of a laser beam aimed at the back of the substrate. If biomolecules are captured at the substrate surface by a recognition event, the instrument detects this event ad a change in this deflection angle.

SPR detection has been previously used to detect the hybridization of DNA targets to surface-bound, complementary capture strands, and scanning SPR imaging instruments have been developed to image SPR response from DNA hybridization at multiple DNA microarray elements. It can be used to detect adsorption of molecules, such as polymers, DNA, or proteins with good sensitivity, but not as good as sensitivities achieved by fluorescence techniques, and fairly good temporal resolution in real-time.



Completely integrated SPR systems have been developed to measure binding kinetics in a protein microarray or to perform immunoassays. SPR has also been coupled with digital microfluidics, thus creating a high-throughput screening system with reliable quantification.

Fig. 3.8: Diagram of a typical Surface Plasmon Resonance imaging (SPRI) system. The transverse magnetic (TM)–polarized incident beam of wavelength λ is refracted through a high-index glass prism of angle θ.

Localized SPR (LSPR) improves the sensitivity of the method and requires a smaller sample volume. This technique relies on a wavelength shift due to a change in the dielectric constant of the substrate. This technique has been applied in microfluidic devices for immunoassays with good results.

Disadvantages to note include the potential difficulty to functionalize metal surfaces, nonspecific adsorption, and immobilized biomolecules potentially losing their native bioactivity. Other negative aspects could be the high cost of equipment and large size of most currently available instruments.

SPR with integrated microfluidics is a widely used technique to study molecular interactions and enables the characterization of molecules and biochemical interactions occurring in diseases. It is a good alternative to optical detection for bio-diagnostics, as it does not require labelling and target modification prior to analysis.

3.4 Piezoelectric biosensors

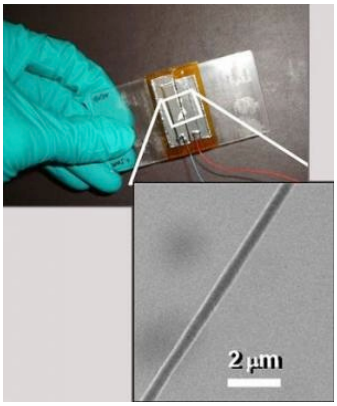
Piezoelectric sensors are based in the observation of resonance frequency changes on a quartz crystal microbalance following mass changes on the probe/transducer surface. In other words they measure a change a in the frequency which results from adsorption of target DNA that hybridized with the DNA probes immobilized on the crystal.

The relation between mass and resonant frequency is given by the Sauerbrey equation:

$$\Delta F = \frac{-2.3 * 10^6 F_0^2 \Delta m}{A}$$

where ΔF is the frequency change in Hertz, F_0 the resonant frequency of the crystal in MHz, Δm the deposited mass in grams and A is the coated area in cm^2.

The use of QCM allows the detection of bacteria using probes modified with immobilised antibodies. An antibody probe immersed for an hour in a solution containing E. coli gives an example of how E. coli may be detected. It is then extracted and dried under nitrogen. The resonant frequency of the probe is finally measured and results are obtained within minutes after drying.



Although the dip-and-dry method is more sensitive, reproducible and reliable than traditional flow-through methods, it is not as suitable for automation.

Fig. 3.9: Nano-sized generators with piezoelectric properties that allow them to convert into electricity the energy created through mechanical stress, stretches and twists.

3.5 Conclusions

Traditional pathogen detection methods, although sensitive enough, are often too slow to be of ay use. Therefore, new methods are needed that exceed their performance. Over the recent years, a lot of effort has gone into the study and development of biosensors of the most diverse nature, but their performance is irregular and still needs improvement.

Over the past two decades, the practice of DNA sequence detection has become more and more ubiquitous. This has been driven partly by the quantity of DNA sequence information that we have collected on humans and other organisms, and partly by the increasingly sophisticated technologies now available to detect specific DNA targets.

Optical detection is currently the preferred detection technique, due to its simplicity, high sensitivity, continuous monitoring capacity, and the absence of contact with the target analytes. The detection of single molecule has been achieved by both frequency-modulated absorption ad laser-induced fluorescence. Because of the low background and high signal-to-noise ratios, laser-induced fluorescence has become the most widely used method.

To enable the development of fast, sensitive and portable POC devices, recent progresses have been made towards a complete integration of sensing mechanism. Therefore, devices can be completely portable and achieve the required sensitivity for a reliable detection and diagnostics. The advantage of using microfluidics is that it allows for confining the processed biomolecules or cells in a region of interest, and as a result, facilitating sensing from small sample volume.

Summarizing the combination of microfluidics with conventional optical detection offers several advantages:

- The tight control of liquid flow enables the concentration of analytes and labels of interest in a small volume, therefore enhancing binding kinetics and overcoming sensitivity issues.
- Microfluidics save precious reagents and time.
- Microfluidics offer automated processing of samples, reducing human error, loss of samples and contamination with the added ability to process samples in parallel.
- Microfluidics can bring the target close to the detection area, enhancing the signal-to-noise ratio and improving the sensitivity.

However, optical detection does not seem to be an optimal solution for detection in Point Of Care devices. It is still difficult to produce low-cost, sensitive, and portable optics. The necessary precise alignment between microfluidic and optical detection components makes it hard for mass production.

First of all, the shelf life of fluorescent reagents or functionalized nanoparticles are limited, hence effort has been devoted in the development of label free detection methods. Then it should be considered that the major components of the microfluidic platforms are the materials they are made of and the methods used to control the fluid flow. While microfluidics hold great promises to the applications of POC medical diagnostics, limitations still exist. First, fabrication cost and material compatibility are the two major considerations in material development. Second, most current methods for fluid control require expensive and complicated off-chip components that are not directly amenable to use outside of the laboratory setting.

Therefore, optical techniques perhaps provide better sensitivity than electrochemical ones, but their cost and complexity makes them unattractive to most end users. Electrochemical techniques, on the other hand, are much easier to use but when it comes to detecting pathogens, their performance is still far from adequate. Current optical-based techniques still require a relatively large amount of power, need a bulky chip reader (optics, lasers and cameras), and thus are less applicable to miniaturization. In order to become attractive, biosensors first need to show that they are capable of reaching at least the same detection levels as traditional techniques, next they need to do so in a fraction of the time without overlooking cost.

4

MODELING AND METHODOLOGY:

from the idea to the application

Due to the complexity in the coding, deciphering DNA code is one of the major problems in diagnostics. In addition, due to very small dimensions, manipulation and observation of DNA is another problem in this work. Therefore, design of an accurate sensor for recognition of DNA hybridization is the most challenging part of the DNA diagnostic chip. In fact, most of the proposed methods for recognition of hybridization involve chemical processes, which convert chemical reactions of hybridization to electrical signals. Since DNA itself, as a biological entity, is in a solution inherently chemical, any unpredicted chemical reaction will influence the signal and therefore, reduce the accuracy of the sensor.

The main objective of this thesis is to investigate a stable and simple method to control DNA hybridization and amplification, which must be as precise as possible, in order to give us a good sample in which it could be possible to easily recognize the presence of diseases. Therefore it was required to create an electronic board able to: collect the sample in a provided chamber; control the injection of the different reagents; maintain a stable temperature; mix the reagents with the sample and eventually to pipe the sample to a microarray detector.

The most appropriate, simple and stable way to guide all these procedures is to use a microcontroller. Microcontrollers can be considered downright systems with a complete microprocessor, integrated in a single chip, and they are projected just to obtain the maximum

functional self-sufficiency, and to optimize the price to performance ratio in a particular application.

For this project it was decided to use an open source electronics prototyping platform, namely Arduino.

4.1 The choice of the electronics platform

Arduino is an open source physical computing platform based on a simple input/output board and a development environment that implements the Processing language. It can be used to develop standalone interactive objects or can be connected to software on a computer. Arduino is composed of two major parts: the Arduino board, which is the piece of hardware you work on when you build your objects; and the Arduino IDE (Integrated Development Environment), the piece of software you run on your computer. You use the IDE to create a sketch, a little computer program that you upload to the board. The sketch tells the board what to do.

To realize the project it was decided to use and to purchase the Arduino Mega2560, because, even if the work was still at the very beginning, it is supposed to become more and more complex, so better to start with a flexible environment to save money, and especially time, in the future if it will be required to amplify the system.

The Arduino Mega2560 is based on the ATMega2560, it has 54 digital input/output pins, of which 14 can be used as PWM outputs, 16 analogue inputs, 4 UARTs (hardware serial ports), a 16 MHz crystal oscillator, a USB connection, a power jack, an ICSP header and reset button. It contains everything needed to support the microcontroller; simply it can be connected to a computer with a USB cable or powered with an AC-to-DC adapter or battery to get started.

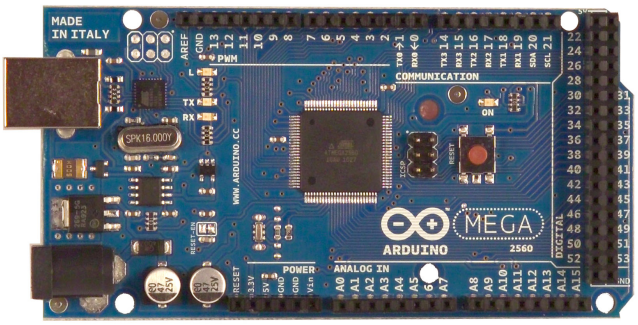


Fig. 4.1: Arduino Mega2560.

To have a better view of the features it is possible to summarize them with this list:

- Microcontroller ATmega2560
- Operating Voltage 5V
- Input Voltage 7-12V (recommended)
- Input Voltage 6-20V (limits)
- Digital I/O Pins 54 (of which 14 provide PWM output)
- Analogue Input Pins 16
- DC Current per I/O Pin 40mA
- Flash Memory 256 KB of which 8 KB used by boot loader
- SRAM 8 KB
- EEPROM 4 KB
- Clock Speed 16 MHz

The Arduino Mega can be programmed with the Arduino software and it comes preburned with a boot loader that allows you to upload new code to it without the use of an external hardware programmer. It communicates using the original STK500 protocol. The firmware source code is available in the Arduino repository and it is loaded with a DFU boot loader, which can be activated by connecting the solder jumper on the back of the board and then resetting.

The software interface is very minimal and easy; it allows you to write sketches for the board in a simple language modelled after the Processing language. The written code is translated into the C language and is passed to the avr-gcc compiler, an important piece of open source software that makes the final translation into the language understood by the microcontroller.

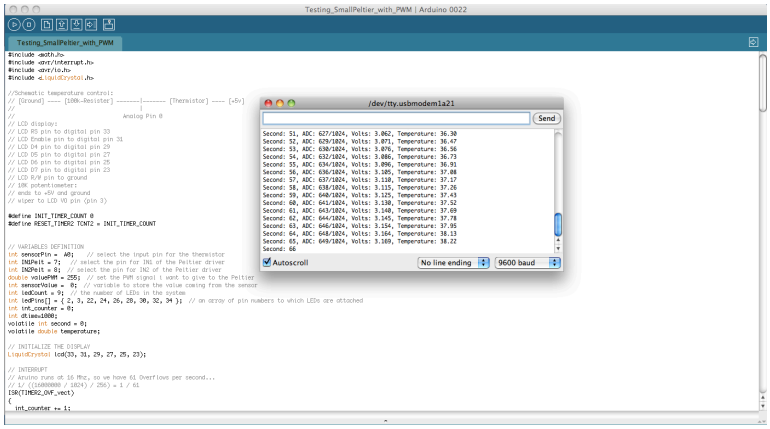


Fig. 4.2: Arduino Software interface.

4.2 The temperature reading

First step was to find out the most appropriate way to read the temperature in the chamber, where the sample is supposed to be collected. After considering using the LM135 sensor or a thermistor, it was decided for the last one because of its more linear characteristics and much better precision.

A thermistor is a type of resistor whose resistance varies significantly with temperature, more so than in standard resistors. It is generally a ceramic or polymer and it can achieve a high precision within a limited temperature range, usually -90°C to 130°C. Unfortunately the relationship between resistance and temperature is not perfectly linear, but what is important is that the characteristic is monotonous and decreasing. So the first thought was that if it could be possible to approximate the function and to work in a very limited range of temperature, we would have been allowed to consider it almost linear. And that is what we got. In fact, the range of temperature, we were working in, varies between 20°C to 40°C.

Before any further experiment it was useful to calibrate the sensor, so, from the graph of the thermistor, taken from the datasheet, it was possible to plot the curve, which expresses the relationship between the temperature and the correspondent value of resistance. The result is shown in fig. 4.4, and the equation of the curve is indicated just next to the graph.

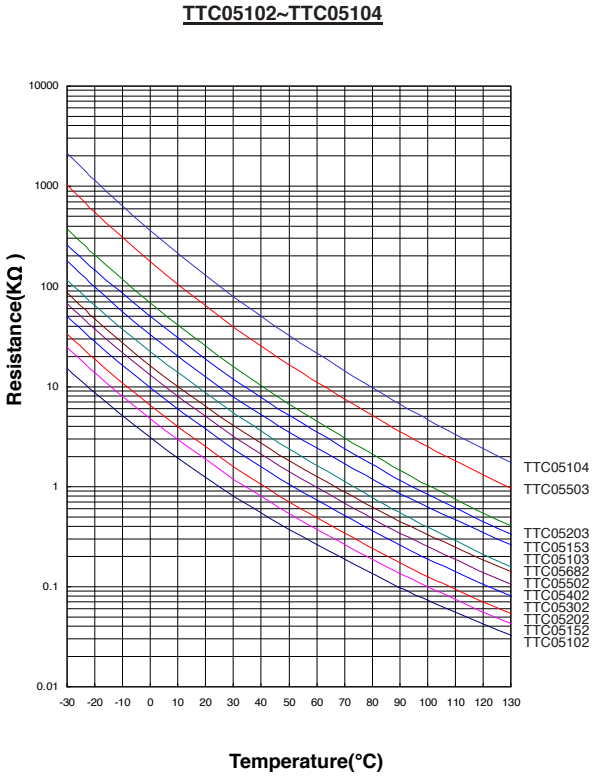


Fig. 4.3: Temperature to resistance chart taken from the datasheet.

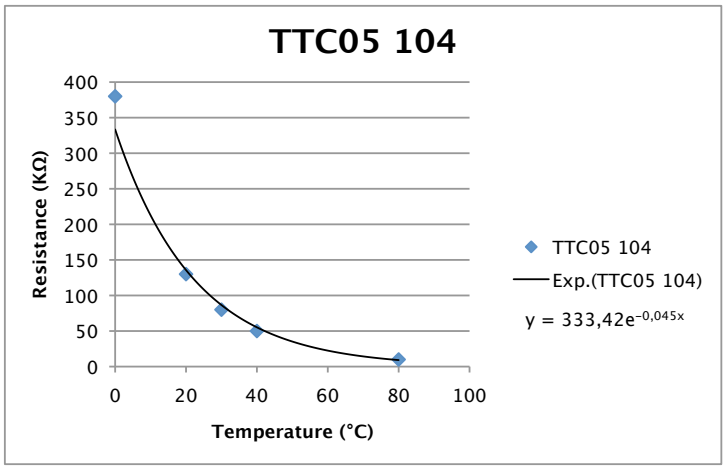


Fig. 4.4: Calibration of the thermistor.

The easiest way to read the value of resistance varying with the temperature is to put the thermistor in series with a standard resistance, in order to create a voltage divider between 0-5V, the Arduino operating voltage range. In this way, we can read the varying value of the node in the middle just monitoring it with the Arduino software serial monitor. This window, shown in fig. 4.5, is simply a tool, available in the open source software, which allows the user to print the values of the analogue input pins, if correctly set.

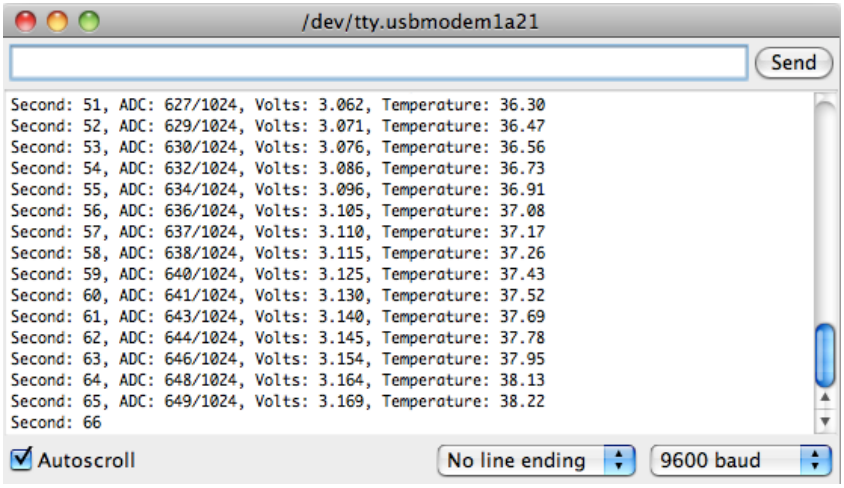


Fig. 4.5: Screenshot from the Arduino Serial Monitor.

The configuration to read the value of temperature is the one shown below in fig. 4.6. As we can see the thermistor is in series with a 100KΩ resistor in a simple voltage divider. The supply is coming from the Arduino, that's why it is 0-5V; and the central node is connected to one of the analogue input pins, so it could be monitored, as just said, using the software.

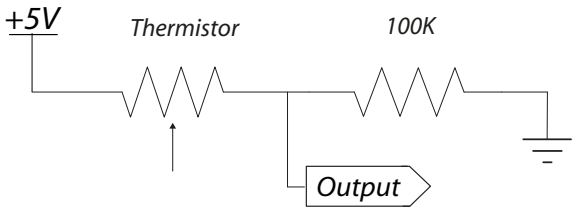


Fig. 4.6: Voltage divider to read the temperature.

Because of the non-linear characteristic of the voltage divider, it was useful to find out the resistance that optimizes the expression. So, plotting different values, function of the output, we observed, as expected, that the maximum of the curve corresponds just to 100KΩ.

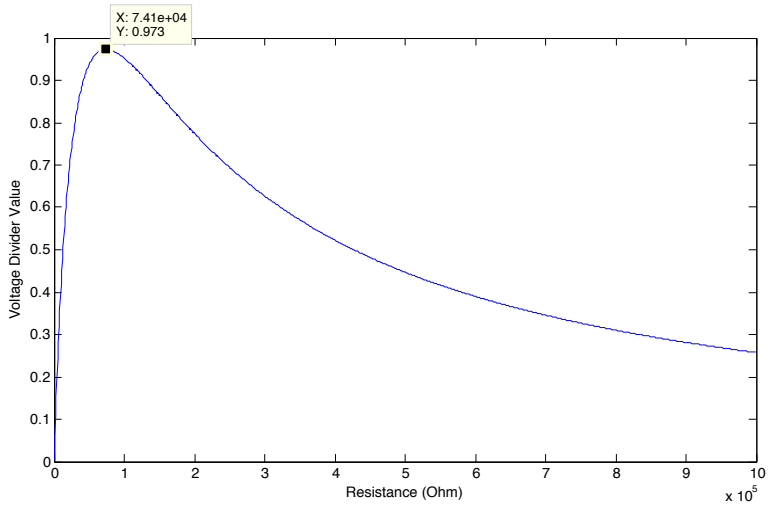


Fig. 4.7: Different values of resistance function of the voltage divider result, from MatLab.

To obtain the equation that describes the relationship between voltage and temperature, the thermistor was brought to different temperatures and the correspondent analogue value was recorded. This is possible because the ATmega controllers contain an on board 6-channel analogue-to-digital (A/D) converter. The converter has 10 bit resolution, returning integers from 0 to 1023. The main function of the analogue pins is to read, like this case, analogue sensors, but they also have all functionality of general-purpose input/output pins. The data recorded from the experiment were plot in the graph below, which shows the voltage divider result function of the temperature. As we can see, in the limited range 10-40°C the relationship is almost linear and described by the equation by its side.

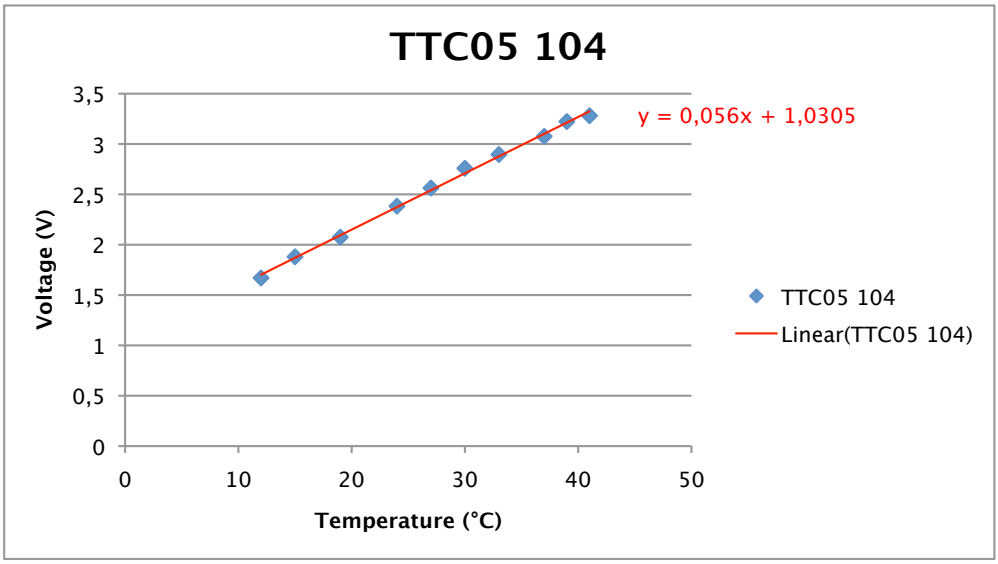


Fig. 4.8: Voltage divider result function of the temperature.

The equation of the straight line, which interpolates the data, was then used to convert the analogue value, coming from the A/D converter, to a proper value of temperature, that really is the one of the chamber we were trying to monitor.

4.3 The cartridge control

It is now possible to monitor the temperature of the chamber. Stepping backwards and focusing on the result we would like to achieve, it is possible to schematize the issue we need, with the picture below in fig. 4.9.

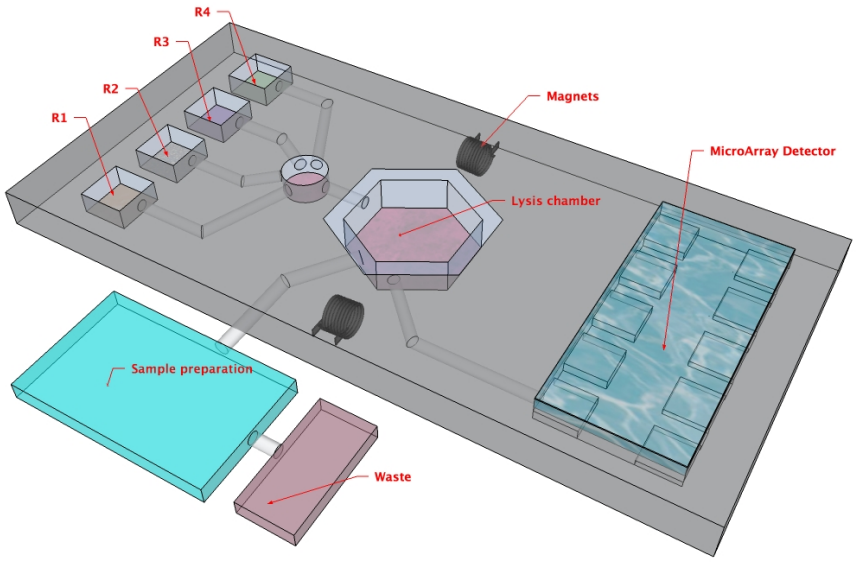


Fig. 4.9: Cartridge prototype.

The design is quite clear and simple. There are different wells, four in this picture, with different reagents useful for the DNA lysis and amplification; the central chamber which contains the DNA sample, with two magnets placed side by side. These perform the mixing of the reagents with the sample and their specific functioning will be explained afterwards. Before going into the central chamber the DNA is extracted from the biological sample (blood, urine or swabs) and well prepared into an external chamber. The waste coming from this operation is washed away and collected into a specific waste well. After all the reactions are well developed and finished, the amplified DNA is pumped into the microarray detector, ready to be analysed.

The code written for the microcontroller is reported below, we can now start commenting the different operations that this controls. From line 1 to 31 all the variables are declared and the display initialized. The main code is very simple; it is just a series of calls to the functions written from line 74 to 134. The program proceeds chronologically, first with the injection of two reagents into the chamber, opening and closing the valves, which block the flux when not needed. At this stage we didn't have the real chip prototype and the valves weren't already chosen, so just to simulate the command, two LEDs are made blinking. After this, the function to set the magnets on and off alternatively is called. This operation is required because the amount of sample we are working with is very small. The system is a microfluidic sensor, and the flow in capillary tubes is laminar, so there is no possibility, for the different reagents, to mix.

To perform a correct mixing some paramagnetic beads are injected, as normal reagents, and moving alternatively from one side to the other of the chamber, according with the magnetic field polarization, they help the reaction to develop. Paramagnetic materials have a small, positive susceptibility to magnetic fields. These materials are slightly attracted by a magnetic field and the material does not retain the magnetic properties when the external field is removed. Paramagnetic properties are due to the presence of some unpaired electrons, and from the realignment of the electron paths caused by the external magnetic field. Paramagnetic materials include magnesium, molybdenum, lithium, and tantalum. In this project the magnets are connected to two different output pins of the Arduino board, which can perform also a PWM signal. This aspect is very important, because the magnets can't work with the low supply coming from the Arduino. They are connected to an amplifier powered with a higher generator, 12 Volts in this case, according to laboratory availability, and a PWM signal swinging from 50% to 0% of duty cycle, powers them with 6 Volts. This way of working allows us to calibrate the supply for the magnets depending on the power consumption requirements, which are not established yet, at this stage. It gives the project a high level of versatility, because the value of duty cycle can be changed just assigning different values from 0 to 255, respectively 0% and 100% of duty cycle, at line 93 of the code. For the experiments here conducted, the magnets were "hand-made", so there isn't a particular model, or datasheet we can refer to; they were just composed of a magnetic core and a coil all around. Problems related to power dissipation and coil overheating aren't considered at this time.

Next step is to wash away the waste coming from the reaction, so the correspondent function is called. It is important to note that the program supposes the action of a pump, which should be responsible of the thrust to help the flow in the capillary tubes. Micro pumps are very expensive

devices, and many evaluations are required to be considered, so at this stage of the project it is impossible to identify the one, which better suits our needs. Only after a consultancy also with the biology team, the pumps will be chosen and purchased; so just for now the command in the code turns on and off a simple LED, which represents this functionality. At the end the sample, once reacted and well prepared, is pumped into the microarray detector in order to be analysed.

In the next paragraph it will be explained the other important function developed by the program, which is the temperature control in the central chamber. What is required to do is to print out on a screen the actual temperature the well is, and to maintain this temperature as constant as possible. The reaction we want to perform hasn't strict conditions about the temperature it should be conducted at, but it should be maintained around 37 degrees. To do so we decided to use a Peltier element that can work both as a heater and a cooler.

As shown in the graph below, the temperature reading and control is assigned to an interrupt routine developed by the microcontroller. So every second it reads the value of temperature, it prints the result on the serial monitor of the Arduino software and it controls if this is higher or lower than a settled point, 37 degrees in our case (lines 43-73).

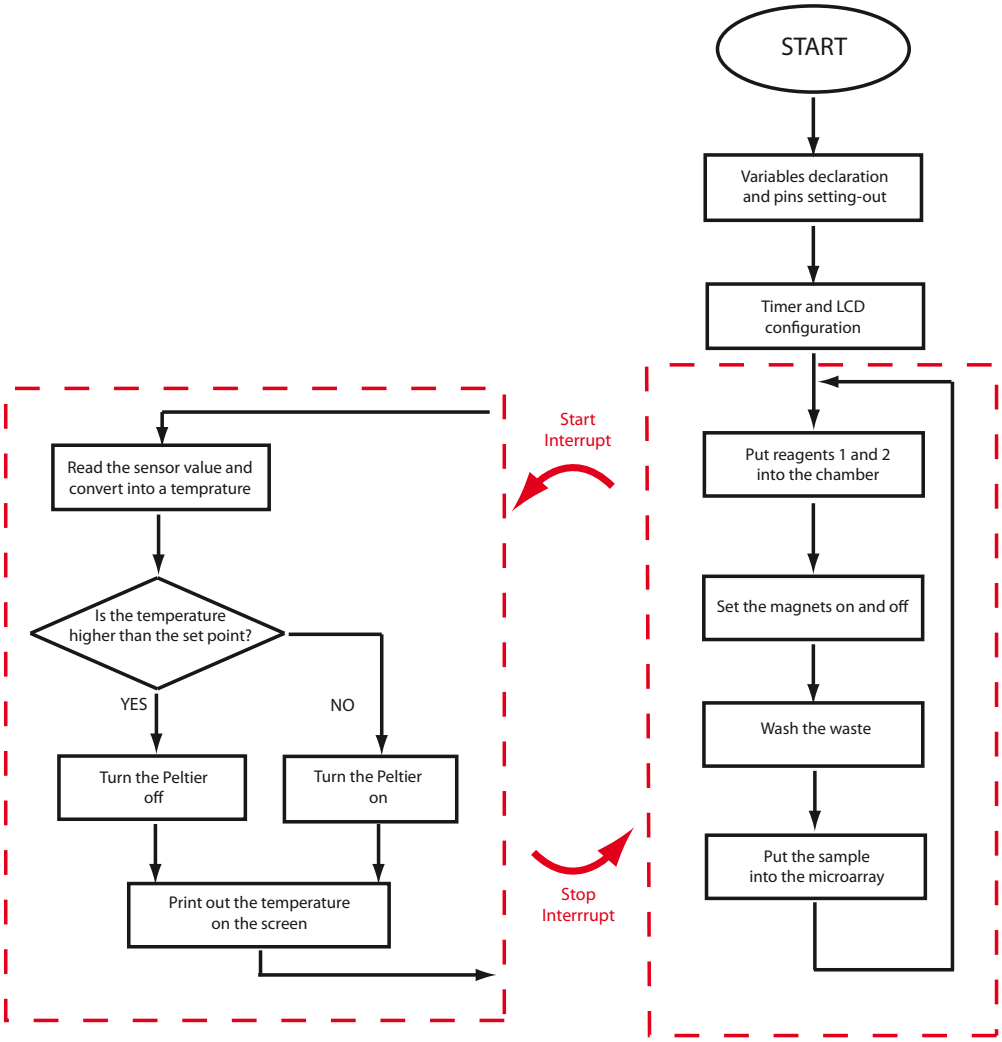


Fig. 4.10: Flowchart of the microcontroller code.

```
1     #include <math.h>
2     #include <avr/interrupt.h>
3     #include <avr/io.h>
4     #include <LiquidCrystal.h>
5     //Schematic temperature control:
6     // [Ground] ---- [100k-Resister] -------|------- [Thermistor] ---- [+5v]
      //                                      |
      //                                 Analog Pin 0
7     // LCD display:
8     // LCD RS pin to digital pin 33
9     // LCD Enable pin to digital pin 31
10    // LCD D4 pin to digital pin 29
11    // LCD D5 pin to digital pin 27
12    // LCD D6 pin to digital pin 25
13    // LCD D7 pin to digital pin 23
14    // LCD R/W pin to ground
15    // 10K potentiometer:
16    // ends to +5V and ground
17    // wiper to LCD VO pin (pin 3)
18    #define INIT_TIMER_COUNT 0
19    #define RESET_TIMER2 TCNT2 = INIT_TIMER_COUNT
20    // VARIABLES DEFINITION
21    int sensorPin = A0;    // select the input pin for the thermistor
22    int ledPinPelt = 4;   // select the pin for the Peltier
23    int sensorValue = 0;   // variable to store the value coming from the
      sensor
24    int ledCount = 9;  // the number of LEDs in the system
25    int ledPins[] = { 3, 4, 22, 24, 26, 28, 30, 32, 34 };  // an array of pin
      numbers to which LEDs are attached
26    int int_counter = 0;
27    int dtime=1000;
28    volatile int second = 0;
29    volatile double temperature;
30    // INITIALIZE THE DISPLAY
```

```
31    LiquidCrystal lcd(33, 31, 29, 27, 25, 23);
32    // INTERRUPT
33    // Aruino runs at 16 Mhz, so we have 61 Overflows per second...
34    // 1/ ((16000000 / 1024) / 256) = 1 / 61
35    ISR(TIMER2_OVF_vect)
      {
36      int_counter += 1;
37      if (int_counter == 61)
       {
38        temperature = check_temperature();
39        int_counter = 0;
40        second++;
41        Serial.print("Second: ");
42        Serial.print(second);
        }
      }
43    // CONVERT FROM ANALOG VALUE TO TEMPERATURE
44    double to_celsius(int RawADC)
45    {
46      double t = (RawADC - 211.05)/11.46;
47      //Print out RAW ADC Number
48      Serial.print(", ADC: ");
49      Serial.print(RawADC);
50      Serial.print("/1024");
51      // Print out Volt value
52      Serial.print(", Volts: ");
53      Serial.print(((RawADC*5)/1024.0),3);
54      // Return the Temperature
55      return t;
      }
56    void show_temperature(double temperature )
      {
57        Serial.print(", Temperature: ");
```

```
58          Serial.print(temperature);
59          Serial.print("\n");
        }
60      // TEMPERATURE CONTROL
61      double check_temperature()
        {
62      double t;
63      double analog_input;
64      analog_input = analogRead(sensorPin);
65      t = to_celsius(analog_input);
66      if(t < 37)
67      {
68        // turn the ledPinPelt on
69        digitalWrite(ledPinPelt, HIGH);
         }
70       else
    {
71        // turn the ledPinPelt off
72        digitalWrite(ledPinPelt, LOW);
    }
73    show_temperature(t);
74     return t;
       }
/***************** MAIN LOOP FUNCTIONS ******************/
75     void reagents12tochamber()
       {
76     int ncount = 2;
77      int n[] = {28, 30};
78       for (int i=0; i < ncount; i++)
         {
79         lcdPrintStringInt("Reagent", i+1);
80         delay(dtime);
81         digitalWrite (n[i], HIGH); //set the LED on
```

```
82          delay (dtime);
83          digitalWrite (22, HIGH); //set the pump on
84          delay (dtime);
85          digitalWrite (22, LOW); //set the pump off
86          delay (dtime);
87          digitalWrite (n[i], LOW); //set the LED off
88          delay (dtime);
        }
      }
89    void setMagnetsOnOff()
      {
90    lcdPrintString("Mixing");
91       for (int j=0; j<5; j++)
        {
92          for (int i=0; i<2; i++)
          {
93            analogWrite (i+2, 127); //set the magnet on
94            delay (2000);
95            digitalWrite (i+2, 0); //set the magnet off
           }
         }
      }
96    void washTheWaste()
      {
97      analogWrite (2, 127); //set the magnet 1 on
98      delay (dtime);
99      lcdPrintStringAt(0, 0, "Wasting cell");
100     lcdPrintStringAt(0, 1, "open");
101     digitalWrite (24, HIGH); //wasting cell open
102     delay(dtime);
103     lcdPrintString("Reagent 3");
104     digitalWrite (32, HIGH); //reagent 3 in
105     delay(dtime);
```

```
106     digitalWrite (22, HIGH); //set the pump on
107     delay(dtime);
108     digitalWrite (22, LOW); //set the pump off
109     delay(dtime);
110     digitalWrite (32, LOW); //reagent 3 stop
111     delay(dtime);
112     lcdPrintStringAt(0, 0, "Wasting cell");
113     lcdPrintStringAt(0, 1, "closed");
114     digitalWrite (24, LOW); //wasting cell close
115     delay(dtime);
116     digitalWrite (2, 0); //set the magnet 1 off
117     delay(dtime);
      }
118   void sampleIntoTheDetector()
      {
119     lcdPrintString("Reagent 4");
120     digitalWrite (34, HIGH); //reagent 4 in
121     delay(dtime);
122     digitalWrite (22, HIGH); //set the pump on
123     delay(dtime);
124     lcdPrintStringAt(0, 0, "Detector chamber");
125     lcdPrintStringAt(0, 1, "open");
126     digitalWrite (26, HIGH); //detector chamber open
127     delay(dtime);
128     digitalWrite (22, LOW); //set the pump off
129     delay(dtime);
130     digitalWrite (34, LOW); //reagent 4 stop
131     delay(dtime);
132     lcdPrintStringAt(0, 0, "Detector chamber");
133     lcdPrintStringAt(0, 1, "closed");
134     digitalWrite (26, LOW); //detector chamber closed
      }
```

```
135   /***************** LCD PRINTING FUNCTIONS ******************/
136   void lcdPrintString( char *string){
137     lcd.clear();
138     lcd.print(string);
      }
139   void lcdPrintStringInt(char *string, int value){
140     lcd.clear();
141     lcd.print(string);
142     lcd.print(" ");
143     lcd.print(value);
      }
144   void lcdPrintStringAt(int row, int col, char *string){
145     lcd.setCursor(row,col);
146     lcd.print(string);
      }
147   void lcdPrintIntAt(int row, int col, int value){
148     lcd.setCursor(row,col);
149     lcd.print(value);
      }
150   void setup() {
151     // set up the LCD's number of columns and rows:
152     lcd.begin(16, 2);
153  // loop over the pin array and set them all to output:
154     for (int thisLed = 0; thisLed < ledCount; thisLed++)
         {
155        pinMode(ledPins[thisLed], OUTPUT);
         }
156     // declare the ledPin as an OUTPUT:
157     pinMode(ledPinPelt, OUTPUT);
158     // initialize serial communications at 9600 bps:
159     Serial.begin(9600);
160     //Timer2 Settings:  Timer Prescaler /1024
161     TCCR2B |= ((1 << CS22) | (1 << CS21) | (1 << CS20));
```

```
162     //Timer2 Overflow Interrupt Enable
163     TIMSK2 |= (1 << TOIE2);
164     //Timer2 set as normal mode
165     TCCR2A |= (0 << WGM22) | (0 << WGM21) | (0 << WGM20);
166     //Timer2 Overflow Interrupt Enabled. The corresponding interrupt is
      executed if an overflow in Timer0 occurs.
167     RESET_TIMER2;
168     sei(); //Enables interrupts by clearing the global interrupt mask
      }
169   void loop() {
170     // Print a message to the LCD
171     lcdPrintString("Starting");
172     delay(dtime*2);
173     // Put reagents 1 and 2 in the chamber:
174     reagents12tochamber();
175     // Set magnets on and off:
176     setMagnetsOnOff();
177     // Wash the waste:
178     washTheWaste();
179     // Put reagent 4 into the chamber and fill the detector:
180     sampleIntoTheDetector();
      }
```

4.4 The temperature control

As just introduced, parallel to the series of functionalities of the main program, we need to check the temperature the chamber is. So one of the three timers available on the microcontroller is set to make its interrupt function working. From line 150 to 168, before the main code, there is a setup section, where the LCD display is initialized and the different pins, it is connected to, are declared to be outputs; then the interrupt function of the Timer2 is enabled.

The Timer/Counter2 is an 8-bit register; it can be clocked internally, via the prescaler, or asynchronously clocked form the TOSC1/2 pins. The Asynchronous Status Register controls the asynchronous operation. The Clock Select logic block controls which clock source the Timer/Counter uses to increment its value, but by default it is equal to the microcontroller clock. The Timer is inactive when no clock source is selected.

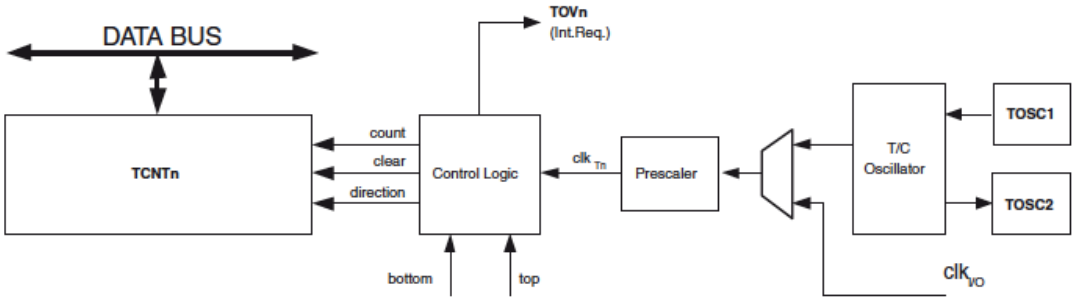
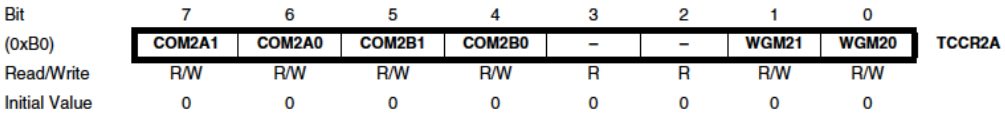


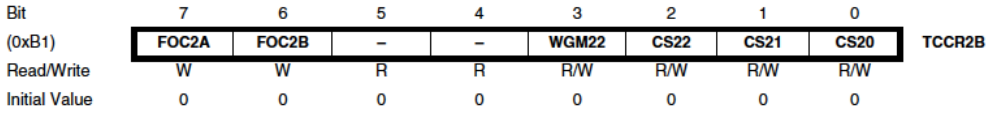
Fig. 4.11: Counter Unit Block Diagram.

Depending on the mode of operation used, the counter is cleared, incremented, or decremented at each timer clock (clk_{T2}). clk_{T2} can be generated from an external or internal clock source, selected by the Clock Select bits (CS22:0). When no clock source is selected (CS22:0 = 0) the timer is stopped. The counting sequence is determined by the setting of the WGM21 and WGM20 bits located in the Timer/ Counter Control Register A (TCCR2A) and the WGM22 located in the Timer/Counter Control Register B (TCCR2B).



Bit	7	6	5	4	3	2	1	0	
(0xB0)	COM2A1	COM2A0	COM2B1	COM2B0	–	–	WGM21	WGM20	TCCR2A
Read/Write	R/W	R/W	R/W	R/W	R	R	R/W	R/W	
Initial Value	0	0	0	0	0	0	0	0	

Fig. 4.12: Timer/Counter Control Register A.



Bit	7	6	5	4	3	2	1	0	
(0xB1)	FOC2A	FOC2B	–	–	WGM22	CS22	CS21	CS20	TCCR2B
Read/Write	W	W	R	R	R/W	R/W	R/W	R/W	
Initial Value	0	0	0	0	0	0	0	0	

Fig. 4 .13: Timer/Counter Control Register B.

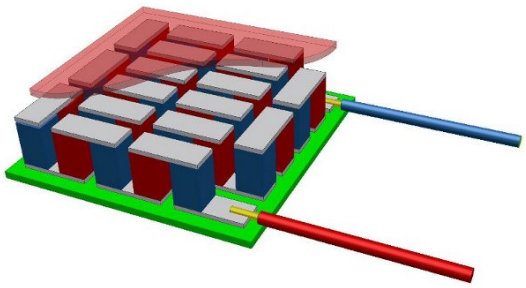
The Overflow Flag (TOV2) is set according to the mode of operation selected by the WGM22:0 bits. The simplest mode of operation is just the Normal mode (WGM22:0 = 0). In this mode, which is also the one chosen for this project (line 165), the counting direction is always up, and no counter is performed. The counter simply overruns when it passes its maximum 8-bit value and then restart from the bottom. In normal operation the Timer/Counter Overflow Flag (TOV2) will be set in the same timer clock cycle, as the TCNT2 becomes zero. The TOV2 Flag in this case behaves like a ninth bit, except that it is only set, not cleared. However, combined with the timer overflow interrupt that automatically clears the TOV2 Flag, the timer resolution can be increased by software.

After these informations about the code, we better focus on the system. As just said, to maintain a constant temperature it has been decided to use a Peltier element working as a heater or a cooler, depending on the requirements.

Peltier elements are devices using the thermoelectric effect, which is the direct conversion of temperature differences to electric voltage and vice-versa. A thermoelectric device creates a voltage when there is a different temperature on each side. Conversely, when a voltage is applied to it, it creates a temperature difference. At atomic scale, an applied temperature gradient causes charged carriers in the material to diffuse from the hot side to the cold side; hence inducing a thermal current. This effect can be used to generate electricity, measure temperature or change the temperature objects. Because the direction of heating and cooling is determined by the polarity of

the applied voltage, thermoelectric devices are efficient temperature controllers. The term "thermoelectric effect" encompasses three separately identified effects: the Seebeck effect, Peltier effect and Thomson effect. These effects are thermodynamically reversible, whereas Joule heating is not.

Peltier effect, which is the one we are interested in, is the presence of heat at an electrified junction of two different metals. When a current is made to flow through a junction made of material A and B, heat is generated at the upper junction at T_2, and absorbed at the lower junction at T_1.



A Peltier thermo-element is a device that utilizes the Peltier effect to implement a heat pump. It has two plates, the cold and the hot one. Between those plates there are several thermo couples. All those thermo couples are connected together and two wires come out.

Fig. 4.14: Schematic and simplified view of a Peltier element.

The device is called a heat pump because it does not generate heat or cold, it just transfers heat from one plate to another, and thus the other plate is cooled. Because they have several thermo couples, as shown in the picture, a lot of heat is transferred between the plates and sometimes it can reach a temperature difference of 80 degrees.

Peltier thermo elements are mainly made of semiconductive material. This means that they have P-N contact within. Actually, they have a lot of P-N contacts connected in series. They are also heavily doped, meaning that they have special additives that will increase the excess or lack of electrons. The following drawing shows how the P-N contacts are connected internally within a Peltier TEC.

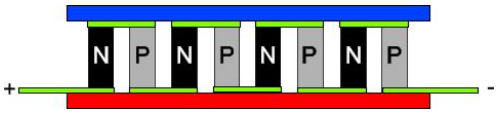


Fig. 4.15: P-N contacts within a Peltier TEC.

For this project the miniature thermoelectric module ET-011-05-15-RS has been chosen. This because it has very small dimensions, comparable with the one of our chip prototype and low current requests. Values about maximum current and power, and dimensions as well, are reported below in the table.

Thot=27 °C (300 °K)					Dimensions, mm			
Imax, A	Umax, V	Qmax, W	dTmax, K	Rac, Ohm	A	B	H	d
0.8	1.4	0.6	74	1.5	6	4	3	n/a
Note 1	Note 2	Note 3	Note 4	Note 5				

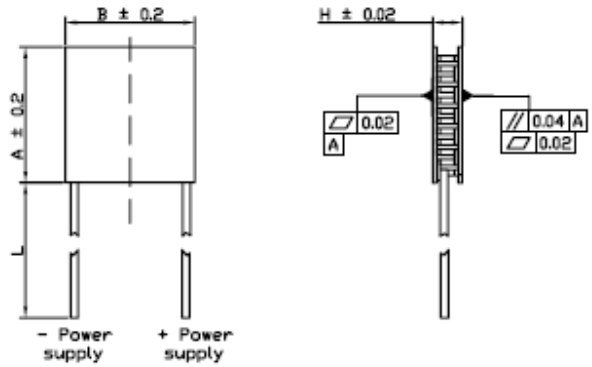


Fig. 4.16: Current, power limits and dimensions of the ET-011-05-15-RS (Peltier element).

Peltier elements can give more than to 80 degrees difference between their plates. But this is not a standard value. Actually, this would be achieved in ideal conditions. The actual difference is usually smaller. The specifications of a TEC usually show the achieved temperature difference in conjunction to the transferred power in Watts. Peltiers come usually with the datasheet that indicates the performance curves of the device operation. The first characteristic curve for a Peltier is the temperature difference vs heat pump capacity. This curve indicates the temperature difference to be achieved in order to pump specific power of heat. It may be one or more curves for different current loads. The second curve is the temperature difference vs voltage. With this, we can calculate the voltage needed to be applied on the TEC in order to achieve the appropriate temperature difference. Our case is the one shown in the following diagrams.

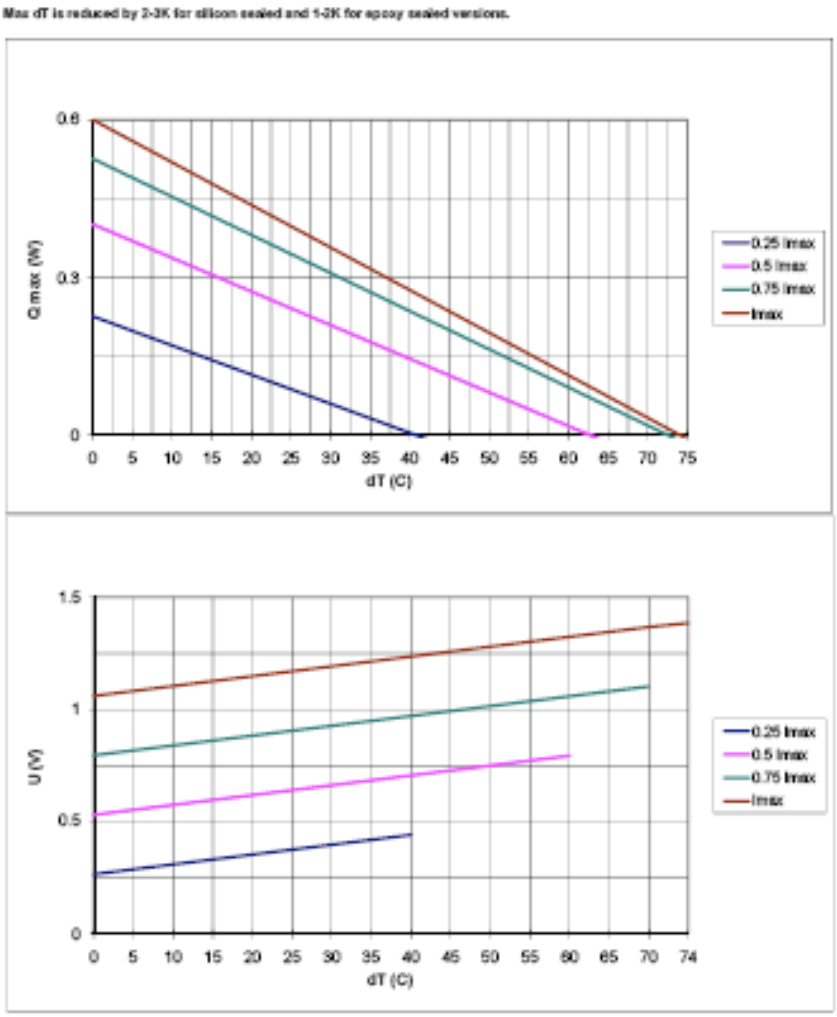


Fig. 4.17: Peltier element's characteristic curves.

The result and the prototype used for the following tests is the one shown in the picture. We can distinguish: the block of polymeric material representing the chip itself; the thermistor glued inside to measure the temperature; the thermoelectric element underneath the plastic piece; and a heat sink to maintain as constant as possible, at room temperature, one of the plates of the Peltier. Just to have an idea of the temperature difference varying during the process, two thermo couples are bonded above and below the heat pump, this way it is easier to monitor the course of the temperature value during the reaction's time.

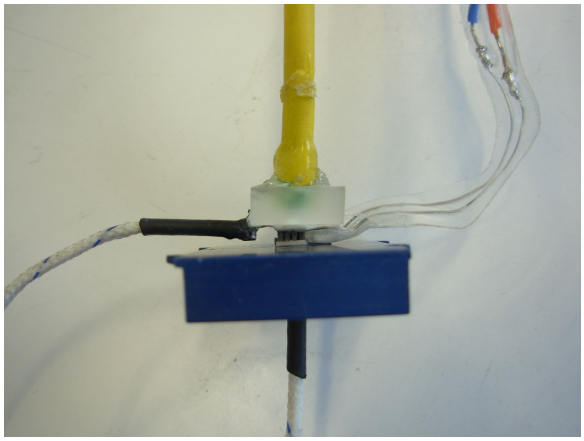


Fig. 4.18: Chip prototype.

The only thing to consider it was the best way to connect the heat pump, this because we chose a device, which requires low current to work, which is small, but it needs a very low voltage. The Arduino can only give 5 or 3.3 Volts, depending on the pin we use; this element, as it is possible to check also in the table above, can be powered with at maximum 1.4 Volts. So a configuration to lower the voltage coming from the Arduino needed to be investigate.

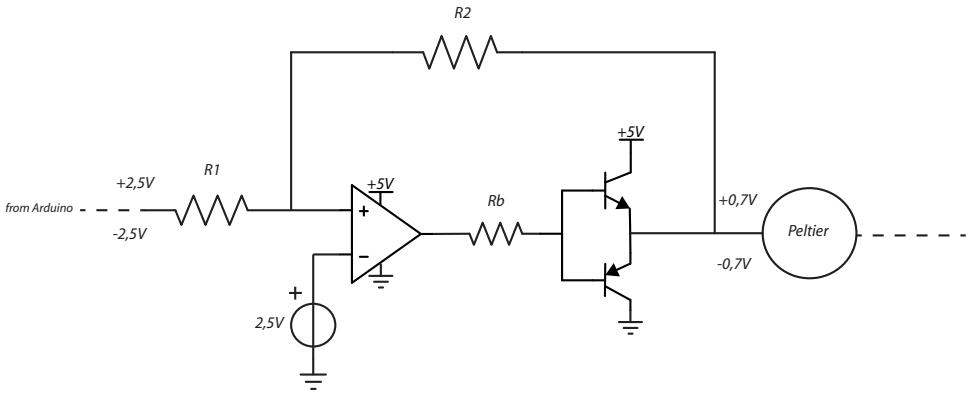


Fig. 4.19: Configuration chosen to power the Peltier element.

The best solution is the one shown in the picture above. The network is connected to an output pin on the Arduino board, able to perform a PWM signal, and then this is involved in a feedback reaction created with the operational amplifier and two resistors R1 and R2. The value of these resistances has been chosen in order to lower the signal coming from the Arduino. The board provides a voltage varying between 0-5 Volts, so setting an offset at 2.5 Volts, it is possible to work around this setpoint and swing up and down depending on the functioning of the thermoelectric component. In conclusion if we use R1=20KΩ and R2=5.6KΩ we can provide the Peltier with ± 0.7 Volts; the structure is symmetric, so the other side is totally the same and it provides ± 0.7 Volts as well. In this way we created a sort of H-Bridge to make the Peltier functioning as a heater or a cooler depending on the direction of the provided voltage across it. The resistor Rb (10Ω) and the push-pull, with the two BJTs working in their linear region, after the amplifier, just create a current buffer to transfer the signal in a correct way.

The amplifier and the transistors to build the circuit have been chosen depending on power and current limits of the Peltier element. In fact the device, ideally, can be represented as a normal resistor of 1.5Ω, and under normal conditions it works with 1.4 Volts, maximum, across its terminals, so a current of 0.93A flows through it. Even considering the worst case, which is the one when the Peltier works with the maximum ideal current, the transistors and the amplifier continue to work within their limits of maximum temperature and power dissipation.

After considering the system configuration, it is now possible to better understand the temperature control part in the Arduino code. In fact the program reads the temperature from the sensor (line 64), if this temperature is higher than 37 degrees it turns the Peltier on to heat the chamber (lines 66-69), otherwise it switches it off, to allow the chamber decrease its temperature, very easily, just with air convection (lines 70-72).

This is a very limited way to control the heat pump; a better performed way to do it is to use an hysteresis control. This because with just one threshold the system is subject to a lot of errors coming from a certain uncertainty of the device around it. It can wobble a lot, putting the Peltier through a damaging functioning. So, as reported below two different thresholds are set, 36 and 38 degrees, and the device is turned on and off alternatively.

```
 // TEMPERATURE CONTROL
double check_temperature()
{
    double t;
    double analog_input;
    analog_input = analogRead(sensorPin);
    t = to_celsius(analog_input);
    if(t < 36)
    {
      // turn the ledPinPelt on
      digitalWrite(ledPinPelt, HIGH);
    }
     if(t > 38)
    {
      // turn the ledPinPelt off
      digitalWrite(ledPinPelt, LOW);
    }
    show_temperature(t);
  return t;
}
```

Hysteresis control is a far better way to regulate the temperature, and a so limited width of the hysteresis range (2 degrees) is enough because the system is very slow to react, but it isn't the best optimized. This kind of control can cause too high ripples on the temperature development diagrams, and this can damage the correct functioning of all the system. So, as better explained in the next paragraph, it was better to opt for a P.I.D. control.

4.5 P.I.D. control

Proportional-Integral-Derivative (PID) control is the most common control algorithm used in industry and has been universally accepted in industrial control. The popularity of PID controllers can be attributed partly to their robust performance in a wide range of operating conditions and partly to their functional simplicity, which allows engineers to operate them in a simple, straightforward manner. As the name suggests, PID algorithm consists of three basic coefficients; proportional, integral and derivative, which are varied to get optimal response.

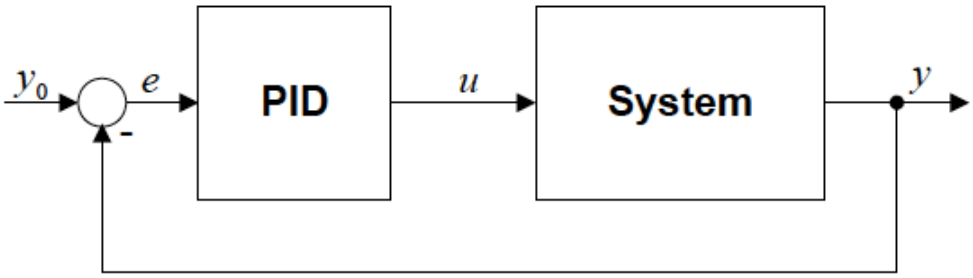


Fig. 4.20: Closed Loop System with PID controller.

In the figure a schematic system with a PID controller is shown. The PID controller compares the measured process value y with a reference setpoint value, y_0. The difference or error, e, is then processed to calculate a new process input, u. This input will try to adjust the measured process value back to the desired setpoint. The alternative to a closed loop control scheme such as the PID controller is an open loop controller. Open loop control (no feedback) is in many cases not satisfactory, and is often impossible due to the system properties. By adding feedback from the system output, performance can be improved. Unlike simple control algorithms, the *PID* controller is capable of manipulating the process inputs based on the history and rate of change of the signal. This gives a more accurate and stable control method.

The basic idea is that the controller reads the system state by a sensor. Then it subtracts the measurement from a desired reference to generate the error value. The error will be managed in three ways, to handle the present, through the proportional term, recover from the past, using the integral term, and to anticipate the future, through the derivate term.

The control design process begins by defining the performance requirements. Control system performance is often measured by applying a step function as the set point command variable, and then measuring the response of the process variable.

Proportional Response

The proportional component depends only on the difference between the set point and the process variable. This difference is referred to as the Error term. The proportional gain (K_c) determines the ratio output response to the error signal. In general, increasing the proportional gain will increase the speed of the control system response. However, if the proportional gain is too large, the process variable will begin to oscillate. If K_c is increased further, the oscillations will become larger and the system will become unstable and may even oscillate out of control.

Integral Response

The integral component sums the error term over time. The result is that even a small error term will cause the integral component to increase slowly. The integral response will continually increase over time unless the error is zero, so the effect is to drive the Steady-State error to zero. Steady-State error is the final difference between the process variable and set point. A phenomenon called integral windup results when integral action saturates a controller without the controller driving the error signal toward zero.

Derivative Response

The derivative component causes the output to decrease if the process variable is increasing rapidly. The derivative response is proportional to the rate of change of the process variable. Increasing the derivative time (T_d) parameter will cause the control system to react more strongly to changes in the error term and will increase the speed of the overall control system response. Most practical control systems use very small derivative time (T_d), because the Derivative Response is highly sensitive to noise in the process variable signal. If the sensor feedback signal is noisy or if the control loop rate is too slow, the derivative response can make the control system unstable.

Using all the terms together, as a PID controller usually gives the best performance.

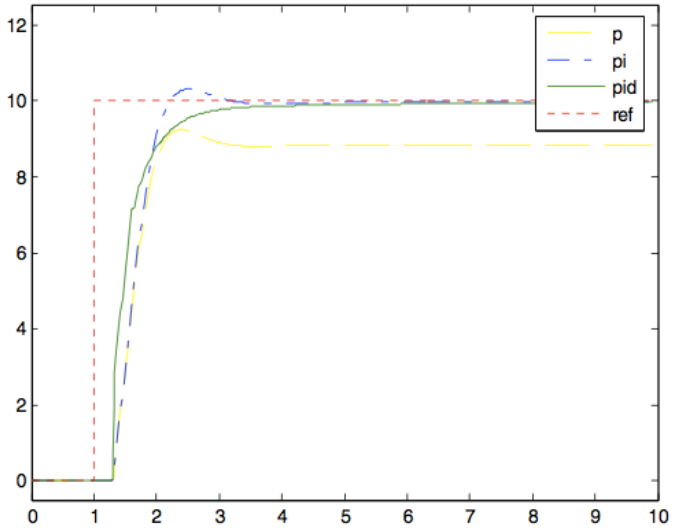


Fig. 4.21: Step response, P, PI and PID controller.

Tuning

The best way to find the needed PID parameters is from a mathematical model of the system, parameters can then be calculated to get the desired response. Often a detailed mathematical description of the system is unavailable, experimental tuning of the PID parameters has to be performed. Finding the terms for the PID controller can be a challenging task. Good knowledge about the systems properties and the way the different terms work is essential. The optimum behavior on a process change or setpoint change depends on the application at hand. Some processes must not allow overshoot of the process variable from the setpoint. Other processes must minimize the energy consumption in reaching the setpoint. Generally, stability is the strongest requirement. The process must not oscillate for any combinations or setpoints. Furthermore, the stabilizing effect must appear within certain time limits.

Several methods for tuning the *PID* loop exist. The choice of method will depend largely on whether the process can be taken off-line for tuning or not. Ziegler-Nichols method is a well-known online tuning strategy. The first step in this method is setting the I and D gains to zero, increasing the P gain until a sustained and stable oscillation, as close as possible, is obtained on the output. Then the critical gain K_c and the oscillation period P_c is recorded and the P, I and D values adjusted accordingly using the table reported below.

Controller	K_p	T_i	T_d
P	$0.5 * K_c$		
PD	$0.65 * K_c$		$0.12 * P_c$
PI	$0.45 * K_c$	$0.85 * P_c$	
PID	$0.65 * K_c$	$0.5 * P_c$	$0.12 * P_c$

Fig. 4.22: Ziegler-Nichols parameters.

In some cases, the response of the system to a given control output may change over time or in relation to some variable. A nonlinear system is a system in which the control parameters that produce a desired response at one operating point might not produce a satisfactory response at another operating point. The measure of how well the control system will tolerate disturbances and nonlinearities is referred to as the robustness of the control system.

The project idea

After this brief overview about PID control, we should go back to our system and analyse our specific situation to understand which is the best solution to be as precise as possible. The reason why nonlinear systems can present problems and errors is clear, and unfortunately this is our case. In fact the device is supposed to work with biological samples, which are never the same; the ambient conditions are difficult to be always repeatable; and after all we are trying to interface a biological system with an electronic one, so nothing comparable. These issues can be overcome schematizing the concrete part, sample, thermistor and Peltier altogether, with a single pole system and then operating on it with a feedback control.

In the picture below this concept is represented; then we just had to find out the characteristics of this system that are time constant and gain.

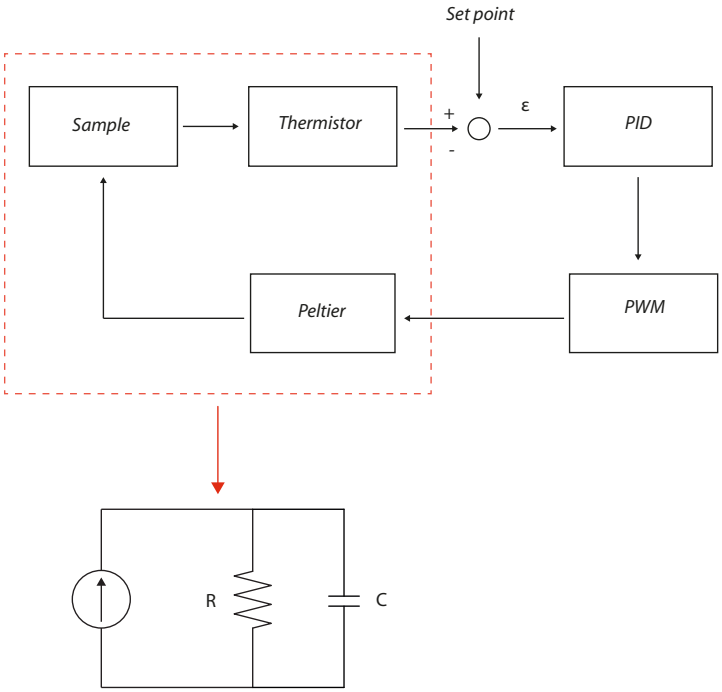


Fig. 4.23: System concept.

First step is to calculate the time constant of this simplified network. To do this, the Peltier has been kept for a long time switched on in order to bring the plastic block to a very high temperature, surely higher than the room one, 43 degrees to be more precise. Then we left the system evolve naturally to see how long it would have been taken to cool down till a stable temperature. The second part of the graph, in this way obtained, is the one we were interested in, and it is the one we used to calculate the time constant of the system. The picture here reported, shows the result and the temperature development. The time constant resulted being 116.432 seconds, so a very long time response system.

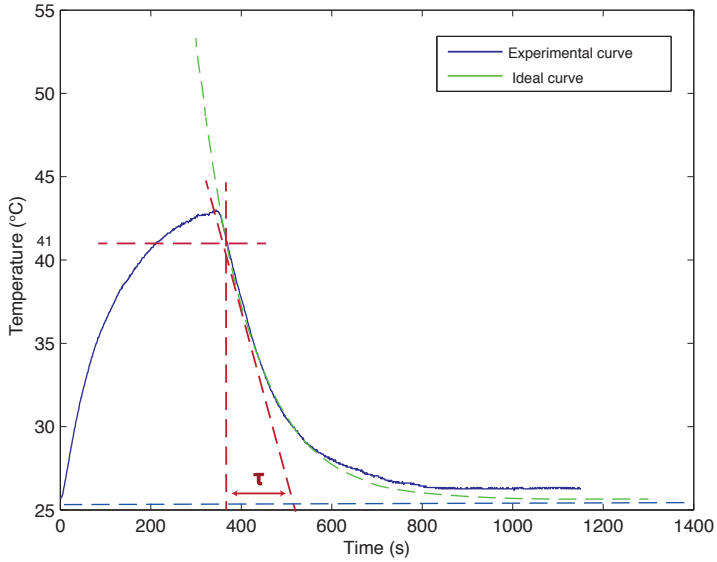


Fig. 4.24: Time constant evaluation.

As already described, the entire configuration to drive the Peltier element is attached to two output pins of the Arduino board, which can perform also PWM signals. To be more precise, the two terminals of the thermoelectric device are powered with complementary signals, in fact the driver realizes a sort of H-bridge, which makes the load (the Peltier) working with the difference of signal between the two terminals. For example, we need to set 40% of duty cycle on one side and 60% on the other one to power the Peltier with a PWM signal with 20% of duty cycle. This aspect is very important for PID control; in fact, just because it is a closed loop control, it can adjust the value of duty cycle depending on the difference between the actual temperature of the chamber and the setpoint. For instance, at the beginning we expect the thermistor to detect a temperature quite close to the room one, so the difference between this reading and the setpoint, imagined to be 37 degrees, is very ample and the program will set a very high value of duty cycle to provide the Peltier a lot of power and accelerate the process of warming up. Then, when the difference is getting smaller we want the heater increasing the temperature much slower, to reverse the polarity if necessary, in order to approach the setpoint very slowly without too high overshoots and errors.

To find the second characteristic of the single pole circuit, we used just this way of functioning. In fact to find the gain of the new network, we imposed different values of duty cycle and then we read the final temperature the thermistor reached in order to plot a curve, possibly linear, whose equation could give us the gain expression.

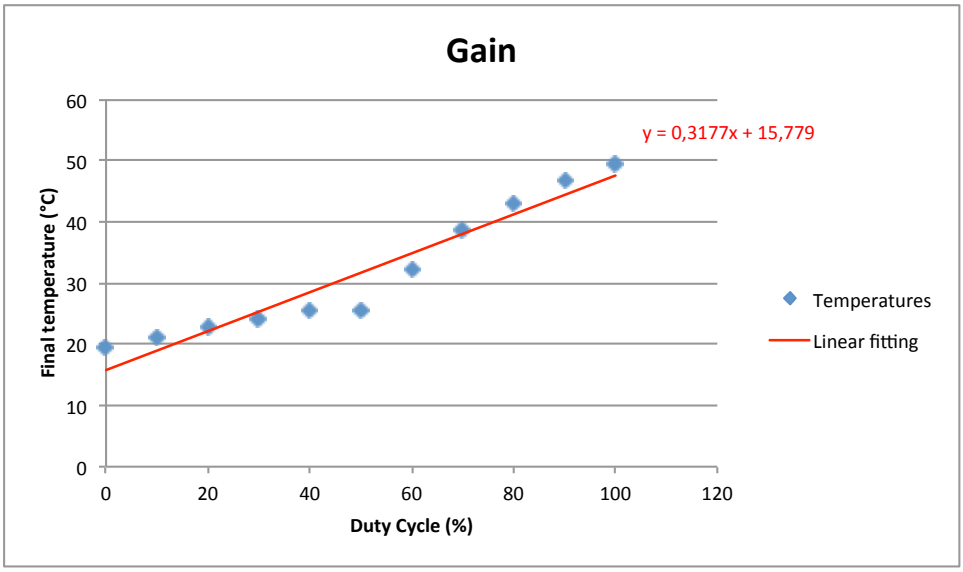


Fig. 4.25: Gain curve.

The result is reported in the picture above and we can read also the curve expression, which better fits the data. We expected a linear curve, and it can actually be considered quite close, but the central part of the graph. This defect is not really worrying, because we understand that around 50%, the Peltier is like it is working with no signal across its terminals, the two signals are almost the same and their difference is close to zero, so a slight gap from the expected behaviour is quite reasonable, because of noises and condition imperfections. This is the reason why a more limited graph is reported below.

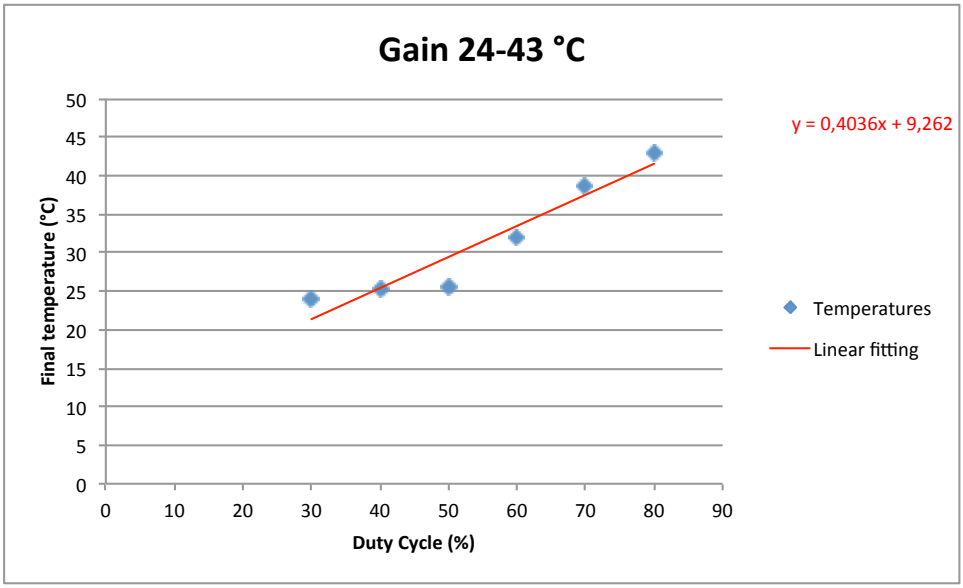


Fig. 4.26: Gain curve in a limited range of temperatures.

We can see that the fitting curve is much more linear and the gain increases a little bit; the defect around 50% is still present, but it is well limited around that position and we can consider it does not really comprise the correct functioning of the system.

The code

After all these considerations we are now able to understand what we need. Surely we should use proportional control by applying pulse width modulation to the heater. The value of K_p will need to be investigated; too low a value and the system will have a poor response; too high a value and it will overshoot and oscillate too much. The optimal K_p will allow a small overshoot. Using proportional control does cause the problem of having a dead-band, which is a delay between when a process variable changes, and when that change can be observed. In this case some integral component will be needed to eliminate it. Again, the value of K_i would need to be carefully considered and will be much lower than K_p. The integral control is needed only if the dead-band is wider than the allowable temperature tolerance. Because of the very long response time of the system, there is no need for any derivative input; in fact it would be a waste of time.

The code written for the Arduino is reported below and the equations, which calculate the correct value of duty cycle needed to be imposed to the signal, are highlighted. In the first part just the proportional control is developed; in the second one also integral control is present; in the next chapter the difference in the response curve between these two situations will be presented; just for now the attention is limited on the functioning.

The program calculates the difference between the actual temperature and the setpoint, then it finds out the ideal value of duty cycle we should impose, depending on the different coefficients we estimated with the Ziegler-Nichols method (lines 8 and 52). If the difference is very high it sets the maximum value of duty cycle, the opposite if the difference is almost zero, otherwise it sets the correct value evaluated with this method.

```
1     // TEMPERATURE P-CONTROL
2     double check_temperature()
      {
3         double t;
4         double analog_input;
5         analog_input = analogRead(sensorPin);
6         t = to_celsius(analog_input);
7         show_temperature(t);
8         idealNEWPWM = ((setPoint-t)*100)+127
9         if (idealNEWPWM > 255)
```

```
        {
10        afterP = 255;
11      // use this new value of PWM and print it
12        analogWrite(IN1Pelt, afterP);
13        analogWrite(IN2Pelt, 255-afterP);
14        Serial.print(", idealNEWPWM: ");
15        Serial.print(idealNEWPWM);
16        Serial.print(", afterP: ");
17        Serial.print(afterP);
18        Serial.print("\n");
        }
19      else if (idealNEWPWM < 0)
        {
20        afterP = 0;
21      // use this new value of PWM and print it
22        analogWrite(IN1Pelt, afterP);
23        analogWrite(IN2Pelt, 255-afterP);
24        Serial.print(", idealNEWPWM: ");
25        Serial.print(idealNEWPWM);
26        Serial.print(", afterP: ");
27        Serial.print(afterP);
28        Serial.print("\n");
          }
29      else
        {
30        afterP = idealNEWPWM;
31        analogWrite(IN1Pelt, afterP);
32        analogWrite(IN2Pelt, 255-afterP);
33        Serial.print(", idealNEWPWM: ");
34        Serial.print(idealNEWPWM);
35        Serial.print(", afterP: ");
36        Serial.print(afterP);
37        Serial.print("\n");
```

```
        }
38      return t;
    }


39  // TEMPERATURE PI-CONTROL
40  double check_temperature()
    {
41      double t;
42      double analog_input;
43      double P_error;
44      double old_error;
45      double I_error;
46      analog_input = analogRead(sensorPin);
47      t = to_celsius(analog_input);
48      show_temperature(t);
49      P_error = (setPoint-t);
50  old_error = P_error;
51      I_error += old_error;
52      idealNEWPWM = ((P_error*90)+127) + (I_error*(90/(0.8*33)));
|
|
|
```

5

TESTS AND RESULTS

This chapter summarizes the different tests and experiments have been developed on the circuit to verify the effective functioning. First of all, the results obtained using just a bang-bang control are reported; then we can observe the temperature behaviour in the time domain, using first a simple hysteresis control and then the PID control, expressed in the previous chapter. This way, it is possible to notice how the different ways of working influence the response of the circuit and how the PID control results being the best choice for our project. Then, the attention will be focused on the operation of the magnets, to see how they actually work and evaluate the power they dissipate.

Before starting analysing the different responses of the circuit we better clarify an aspect of the Peltier functioning because at first sight it could generate some doubts. In fact observing with a simple oscilloscope (fig. 5.1) the two PWM signals coming from the Arduino, which are responsible to power the heater, we can notice they are not working synchronously. This goes against what we said so far because we wanted the two branches, that drive the heater, working with complementary signals, in this way the Peltier can be powered with the difference between them. But if they are asynchronous it is reasonable to think this "game" doesn't work properly.

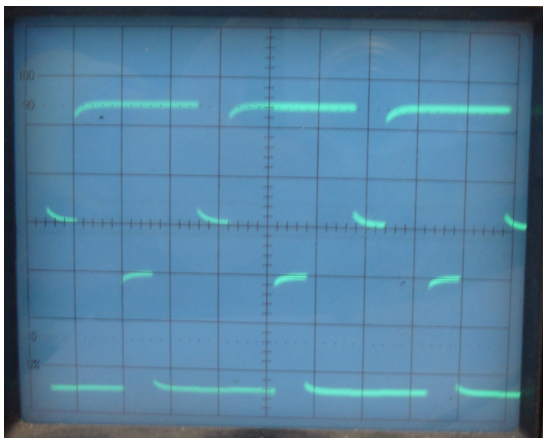
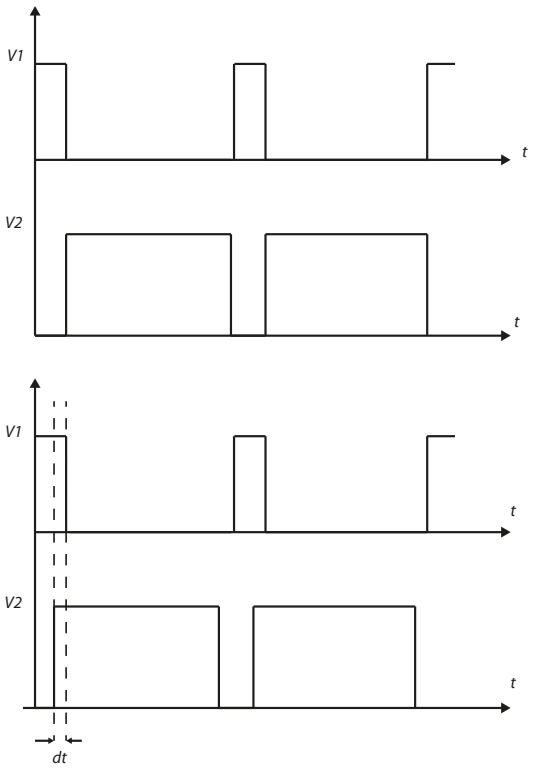


Fig. 5.1: PWM signals on the oscilloscope screen.



This idea is immediately proved to be wrong looking at the picture on the left. In fact even if there is a slight overlap between the two signals, doing the difference this effect is going to be cancelled, allowing the system to work in the way it was originally thought. We can confirm this also solving the equation below:

V1(10%-dt) + (V1+V2)dt + V2(90%-dt) + 0*dt =

= V1*10% - V1*dt + V1*dt + V2*dt + V2*90% - V2*dt =

= **V1*10% + V2*90%**

Fig. 5.2: Asynchronous PWM signals.

Tests on temperature control

In the picture below we can see how the temperature evolves in the time domain when the control board is set with just a bang-bang control. The setpoint is 37 degrees, like in all the others cases, and if the read value is lower than 37 the Peltier will be switched on, otherwise it will be turned off, leaving the system cooling down with just natural convection.

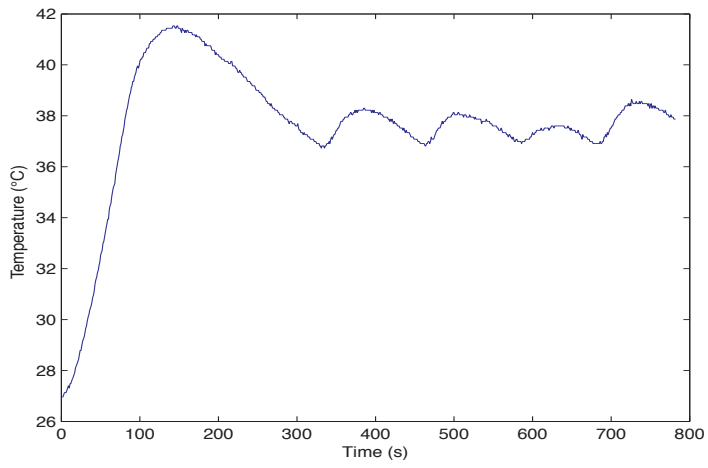


Fig. 5.3: Temperature in the time domain with bang-bang control.

The initial overshoot is very high and the system keeps oscillating with no appearance of stabilization at some point; and it takes also a long time (300 seconds) to the first overshoot to last. So it is very easy to understand that this is not the correct way to operate, there are too many errors in the measures and the system is not stable at all.

The response coming from an hysteresis control on the temperature is the one reported below. The two thresholds are fixed at 36 and 38 degrees and the system oscillates between these values indeed. There is no initial overshoot, the time response seems to be shorter, but like in the first case there is no sign of stabilization, the temperature continues to oscillate. This aspect is not acceptable, even if the reaction we want to perform isn't very strict about temperature conditions, we can't accept this kind of behaviour.

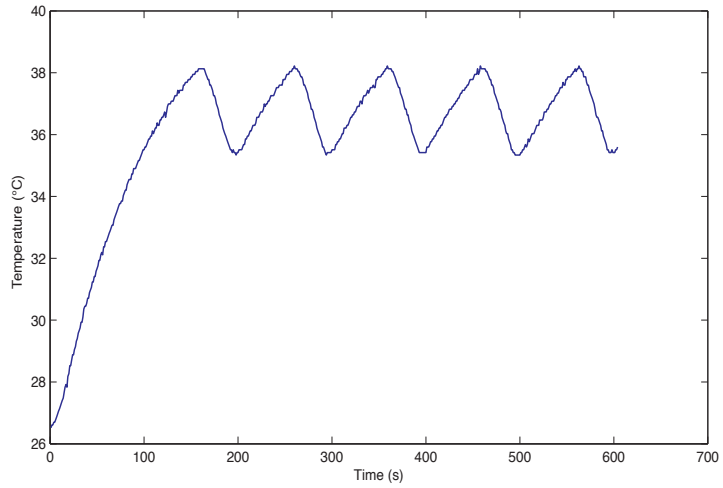


Fig. 5.4: Temperature in the time domain with hysteresis control.

Without doubt the best result is obtained with PID control and the related response curves are reported below, first only with a proportional control and then also with integral control, respectively fig. 5.5 and 5.6.

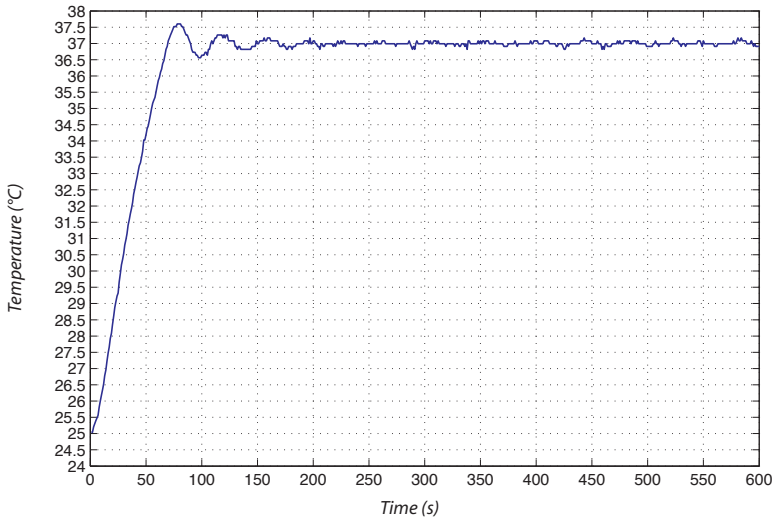


Fig. 5.5: Temperature in the time domain with P Control.

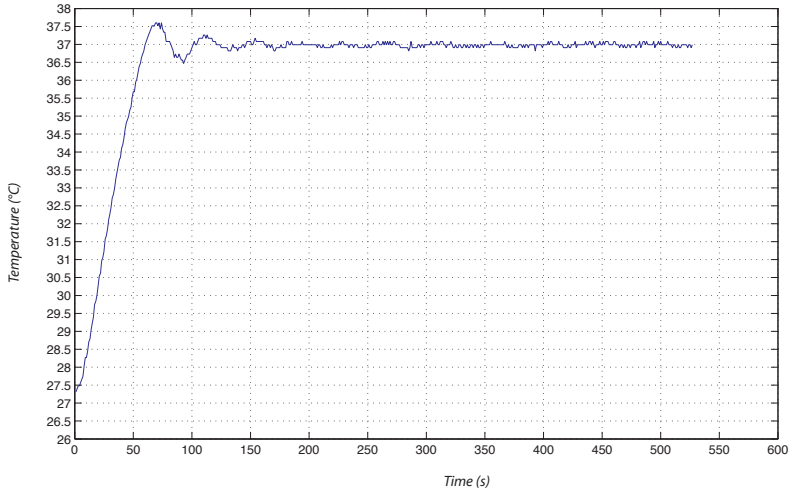


Fig. 5.6: Temperature in the time domain with PI control.

Definitely better the last solution. As we can see the temperature stabilizes around 37 degrees in a way shorter time than the first two solutions, around 100 seconds, and once reached the setpoint it can be considered more than satisfactorily constant. There is no a big difference between the two types of control, P and PI control gives more or less the same result, but it is always better to keep the safe direction and use the PI control, also because it keeps track of the previous data to calculate the correct value of duty cycle to apply.

The only problem in this case is the presence of an offset signal; in fact during the tests we noticed that, even if the setpoint was 37 degrees, the system tended to stabilize around 36.5 degrees; same behaviour for both the controls. So, it was better to consider this aspect, also because an error of 0.5 degrees can't be considered insignificant. Setting different values of temperature we kept track of the final temperature the system reached and then we plotted the curve below.

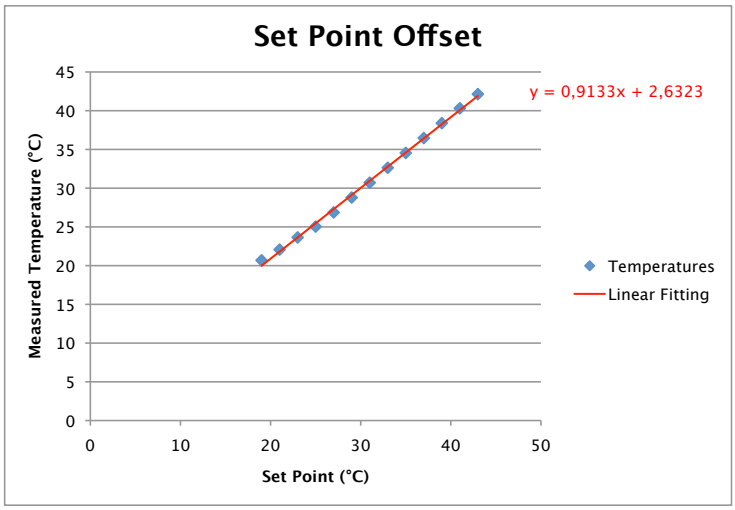


Fig. 5.7: Setpoint offset curve.

Fortunately all the data are fitted by a linear curve, whose expression is reported next to the graph and we can use this to calculate the offset signal and write the correct value in the Arduino code to set the temperature we need.

After all these considerations, just to evaluate if the control is actually stable and effective, we tried to put the entire system through a test, where the room temperature is 30 degrees in one case and 40 degrees in the other one. What resulted is that in the first case the Peltier is able with no problems to warm the sensor up and to keep a constant temperature around 37 degrees. On the other hand, in the second case, the result isn't really satisfying; in fact the Peltier should work like a

cooler and decrease the temperature from 40 to 37 degrees, but the system stabilizes around 38 degrees. So we think there is something wrong, or not totally precise, in the cooler behaviour of the Peltier that should be better investigate.

Magnets functioning

Other important function of the electronic board, subject of this work, is the mixing control realised by two magnets placed next to the central chamber. In the previous chapter we said they are "hand-made", meaning that there isn't a datasheet or model we can refer to, and the project is still at the beginning and many aspects are still to be investigated. This means we still don't have particular requirements about, for example, maximum current or power dissipation.

Just for now, we limit our analysis to the measure of some parameters function of the duty cycle, characterizing the signal applied to the magnets. In the two pictures below we can see, in fact, the magnetic field, the current and voltage functions of the duty cycle (fig. 5.8), which are all almost linear curves; and the power, function of the duty cycle as well (fig. 5.9).

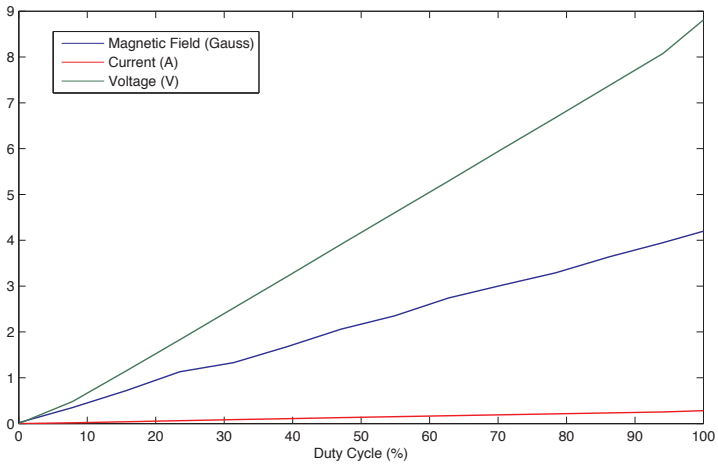


Fig. 5.8: Magnetic field, current and voltage vs duty cycle.

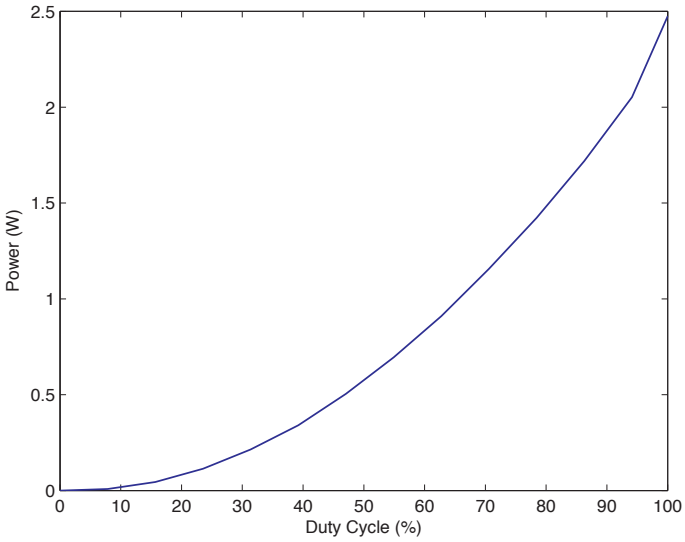


Fig. 5.9: Power vs duty cycle.

The magnets used for the experiments look like the one reported in the picture 5.10. With these we arranged a good setup to verify their effect on a liquid injected into a capillary tube. The system is reported in the picture below (fig. 5.11) and it gave the results we expected.

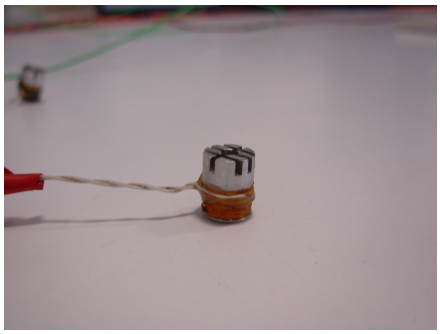


Fig. 5.10: Magnet used for the experiments.

The picture here on the right has been taken using a camera placed on a microscope. The image is quite clear and we can see that in the liquid there are some magnetic beads, just like the ones we talked about previously. In this case the magnet on the right is turned on and all the beads are attached on that side. Switching on and off alternatively the two magnets we can perform the mixing we need, otherwise the flow in the tube would be laminar and the reagents are not going to mix.

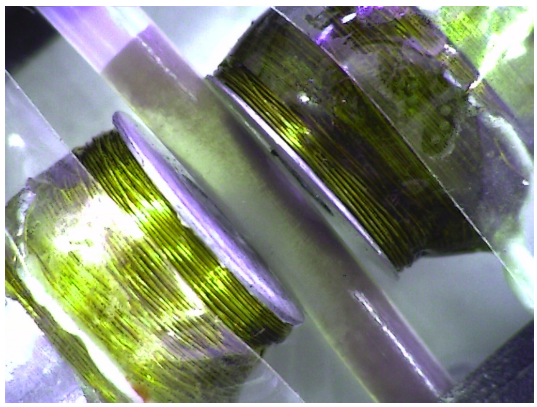


Fig. 5.11: Setup to verify the reagents mixing.

The experiment has been repeated several times and it gave all the times good results; we tried also to use two particular liquids that, if mixed, are going to become fluorescent and also in this case we could see that the system works properly. The only one problem, which needs to be further investigate, is that after several cycles of switching, the magnetic beads are going to concentrate, leaving good part of the tube with just the liquid, so we wouldn't be able to perform a correct and homogenous mixing. This issue is left to the next analyses.

The entire system, object of this work, is shown in the picture below. We can see the Arduino control board; two breadboards, with all the LEDs representing the different devices, the LCD display and the components used to drive the Peltier. The chip prototype is the one placed on the metallic block, with the thermistor glued inside, the heater underneath and the blue heat sink. Two thermocouples connected to the device on the left are going to monitor the temperature difference between the two plates of the Peltier.

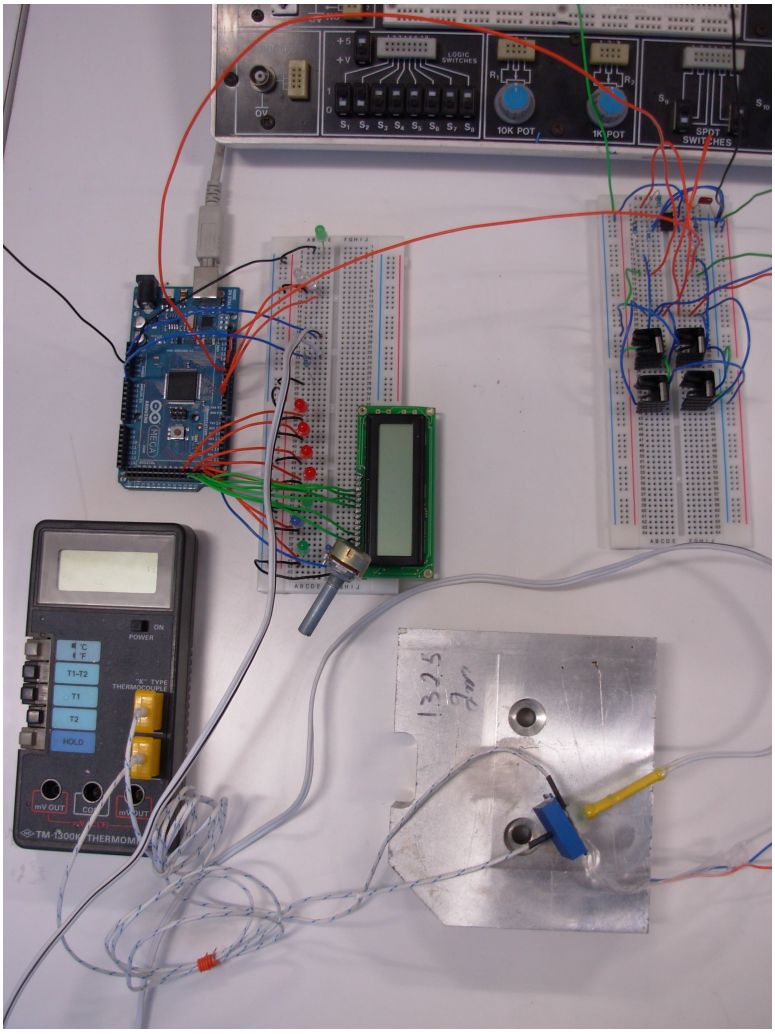


Fig. 5.12: Entire system.

CONCLUSIONS AND FUTURE WORK

In the introduction it has been presented, in a very general way, the system realized for this project. It laid the foundations for a conscious and critical approach for what concerns a project work. We were still at very beginning, but we tried to handle the problem taking a look also to the future, in fact we tried to design a circuit that can be easily changed and improved. There are still many devices and peripherals which haven't been used, for instance on the Arduino board many pins are still free and there are other two timers, and the converter as well, that remain unused. The code is flexible and it can be integrated whenever it needs to be.

The final goal is still very far to reach, but the importance and the complexity of this issue is too big to last in a very short time. As we said, many professional figures have to collaborate to sort this device out and many different aspects have to be evaluated. The design is important and the engineer has a very challenging role, but he can't do anything without the biologist's advice and supervision, and the economic aspect needs to be always kept in mind. This, not less important requirement, can't be underestimate, because we said we want to achieve a device that detects sexually transmitted diseases, and we want it to do it in a very short time, in order to treat the problem as it arises. These kinds of situations are so largely spread, especially between not educated people, that at the end the cost has to be affordable by almost everyone. So it is fine to use the most advanced technology in order to be competitive on the market and to give a result as reliable as possible, but we have to meet the user's needs as well.

Now we can think of what we left behind and what still needs to be done. Considering the responses of the temperature control obtained with the tests we can ask if the slight oscillations around the setpoint can compromise the good result of the DNA amplification; and if the initial overshoot can represent a problem for the entire system. For what concerns the first question, the system we will be using for amplifying DNA is the TwistAmp Basic Kit, this uses recombinase polymerase amplification (RPA), which represents a complete revolution in DNA diagnostics, combining superiority in speed, portability and accessibility with exquisite sensitivity and specificity compared to other diagnostic testing systems now on the market. It is usable in nearly any setting, eliminating the need for a trained technician or laboratory environment. The reaction progresses

rapidly and results in specific DNA amplification from just a few target copies to detectable levels typically within 5 - 10 minutes. The entire reaction system is stable as a dried formulation and can be transported safely without refrigeration. RPA can be used to replace PCR (Polymerase Chain Reaction) in a variety of laboratory applications and end-users can design their own ultra-sensitive assays. This operates at an optimal temperature of 37-39 degrees and it is flexible for temperature, but performance decreases the lower and higher the temperature so a tight control will lead to reliable results, as if each time we use the device the temperature is different then we may get different quantitative result for the same sample thus this is a reliability issue. Proteins, like those used for isothermal amplification (RPA), generally, work faster the hotter they are, however above a certain temperature they suddenly change shape, denature, and stop working, even if you cool them down again. This temperature is usually between 40-70 degrees.

So we can rapidly answer that even if the temperature oscillates around the setpoint, as in the graphs reported above, it doesn't really compromise the good result of the reaction. And even if the initial overshoot reaches a higher temperature, 38 degrees in our case, we are still in the optimal range of temperature.

Talking about future developments for the system it is interesting to mention a new deal that Android has recently arranged with Arduino. In fact the Android 3.1 platform introduces Android Open Accessory support, which allows external USB hardware to interact with an Android-powered device in a special "accessory" mode. When an Android-powered device is in accessory mode, the connected accessory acts as the USB host and the Android-powered device acts as the USB device. Android USB accessories are specifically designed to attach to Android-powered devices and adhere to a simple protocol that allows them to detect Android-powered devices that support accessory mode. Accessories must also provide 500mA at 5V for charging power. Simply this is going to allow Android related devices receive data from different sensors, just via USB for now, via Bluetooth in the future, and the board Arduino Mega2560 (used for this project) is one of them. So it seems like the user will be able to analyse his own sample, using the device we want to build, and then send the results to the phone and thus directly to the doctor or an analysis laboratory. In this way the dialogue between patient and medicine is more direct and we hope it can avoid the bad consequences caused by late cures.

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