DETECTION OF COELIAC DISEASE PREDISPOSITION USING DNA BIOSENSOR ARRAYS

Hamdi Abdelazim Osman Joda

Dipòsit Legal: T. 1055-2012

DOCTORAL THESIS

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UNIVERSITAT ROVIRA I VIRGILI

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UNIVERSITAT ROVIRA I VIRGILI

Tarragona

2012

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CERTIFY:

The Doctoral Thesis entitled: "**Detection of coeliac disease predisposition using DNA biosensors arrays**", presented by **HAMDI ABDELAZIM OSMAN JODA** to obtain the degree of Doctor with European mention by the Universitat Rovira i Virgili, has been carried out under my supervision at the Department of Chemical Engineering.

Tarragona, 25 May 2012

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CERTIFICO:

Que el present treball, titulat "**Detection of coeliac disease predisposition diagnosis using DNA biosensor arrays**", que presenta **HAMDI ABDELAZIM OSMAN JODA** per a l'obtenció del títol de Doctor amb menció europea, ha estat realitzat sota la meva direcció al Departament d'Enginyeria Química d'aquesta universitat.

Tarragona, 25 Maig, 2012

Dr. Ioanis Katakis Dra. Ciara K. O´Sullivan Dr. Valerio Beni

ACKNOWLEDGEMENT

First of all, all praises and thanks are due to *Allah*, for giving me the power, health and patience to finish my PhD thesis.

I would like to express my sincere gratitude to my supervisors Dr Ioanis Katakis, Dr. Ciara K. O'Sullivan and Dr. Valerio Beni for their valuable guidance and advice, for helping me to broaden my view and knowledge, and always open for discussing.

I would like to thanks Dr. Jukka Partanen and Noora Alakulppi for their guidance and hospitality during my research stay at Finnish Red Cross Blood Service (Helsinki, Finland).

My special thanks to Andreas Willems (Inno-Train Diagnostik GmbH, Germany), Kristina Lind and Linda Strömbom (TATAA Biocenter AB, Sweden) for providing PCR samples and for their scientific discussions.

I would like to thanks all NBG and the BBG group members (and former members): M.Carmen, Jos, Mary Luz, Diego, Valerio, Pablo, Mayreli, Alex, Tete, Oli, Magda, Tesfaye, Hany, Hossam, Rukan, Ale, Laia, Carlos, Viji, , Marketa, Mabel.

I am thankful to Ms. Barbara Vastenavond, Ms. Núria Juanpere Mitjana, Ms. Sira Durán Cothenet for their assistance in all the administrative works.

I owe a great thanks to my friends: Obai, George, Hany-2, Hatem and Radoune and all the others with whom I shared my time in TGN

Special thanks to my mother, brothers and sisters for their love and support. I am personally grateful to my wife Sarah and my daughter Malak for their love, patience and support.

Dipòsit Legal: T. 1055-2012

Summary

Coeliac disease (CD) is a chronic immune-mediated enteropathy of the small intestine affecting genetically susceptible individuals. The main feature of CD is bowel mucosal inflammation, crypt hyperplasia and villous atrophy, causing malabsorption syndrome. CD is elicited by the ingestion of gluten and similar proteins that are present in wheat, barley and rye.

Human Leukocyte Antigens (HLA) DQ2 and DQ8 genes have been identified as key genetic factors in coeliac disease as they are present in almost 100% of coeliac disease sufferers. These genes are encoded by the combination of certain alleles in the DQA1 and DQB1 region of chromosome 6. Specifically, DQA1*05:01 and DQB1*02:01 alleles for serologically defined leukocyte antigen DQ2 cis, DQA1*05:05 and DQB1*02:02 for DQ2 trans and DQA1*03:01 and DQB1*03:02 alleles for the DQ8.

In recent years, molecular methods have replaced the serological HLA typing methods initially used, with the most common HLA typing methods being the use of sequence-specific primers (SSP), sequence-specific oligonucleotide probes (SSOP), single strand conformation polymorphism (SSCP), restriction fragment-length polymorphism (RFLP) and sequencing based typing (SBT).

The work reported in this thesis focuses on the development of a genosensor array for HLA DQ2/DQ8 typing for screening of CD predisposition. Two different methods have been proposed and investigated in parallel. In the first method (Chapters 2 and 3), the genosensor array was developed employing the SSOP method, by designing different allele specific probes, whilst in the second approach, the SSP technique exploiting a novel approach for the direct detection of a double stranded PCR amplicon was investigated (Chapters 4 and 5).

The PhD thesis is organised in separate chapters, and the summary of each chapter is described below:

Chapter 1, is a general introduction, which gives an overview of HLA, coeliac disease, biosensors and also discusses the state of the art and the thesis objectives.

In **Chapter 2**, the development of a multiplex colorimetric assay for the low to medium HLA typing of the CD associated DQ2 and DQ8 genes is presented. A set of probes for the low to medium resolution genotyping of CD associated genes was designed, and then the probe design and hybridisation temperature was studied using surface plasmon resonance (SPR). SPR results indicated that the various probes behaved differently with temperature, but the optimal temperature of the different probes for the selective detection of DQ2/DQ8 alleles was 37 °C. Furthermore, the hybridisation conditions and timing of the different steps involved in the detection were investigated using enzyme-linked oligonucleotide assay (ELONA), and optimised to be 5 minutes for allele hybridisation and 20 minutes for reporter probe capture, limiting the total assay to less than 30 minutes at 37 C°. Finally, the performance of the developed genotyping platform was validated using real patient samples and an excellent correlation was obtained with hospital based typing technology.

In **Chapter 3**, a multiplex electrochemical genosensor array integrated in a microfluidic platform for rapid HLA-DQ2/DQ8 genotyping is presented. In this work we extended the number of the probes to a total of 10 sequence specific probes, to achieve medium to high resolution DQ2/DQ8 genotyping. These probes were immobilised on 36 gold electrodes of an electrode array and integrated within a microfluidics set-up for sample injection and manipulation. A study of the optimum hybridisation time for both the target allele and reporter probe demonstrated 10 minutes at 37 °C to be adequate for each step, taking sensitivity and selectivity into consideration. The stability of the probes on the gold electrodes was explored and exhibited an excellent stability in both real time and Arrhenius thermal accelerated stability studies, demonstrating the suitability of the developed genosensor array as a point of care diagnostic tool.

An evaluation of the specificity of the designed probes was performed by cross hybridisation studies using synthetic analogues of real PCR amplicons and good selectivity over the potentially interfering alleles including single base mismatches, was observed. Real human samples were analysed using the developed electrochemical genosensor array for the presence of HLA-DQ2/DQ8 alleles, and an excellent correlation with laboratory based HLA typing technology was achieved again,

highlighting the true application of the developed packaged sensor array for the easy, rapid and precise diagnostic tools for coeliac disease predisposition.

In the above mentioned chapters, DQ2/DQ8 genotyping was performed by designing an allele or allele group specific probe, namely a sequence specific oligonucleotide probe (SSOP), where post-PCR single stranded DNA generation was necessary to perform the genotyping. In **Chapter 4,** a method for the direct detection of dsDNA PCR product for CD associated HLA genotyping was developed, taking advantage of an existing HLA typing method (SSP), which depends on the design of special primers for selective amplification of the desired allele/alleles, modified with an extra ssDNA tail and PCR stopper. The use of the proposed primer design resulted in the production of double stranded PCR product with double single stranded tails that can be detected directly via hybridisation. The production of amplicon with the above mentioned characteristics was made possible by the different functional elements implemented in the developed primer design: a ssDNA tail for amplicon capture by surface immobilised probe or hybridisation of HRP labelled reporter probe; a PCR stopper for restriction of polymerase mediated extension; DNA sequence for sample purification and finally a DNA sequence having the functionality of conventional PCR primers. The influence of un-reacted primers on the efficiency of PCR detection was also investigated, and an approach for the removal (fishing) of excess un-reacted primer from the PCR product was demonstrated. However, changing PCR protocol to two-temperature and use of higher concentration of PCR product for hybridisation showed that the fishing step can be avoided and hybridisation can be performed directly from un-purified PCR product. Furthermore, different hybridisation times for direct PCR detection was assessed and showed the possibility to decrease the colorimetric assay time to less than 5 minutes. As a proof of concept, amplification via sequence specific primers (SSP) method, and direct colorimetric detection of the HLA-DQB1*02 allele, is presented.

This work was extended in **Chapter 5**, increasing the number of primers for multiplex PCR to allow low resolution genotyping of DQ2/DQ8. The three different primer-pairs were designed for the selective amplification of the coeliac disease associated alleles DQA1*05, DQB1*02 and DQB1*03:02, in addition to the primer for human growth hormone as positive control. As we demonstrated that removal of un-reacted primers is not a critical step, primers with only two parts separated with a PCR were used.

Amplicons with double single stranded tails resulted from these primers, facilitating direct electrochemical detection. Hybridisation time and temperature were optimised, and the entire assay could be completed in less than 5 minutes at 37 °C. Finally, real clinical samples were amplified using a mixture of the four primer-pairs and analysed using the developed electrochemical genosensor array and the results were demonstrated to correlate excellently with results obtained in a reference tissue-typing laboratory.

Chapter 6 outlines the overall conclusions and future work.

List of Publications:

1. Valerio Beni, Taye Zewdu, Hamdi Joda, Ioanis Katakis and Ciara K.O'Sullivan. Gold nanoparticle fluorescent molecular beacon for low resolution DQ2 gene HLA typing. Analytical and Bioanalytical Chemistry, 2012, 402:1001-1009.

2. Hamdi Joda, Valerio Beni, Deirdre Curnane, Ioanis Katakis, Noora Alakulppi, Kristina Lind, Linda Strombom, Ciara K. O´Sullivan. Low-Medium resolution HLA-DQ2/DQ8 typing for coeliac disease predisposition analysis by colorimetric assay. Analytical and Bioanalytical Chemistry, 2012, 403: 807-819

3. Hamdi Joda, Valerio Beni, Ioanis Katakis, Noora Alakulppi, Kristina Lind, Linda Strombom, Ciara K. O´Sullivan. Medium-high resolution electrochemical genotyping of HLA-DQ2/DQ8. In preparation.

4. Hamdi Joda, Valerio Beni, Marketa Svobodova, Andreas Willems, Rainer Franc, Ioanis Katakis, Ciara K. O'Sullivan. Modified primers for direct detection of double stranded PCR product. In preparation.

5. Hamdi Joda, Valerio Beni, Kristina Lind, Linda Strömbom, Andreas Willems, Rainer Franc, Ioanis Katakis, Ciara K. O'Sullivan. Rapid electrochemical HLA typing of coeliac disease predisposition. In preparation.

Others:

Patent:

Inventor: Ciara K O'Sullivan, Ioanis Katakis, Valerio Beni, Hamdi. Joda, Andreas Willems, Thomas Hansen-Hagge.

Title: *Cebadores especiales para la reacción en cadena de la polimerasa*.

Application number: P201230120

Application date: January 30th, 2012

Country: Spain

List of figures

List of Schemes

List of Tables

List of Abbreviations

A: Adenine.

AGA: Antigliadin antibody.

ARMS: Amplification refractory mutation system.

AuNP: Gold nanoparticle.

CD: Coeliac disease.

CF: Cystic fibrosis.

CG%: Cytosine and Guanine content.

CV: Cyclic voltammetry.

C: Cytosine.

DGP: Deamidated gliadin peptide

DNA: Deoxyribonucleic acid.

dsDNA: Double stranded DNA.

DT1: (10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol.

EIS: Electrochemical impedance spectroscopy.

ELISA: Enzyme linked immunosorbent assay.

ELONA: Enzyme linked oligonucleotide assay.

EMA: Endomysium antibodies.

ESPGHAN: European Society of Paediatric Gastroenterology, Hepatology and Nutrition

G: Guanine.

GFD: Gluten-free diet.

HGH: Human growth hormone

HLA: Human leukocyte antigens.

HRP: Horseradish peroxidise.

IHWG: International Histocompatibility Working Group.

IMGT: International Immunogenetics information system.

IUPAC: International Union and Applied Chemistry.

LNA: Locked nucleic acid.

MB: Molecular beacon.

MCH: Mercaptohexanol.

MHC: Major histocompatibilty complex.

PCR: Polymerase chain reaction.

PBS: Phosphate buffer saline.

PBS-T: Phosphate buffer saline - Tween.

PNA: Peptide nucleic acid.

POC: Point-of-care.

RFLP: Restriction Fragment Length Polymorphism.

RT-PCR: Real time polymerase chain reaction.

RU: Resonance unites.

SAM: Self assembled monolayer.

SBT: Sequence based typing.

SNP: Single nucleotide polymorphism

SPR: Surface plasmon resonance.

SSOP: Sequence specific oligonucleotide probe.

SSP: Sequence specific primers.

ssDNA: Single stranded DNA.

T: Thymine.

TEM: Transmission electron microscopy.

TFO: Triplex-forming oligonucleotide.

Tm: Melting temperature.

TMB: 3, 3′, 5, 5′-tetrame-thylbenzidine.

tTG: Tissue transglutaminase.

µTAS: Micro-total analysis system.

Table of Contents

CHAPTER 1

UNIVERSITAT ROVIRA I VIRGILI
DETECTION OF COELIAC DISEASE PREDISPOSITION USING DNA BIOSENSOR ARRAYS
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1. INTRODUCTION

1.1. Human Leukocyte Antigen (HLA)

1.1.1. General introduction

The human leukocyte antigen (HLA) system is the human equivalent of the major histocompatibilty complex (MHC) [1]. HLA region is known to be the most polymorphic region in the human genome [2], and is controlled by a group of genes located in the short arm of chromosome six (6p21.3) and can be divided into three different regions: HLA class I loci (subdivided to HLA-A,-B,-C), class II loci (subdivided to HLA-DR, DQ, and DP) [2-5].

HLA class I molecules are glycoproteins present on the surface of all nucleated cells in the body. HLA class I consists of a heavy chain glycopeptide (α) encoded by HLA and linked noncovalently to the β2-microglobulin light chain (Figure 1.1). The main function of class I is to present endogenous antigens to cytotoxic T-cells (CD8+ T Cell) [2, 3]. HLA class II molecules are present on the surface of "antigen-presenting cells" (APCs) like dendritic cells (DCs), macrophages and B-cells and are composed of two transmembrane glycoproteins, Alpha (α) and Beta (β) chains as heterodimers (Figure 1.1). The key role of Class II molecules is to present exogenous antigens to helper T-cells (CD4+ T cells) for initiation of the immune response [2, 3]. There are also class III molecules that are not part of the HLA complex but are located in the region between HLA class I and class II on chromosome 6, and they encode genes related to the complement system and cytokines [5].

HLA was first identified in 1958 by Dausset when he found donor leukocyte agglutination upon mixing with sera of patients with multiple blood transfusions [4, 6], and in the following years, more leukocyte antigens were reported. To standardise detection techniques and to combine know-how and findings, the first series of histocompatiblity workshops was held at Duke University in mid 1964 and this was followed by a series of workshops to update the information available on HLA [6].
The HLA Nomenclature Committee was established in 1968 and was sponsored by the World Health Organisation to take the responsibility of official HLA names.

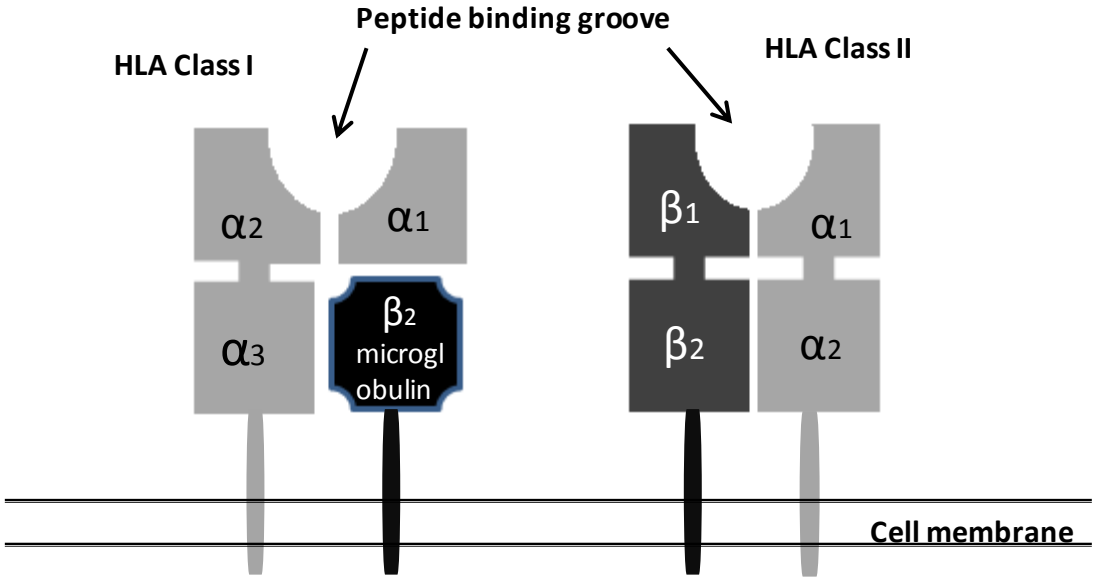

Figure 1.1. Structure of HLA class I and class II molecules.

1.1.2. HLA nomenclature

Before the era of molecular typing, HLA naming was based on serological defined antigens and this is still very common. In this system, HLA is followed by the genetic loci, assigned as one or more letters and then the specific allele designated by numbers [7] (e.g. HLA-DQ2).

For molecular nomenclature, alleles name are initiated with the letters 'HLA' that specifies the gene region, followed by letter/s (e.g. A, DR or DQA1) referring to the locus within the gene region. The first two digits define the allele group, usually equivalent to the serological antigen. The third and fourth digits used to assign the allele subtypes, was first introduced in 1987. Numbers are defined to each allele in the order of which DNA sequences have been resolved. Alleles with different numbers in the first four digits have one or more different nucleotide substitutions that thus change the amino acid sequence of the encoded protein. The use of additional fifth and sixth digits was implemented in 1990s, to specify alleles that differ only by synonymous nucleotide substitutions without a consequent change in amino acid (non-coding substitutions) [8].

Alleles that only differ by sequence polymorphisms in the introns or in the 5' or 3' untranslated regions that flank the exons and introns are distinguished by the use of the seventh and eighth digits [9]. As the number of new HLA alleles discovered every year increased rapidly until reaching the maximum possible current naming capacity. The need for a new nomenclature system was decided upon employs a new system using

colons (:) in the allele names to act as delimiters of the separate fields and was introduced in 2010 [9, 10] (Figure 1.2). International ImMunoGeneTics (IMGT) database is responsible for updating the information on HLA alleles (http://www.ebi.ac.uk/imgt/hla/) [11].

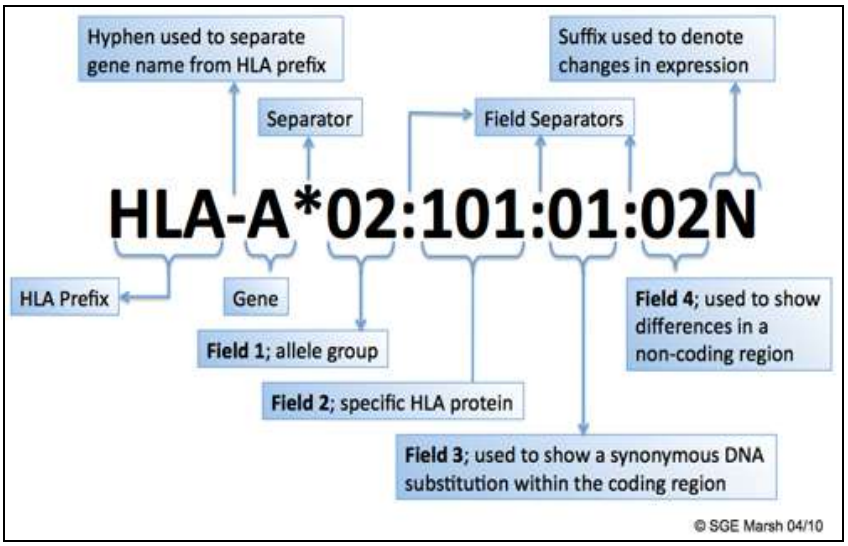

Figure 1.2. Schematic of HLA nomenclature system [9].

1.1.3. HLA typing methods

HLA typing or tissue typing refers to the identification of HLA alleles. There are several methods that have been used for HLA typing, as described below.

1.1.3.1. Serology

Serology was the first method used for HLA typing by identifying the antigen on white blood cells, using the lymphocytotoxicity test, which was developed by Terasaki and McClleland in 1964 [12]. Sera of multiple blood transfused patients or multiparous women containing antibodies to most HLA antigens were mixed with lymphocytes of unknown HLA genotype and complement. In the presence of a particular HLA antigen, antibody will bind leading to cell damage by the complement system and these damaged cells were then visualised under a microscope after staining with eosin or fluorescent.

Serology was the gold standard method for HLA typing for decades until the discovery of the Polymerase Chain Reaction (PCR) in the 1980s. The main drawback of serological typing is that it cannot resolve many of the HLA alleles existing in the population as many HLA polymorphisms can be serologically silent.

1.1.3.2. Restriction Fragment Length Polymorphism (RFLP)

RFLP was first described in 1982 by Wake et al [13], and is performed using specific restriction enzymes that recognise and cut specific DNA sequences. These DNA fragments are then separated according to their length using gel electrophoresis [14]. However, this technique was not used extensively as it is time consuming, expensive and presents difficulty in typing of some allele groups.

1.1.3.3. Molecular HLA typing

The discovery of PCR by Kary Mullis in the 1980s was a revolution for HLA typing [15, 16], as the recognition of HLA polymorphisms was detected at the genetic molecular level instead of the antigenic level used in serology. The most common molecular techniques are: Sequence-Specific Oligonucleotide Probe (SSO), Sequence Specific Primer (SSP) and Sequencing Based Typing (SBT).

a) Sequence Specific Oligonucleotide Probes (SSOP)

For this method, first the region of interest of HLA is amplified using generic or group specific primers. Historically, SSOP was performed by means of dot blot assay by immobilisation of amplified DNA on a nylon membrane using UV irradiation. After generation of single stranded DNA (ssDNA), hybridisation with labelled short oligonucleotide probes (allele specific or group-specific probes) was carried out under stringent conditions to detect the presence of specific alleles [17]. Detection of hybridisation was performed using SSO probes labelled with radioactive material (P^{32}), which was later replaced by enzyme [18] or fluorescent labels [19].

Dot blot is a time-consuming procedure, but it is suitable for the analysis of large samples. An alternative approach is "reverse dot blot" [18], which is now more commonly used. In reverse dot blot approach, a panel of short oligonucleotide probes (allele specific or group-specific probes) are attached to a solid support instead of the long PCR product. The amplified DNA, usually labelled with biotin, is then hybridised to the immobilised probes under stringent conditions. Detection of hybridisation is carried out by colorimetric reaction subsequent to binding of streptavidin-horseradish peroxidase (HRP) to the biotinylated allele and substrate addition.

b) Sequence Specific Primers (SSP)

SSP, the most popular HLA typing method, was introduced in the early 1990s [20, 21]. SSP is based on primers designed to amplify a specific allele or allele group. DNA amplification is more efficient in the presence of fully complementary primers and the existence of one or more mismatches at the 3' end will hinder 3' to 5'exonuclease activity. The presence of the desired allele is indicated by the presence of PCR product of a particular size using gel electrophoresis [20, 22].

However, presence of 3' terminal mismatches does not prevent primer extension in some cases. Thus, another mismatch was created at the second, third, or even fourth nucleotide from the 3' end in order to improve specificity of the primer. This method is called amplification refractory mutation system (ARMS) and was first introduced by Newton et al. [23] and used for the first time in SSP for HLA DQ genotyping by Picard [24]. The specificity of SSP relies on primer design while the specificity of the SSOP approach depends on probe design.

c) Sequence Based Typing (SBT)

Sequence based HLA typing is a powerful tool and considered as the gold standard method for identification of HLA alleles, providing high resolution HLA typing and being the only method that can identify new alleles [25, 26].

Two different DNA sequencing methods were developed independently in the late 1970s. The chain termination method (or Sanger method) was developed by Sanger in 1977 [27], and is based on the use of a DNA template, DNA primer, DNA polymerase, deoxynucleotidetriphosphates (dNTPs), and dideoxynucleotides (ddNTPs). Random incorporation of ddNTPs into the DNA sequence leads to termination of the strand extension at specific bases (either A, C, T, or G), forming DNA fragments of different lengths that can be visualized in gel electrophoresis.

The Maxam and Gilbert method or chemical degradation method [28] involves radioactive labelling at one 5' end of the DNA followed by chemical treatment to cleavage of the DNA at specific nucleotide position, then the sequence is determined by analysing the DNA length by gel electrophoresis. However, the chain termination method is the most popular and commonly used technique for DNA sequencing, and has been refined during the last years and is now automated utilizing fluorescent labelling [29].

d) Other techniques for HLA typing

DNA microarrays

Microarrays provide high throughput, rapid, simultaneous analysis and require low amounts of sample. There are several reports on the use of DNA microarrays for HLA typing. Zhang et al. [30] genotyped the HLA-DRB1 gene using microarrays, where 19 SSO probes were immobilised on a silylated slide. The second exon of the HLA-DRB1 gene was amplified using fluorescein-labeled primer, and then hybridised to SSO probes immobilised on the microarray. Lee and co-workers [31] developed a microarray for the genotyping of HLA-A and DRB1. PCR amplification was carried out for exons 2 and 3 of the HLA-A locus using symmetric PCR, while asymmetric PCR was used for exon 2 of the HLA-DRB1 using 4 pairs of primers for each region, followed by hybridisation and fluorescence detection. Using the same principles, Yoon and et al. presented simple and rapid HLA-C genotyping using a DNA microarray [32].

Luminex genotyping

Luminex xMAP technology has emerged from the existing flow cytometry technology and is based on a suspension of 5.6 μm polystyrene microsphere containing red and infrared fluorophores. Up to 100 microsphere sets can be created using different proportions of the two dyes (Figure 1.3), facilitating the possibility of performing 100 multiplex tests in a single reaction well [33]. HLA genotyping is performed by reverse SSO DNA typing method, through hybridisation of biotinylated PCR product to the microsphere

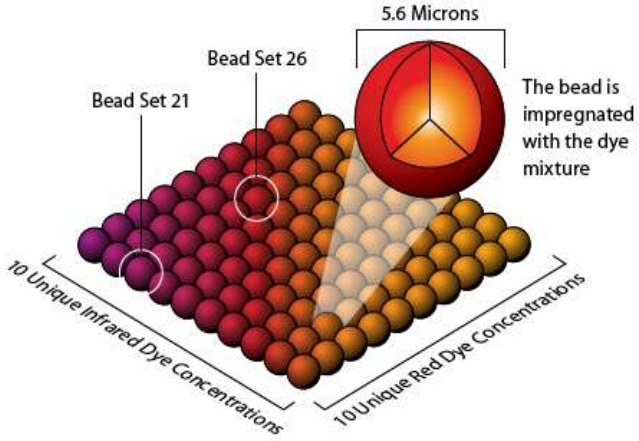

Figure 1.3. Schematic of 100 sets of luminex color-codes microspheres [34].

beads modified with sequence-specific oligonucleotide probes (SSOP). Subsequently, R-Phycoerythrin-conjugated Strepavidin (SAPE) is added and detection is performed using the Luminex instrument, which is able to quantify the relative amounts of PCR product hybridised to each microsphere [33, 35-37].

Real time PCR (RT-PCR)

One of the reported RT-PCR based HLA typing uses a dual labelled fluorescent TaqMan probe, which is labelled at its 5'end with a fluorophore reporter dye, and at 3' end with a quencher dye [38, 39]. During the PCR annealing step the probe hybridises to its complementary sequence on the DNA template and when the forward primer is extended and reaches the probe the exonuclease activity of the Taq polymerase digests the probe, leading to a separation of reporter and quencher and generation of fluorescent signal (Figure 1.4). The presence of a specific allele is indicated by an increasing fluorescence signal each PCR amplification cycle [38-40].

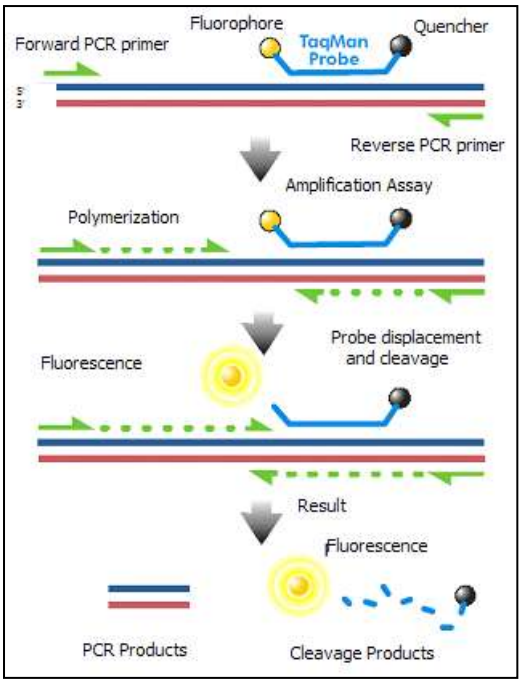

Figure 1.4. The principle of TaqMan probe assay.

1.1.4. HLA and disease association

Susceptibility to some diseases with inheritance of specific HLA genes was postulated more than 40 years ago[41]. The association was found to be weak in some diseases and quite strong in other diseases, these latter diseases mainly being autoimmune diseases.

One of the first and strong associations discovered in 1973, was that of HLAB27 and ankylosing spondylitis [42]. Since then, an increasing number of diseases have been observed to be associated with specific HLA genes. Most autoimmune diseases are associated with HLA class II genes and a few are associated with HLA class I genes. Susceptibility to the following diseases increase with specific HLA genes: insulin dependent diabetes mellitus [43, 44], rheumatoid arthritis [45], pemphigus vulgaris [46], multiple sclerosis [47, 48], IgA deficiency [49, 50], cancer susceptibility [51, 52], ankylosing spondylitis [42, 53] and idiopathic nephrotic syndrome [54].

1.1.5. HLA and transplantation

After the introduction of organ transplantation in the 1960s, the use of HLA matching has been demonstrated to improve transplantation survival rate [55]. One of the main functions of HLA antigens is recognition and differentiation of self from non-self (foreign substances). In the case of weak or non-matched organ transplantation, the presence of non-self HLA induces an immune response and graft rejection [2]. In the early years, serological methods were used for HLA matching between the donor and the recipient, and subsequently used molecular HLA typing, studies have resulted in an improvement in the transplantation outcome[56].

1.2. Coeliac Disease (CD)

1.2.1. General introduction

Coeliac disease (CD) or gluten-sensitive enteropathy, is a permanent immune-mediated disorder of the small intestine, characterized by bowel mucosal inflammation, crypt hyperplasia, and villous atrophy [1-3] (Figure 1.5). CD occurs in genetically susceptible individuals triggered by the ingestion of dietary gluten present in wheat and barley and rye [57-59].

Gluten proteins are composed of two main portions; gliadins and glutenins [60] where gliadin are a monomeric alcohol soluble compound, while glutenins are polymeric alcohol insoluble compounds and both have found to induce CD [61]. Gliadin can be further divided to different subfractions based on mobility in electrophoresis and amino acid sequence [62]. Gluten proteins contain high glutamine and proline residues, which

makes gluten highly resistant to enzymatic degradation during the digestive process in the gastrointestinal tract [63].

The first clinical description of CD was reported by Dr Samuel Gee in 1888 at St Bartholomew's Hospital in London. Later, in 1950 Dicke and co-workers discovered that wheat is the toxic dietary component trigger of CD, while the genetic association of CD with HLA-DQ was reported during the 1980s[64].

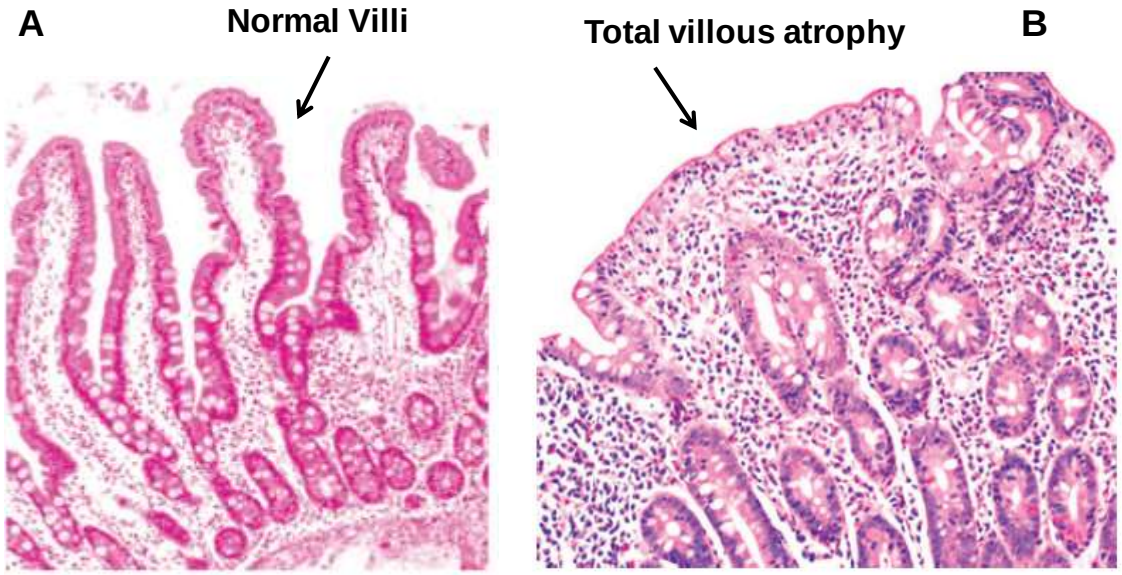

Figure 1.5. Histology of the small intestine **(A)** normal **(B)** coeliac disease affected small intestine [65].

1.2.2. Epidemiology (CD iceberg)

In the past, it was believed that CD as a rare disorder of children, but recent studies have demonstrated that CD is more common than previously thought and the prevalence of CD in Europe and US has been demonstrated to be 1% of the general population [66, 67] and similar levels are expected in many other parts of the world, including Arabian countries [68, 69], India [70], Iran [71], with the highest prevalence being among the Saharawi population [72]. Despite the high prevalence, it has been reported that a ratio between diagnosed to undiagnosed is about 1:7 [73], and the average time form symptom onset to diagnosis is 11 years [74], as reflected in CD iceberg first described by Richard Logan in 1991 [75] (Figure 1.6).

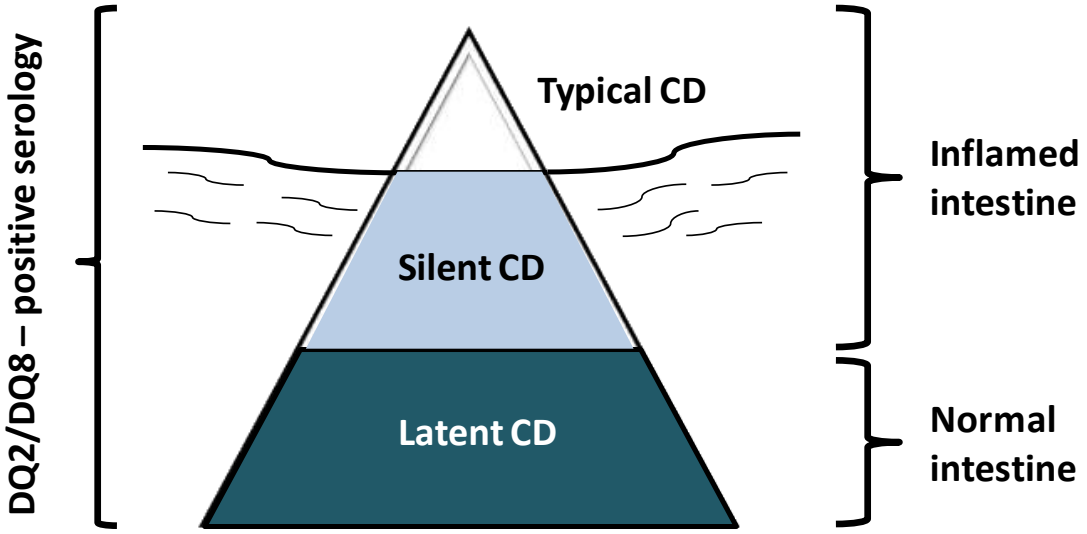

Figure 1.6. Iceberg model of coeliac disease.

1.2.3. Pathogenesis

In CD affected patients, the intestine is more permeable or leaky to gliadin peptide [76, 77]. Inside the lamina propria, deamidation of gliadin is initiated through the conversion of glutamine residues of the peptide to glutamic acid by the tissue transglutaminase (tTG) enzyme [63, 77]. This deamidation process increases gliadin affinity to the HLA DQ2 or DQ8 peptide-binding groove of the antigen-presenting cell (APC), this interaction which leads to CD4 T-lymphocyte activation. Upon continuous exposure to gliadin, the activated T-cells produce proinflammatory cytokines such as interferon-γ, interleukin-4, and tumor necrosis factor *a* (TNF*a*), which induce damage to the enterocyte, increased proliferation in the intestinal crypts and ultimately severe damage to the intestinal mucosa. Activated CD4 T-lymphocyte induces activation of B-cells to produce autoantibodies [78-81] (Figure 1.7). Due to the fact that the tTG enzyme plays a role in epithelial cell proliferation, it in itself may cause further damage to the small intestine [82].

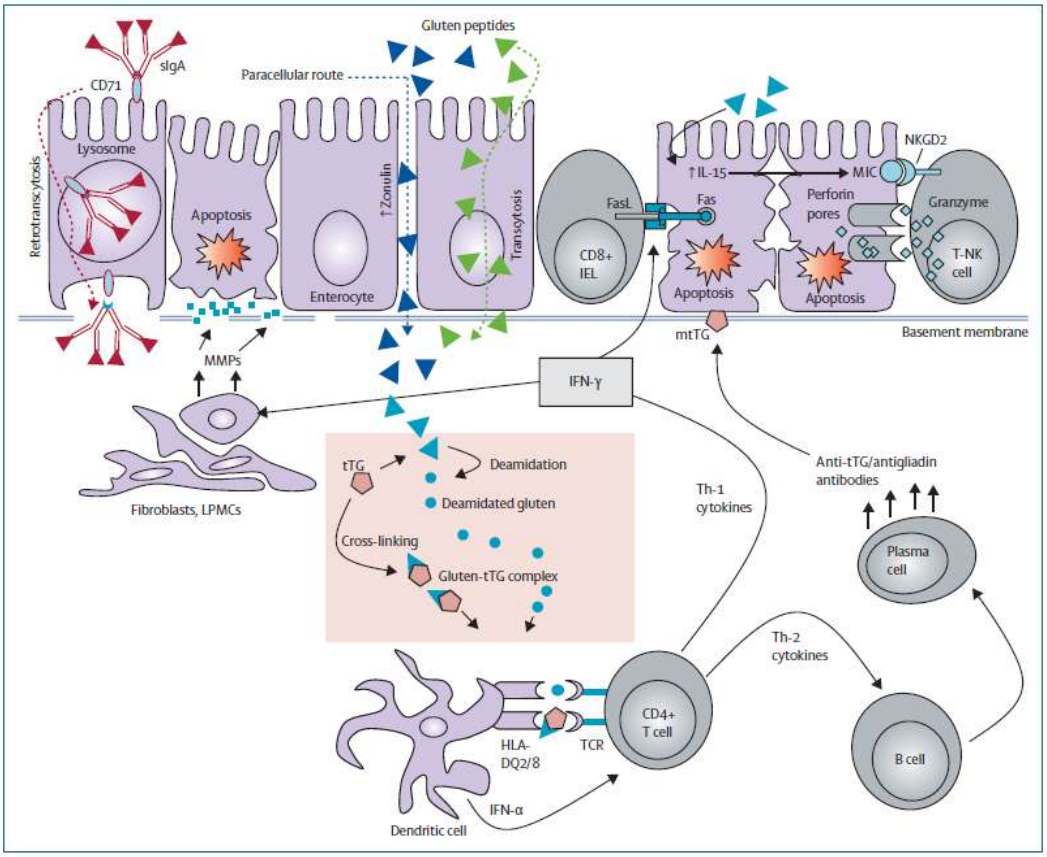

Figure 1.7.Pathogenesis of coeliac disease [80].

1.2.4. Clinical presentation:

The clinical presentation of CD varies considerably [78, 79, 83], making a correct diagnosis extremely difficult. CD may present with typical or atypical symptoms, and can be present as silent or latent CD [81].

a) Typical

Presentation with typical gastrointestinal symptoms is mainly common in children after introduction of gluten containing food, with symptoms such as chronic diarrhoea, failure to thrive, anorexia, abdominal distension, muscle wasting, poor appetite and irritability [81]. In older children and adults the typical presentation is intermittent, including recurrent diarrhoea, abdominal bloating, abdominal discomfort and weight loss. Histological changes in typical CD are present in the proximal small intestine from minor villous blunting to subtotal or total villous atrophy, crypt hyperplasia and / or significantly increased plasma cell and lymphocyte infiltration [78].

b) Atypical

Atypical coeliac disease generally presents as late onset of the disease in older children or adults, and typical gastrointestinal symptoms are minimal or absent, and instead extraintestinal manifestations [78, 81] such as dermatitis herpetiformis, short stature, puberty delay, iron deficiency and resistance to oral iron therapy as well as dental enamel defects are observed.

c) Silent

In silent CD, the patient appears healthy and symptomless. Antibody serology tests may be positive but typical CD intestinal histology change is seen in intestinal biopsy [84]. For silent patients, diagnosis is usually made due to screening of high-risk individuals.

d) Latent

In this case, the patient is entirely asymptomatic or has some varying degrees of signs and symptoms. The intestinal mucosal is normal or minimally abnormal and antibody tests for EMA and/or anti-tTG may be positive with presence of the typical genetic predisposition (HLA-DQ2 or DQ8) and these individuals are at high-risk to develop typical CD in the future [84, 85].

1.2.5. Diagnosis

1.2.5.1. Serology

The main serological markers for CD include anti-tissue transglutaminase (tTG), endomysial antibodies (EMA), and antibodies against deamidated gliadin peptides (DGP). These tests are used to examine suspected patients and for patient monitoring following introduction of a gluten-free diet [84]. These serology test are especially helpful in individuals with atypical symptoms and for screening asymptomatic first degree relatives [81].

Antigliadin antibody (AGA) was the first serological marker for CD introduced during the 1960s [86], and the IgA immunofluorescent assay for detection of the endomysial antibody (EMA) was developed in 1984 [87]. Dieterich et al. identified the tTG autoantigen in 1997 [88], and the measurement of IgA tTG is currently the routine assay used for serology screening for CD, having the highest sensitivity and specificity. In some cases, the detection of antibodies against deamidated gliadin peptide has been shown to have better sensitivity [89]. Additionally, the serum level of IgA should be measured in CD suspected patients as IgA deficiency is frequently associated with CD.

AGA is no longer used routinely for the diagnosis of CD because of low sensitivity and specificity [90].

1.2.5.2. Genetic testing

Genetic predisposition in the pathogenesis of CD is evident with a high correlation in monozygotic twins (86%) and 20% in dizygotic twins [91]. Moreover, CD genetic susceptibility has been demonstrated to be strongly associated with certain HLA alleles, as 95% of patients carry the HLA-DQ2 heterodimer, while most of the remaining carry the HLA-DQ8 [57, 92, 93] (Figure 1.8) and individuals not carrying neither of HLA-DQ8 or HLA-DQ2 rarely develop CD[93, 94]. However, 20-30% of general Caucasian populations are DQ2/DQ8 positive, therefore the presence of DQ2 or DQ8 is essential for developing CD, but being DQ2/DQ8 positive does not necessary result in onset of the disease [81, 85, 95].

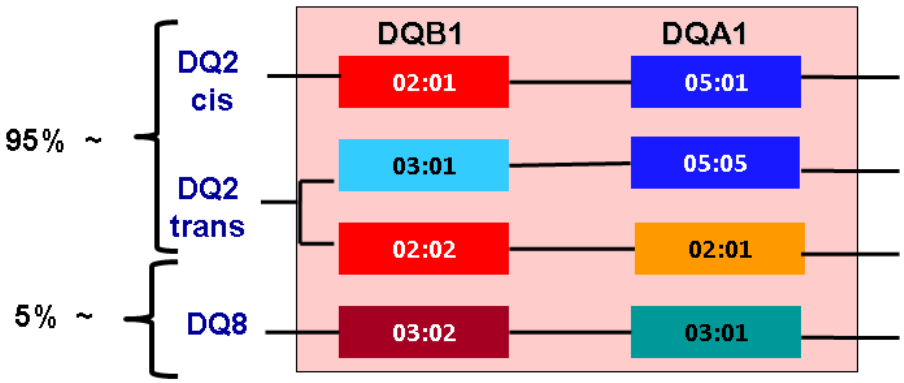

Figure 1.8. CD associating HLA alleles encoding DQ2 and DQ8 heterodimers.

The HLA-DQ2 heterodimer is encoded by (DQA1*05:01/DQB1*02:01) in cis (on one chromosome), or by DQA1*05:05 and DQB1*02:02 in trans (the two DQ2 alleles being encoded on one chromosome from each parent). HLA-DQ8 is encoded by DQA1*03:01/ DQB1 *03:02 [57, 82, 96] (Figure 1.8).

The risk of CD is affected by the presence of allele combinations and it has been reported that susceptibility to CD is sufficient with allele of ADQA1*05-DQB1*02, and a higher risk is associated with the presence of 2 copies of DQB1*02 [97]. For detailed HLA-DQ risk gradient please refer to Table 1.1.

Table 1.1. CD HLA-DQ risk gradient [98, 99].

Haplotype 1		Haplotype 2			**Risk**
DQA1	**DQB1**	**DQA1**	**DQB1**		
05:01	02:01	03:01	03:02	DQ2 *cis* and DQ8	1:7
05:01	02:01	X[a]	02:01[b]	DQ2 *cis*, β2	1:10
03:01	03:02	X[a]	02:01[b]	DQ8, β2	1:24
X[a]	02:01[b]	X[a]	02:01[b]	β2 / β2	1:26
05:01	02:01		02:01[b] neg	DQ2 *cis*	1:35
02:01	02:02	05:05	03:01	DQ2 *trans*	1:35
03:01	03:02		02:01[b] neg	DQ8	1:89
X[a]	02:01[b]		02:01[b] neg	β2	1:210
05:01	02:01 neg		02:01[b] neg	α5	1:1842
Other				Others	1:2518

β2: DQB1*02 in the absence of DQA1*05. α5: DQA1*05 in the absence of DQB1*02.
X[a]: different from DQA1*05. 02:01[b]: either DQB1*02:01 or DQB1*02:02.

1.2.5.3. Biopsy

Until recently, the biopsy was the golden standard for CD diagnosis and is taken from proximal small intestine using upper gastrointestinal endoscopy. The biopsy is then examined for the typical histological lesion associated with CD. Histological characteristics of the small intestinal mucosa in CD has been graded by Marsh [100] and modified by Oberhuber [101] (Figure 1.9) as:

• Type 0 or 'preinfiltrative' stage (normal);
• Type 1 or 'infiltrative' lesion (increased intraepithelial lymphocytes);
• Type 2 or 'hyperplastic' lesion (type 1 + hyperplastic crypts);
• Type 3 or 'destructive' lesion (type 2 + villous atrophy, denominated as 3a (partial villous atrophy), type 3b (subtotal villous atrophy), and type 3c (total villous atrophy).

A biopsy revealing infiltrative changes alone (Marsh type 1) alone does not confirm CD, while a biopsy with infiltrative changes with crypt hyperplasia (Marsh type 2) and positive serological tests, CD diagnosis is highly supported. In negative serology, other intestinal conditions may be considered. The presence of villous atrophy (Marsh type 3) is a definitive diagnostic feature for CD.

Recently, the *European Society of Paediatric Gastroenterology, Hepatology and Nutrition* (ESPGHAN) has revised the guidelines for coeliac disease diagnosis stating

that histological assessment can be omitted in symptomatic cases with high IgA anti-tTG levels (10 x above upper normal limit), and the presence of HLA DQ2 and/or DQ8 heterodimer [102], and also recommend the measurement of total IgA.

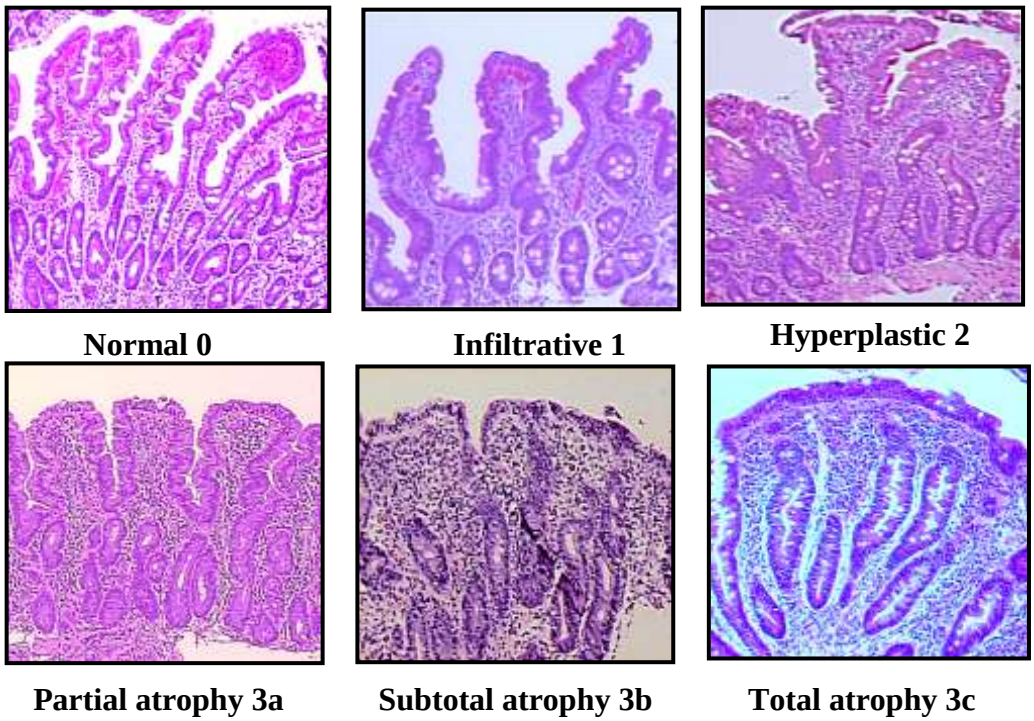

Figure 1.9. Small intestine histological grades in coeliac disease (Marsh classification).

1.2.6. Treatment

CD is treated by a strict lifelong gluten-free diet (GFD) [75, 90, 92]. Relief of symptoms may take several months to resolve completely, and it may take 1 to 2 years for serum antibodies and histological changes to disappear [75]. Recently, several approaches have been explored to develop other alternative therapies, including oral treatment using prolyl-endopeptidase enzymes for the degradation of the toxic gluten peptides in the gastrointestinal tract [103]. A zonulin inhibitor has also been explored as another choice for treatment [104], which decreases intestinal permeability, potentially blocking the entry of gluten to the lamina propria.

1.2.7. Complications

Undiagnosed or inadequately treated CD is associated with several complications from the longstanding malabsorption [75, 78], and there is a higher incidence of other

autoimmune diseases in CD patients. However, it is not clear if they are secondary to CD or related to the common genetic background [78] (table 1.2).

Table 1.2. Complications and diseases associated with CD [78]

Secondary to untreated CD	Associated diseases or secondary to untreated CD	Associated diseases
Anaemia	Autoimmune diseases (Type I	Down syndrome
Osteoporosis	diabetes meleitus, Thyroiditis	
Short stature		Turner syndrome
Infertility	Sjogren's syndrome)	
Pubertal delay	Epilepsy	Williams syndrome
Abnormal liver function	Ataxia and other neurological	
Hyposplenism		IgA deficiency
Tetany	disturbance	
Dermatitis herpetiformis	Intestinal lymphoma	
Dental enamel defects	IgA nephropathy	

1.3. BIOSENSORS

1.3.1. Definition

A biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with a transduction element [105].

Classification of biosensors can be based either on the biorecognition element or on the mode of physico-chemical signal transduction. According to the biorecognition element, biosensors can be classified as bioaffinity and biocatalytic biosensors. Bioaffinity biosensors are based on selective binding of the target analyte to a recognition element (oligonucleotide, antibody or cell receptor). On the other hand, biocatalytic biosensors generally depend on the use of enzymes as recognition element, which promote a reaction involving target substrate [106, 107]. The most widely used biocatalytic biosensor is the glucose biosensor using glucose oxidase as the recognition element.

The most common biosensor transduction systems are: electrochemical, optical, piezoelectric, acoustic, gravimetric, and calorimetric.

Biosensors are promising alternative tools to conventional analytical instruments because they provide simple, fast, and cheap analysis in a wide range of applications such as medicine, environmental monitoring, and food processing industries.

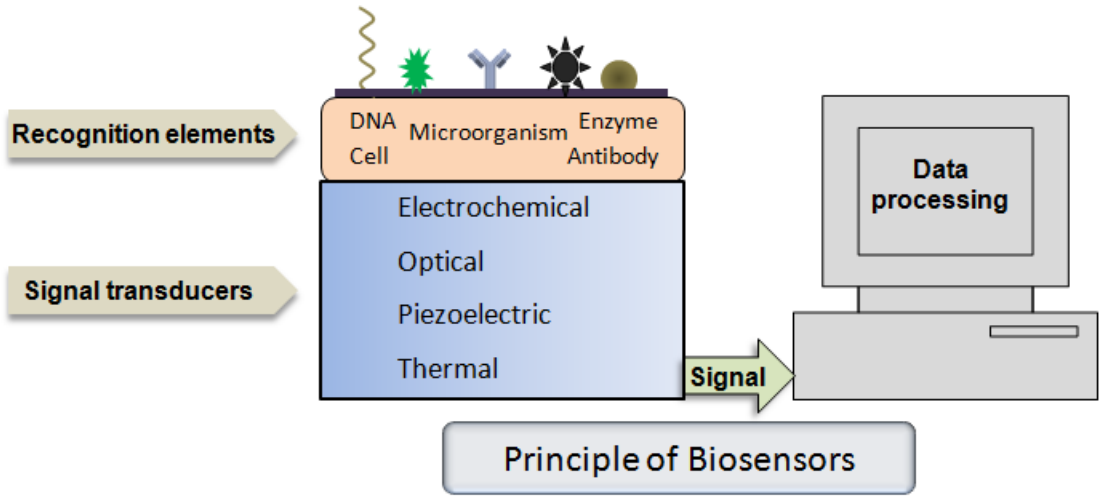

Figure 1.10. Schematic representation of a biosensor.

1.3.2. DNA biosensors (Genosensors)

DNA biosensors (or genosensors) are generally based on the use of short oligonucleotide probes immobilised over the signal transducer, for the hybridisation to specific target sequences, converting it to a quantifiable signal [108-110].

DNA biosensors have gained considerable attention, with a surge in interest following the completion of the human genome project. In the last decade DNA biosensors have been extensively studied for a wide range of applications, such as diagnosis of genetic diseases [111, 112], detection of infectious pathogens [113, 114], tissue matching [31], genetic expression monitoring [115], forensic medicine [116], and drug screening [117].

Several factors play an important role in successful the development of a DNA sensor including probe design and immobilisation method, hybridisation conditions and transduction. Each factor requires optimisation to improve the overall performance of the sensor.

1.3.3. Probe design

Probe design is a crucial step in the development of DNA biosensors particularly when trying to differentiate SNPs and nutations. Probe sequence and length, GC content, and mismatch position play an important role in the sensitivity and selectivity of the genosensor. The probe is typically a single stranded oligonucleotide 15 to 50 bases in length and complementary to a specific region in the target DNA [110]. Usually the probes

are linear oligonucleotides although hairpin oligonucleotides are also frequently used [118-120]. Hairpin probes are structured oligonucleotides composed of a stem-and-loop structure. The stem is a double-stranded part formed by intramolecular base pairing, whereas the loop is a single-stranded portion containing the capture sequence [121, 122]. Normally, probe is single stranded DNA (ssDNA), although peptide nucleic acid (PNA) [123] and locked nucleic acid (LNA) probes [124] have been used to increase sensitivity and selectivity of genosensors.

1.3.3.1. Peptide nucleic acid (PNA)

Peptide nucleic acid (PNA) (Figure 1. 10 B) is an analogue of DNA composed of a "peptide-like" backbone consisting of repeated N-(2aminoethyl)glycine units linked by peptide bonds, with the four purine and pyrimidine bases linked to the backbone by methylene carbonyl bonds [125, 126]. In contrast to the negatively charged DNA backbone, PNA has a neutral backbone. As PNA lacks electrostatic repulsion, the PNA/DNA duplex is more stable than the DNA/DNA duplex and moreover is relatively insensitive to ionic strength and has a higher selectivity against single base mismatches. Moreover, it is also chemically stable over a wide range of pH, and to nuclease and protease degradation [127-129].

1.3.3.2. Locked Nucleic Acid (LNA)

Alternatively, the capturing probe can be modified at a specific position with one or more locked nucleic acids (LNA) (Figure 1. 10 C), which are nucleic acid analogues, wherein the furanose ring of the ribose sugar is locked by methylene linkage between the 2'-oxygen and the 4'-carbon. The melting temperature per LNA modification is higher than unmodified duplexes ranging between +3.0 °C to +9.6 °C [130-132].

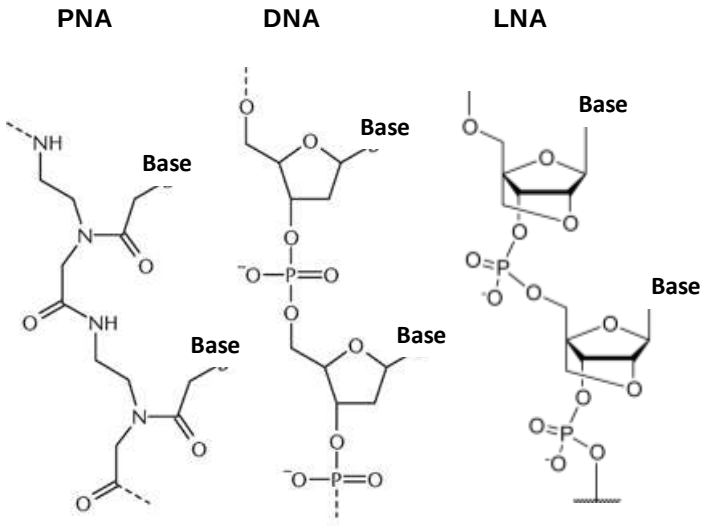

Figure 1.11. Structures of PNA, DNA and LNA oligonucleotides.

1.3.4. Probe immobilisation methods

Probe immobilisation is a key factor in the development and overall performance of genosensors. The essential function is to acquire high sensitivity and selectivity via maximisation of the hybridisation efficiency and minimisation of non-specific adsorption. This can be achieved by controlling the surface chemistry of the recognition layer through optimisation of packing density and orientation of probes to facilitate the hybridisation event [133, 134]. Several techniques have been used for probe immobilisation, and the choice of method usually depends on the transducer surface characteristics.

1.3.4.1. Adsorption

Adsorption is the simplest approach for probe immobilisation, as it does not require any modification of the nucleic acid probe. Adsorption is usually assisted by applying a positive potential to enhance stability by electrostatic interaction between the negatively charged phosphate backbone of DNA probe and the positively charged transducer surface [135-137]. Adsorption presents several drawbacks such as random orientation of the probes, which might affect the accessibility of target complementary strand to the immobilised probe. In addition, high sensitivity and selectivity cannot be achieved as stringent washing may in fact desorb the probe from the sensor surface [133, 138].

1.3.4.2. Avidin/Streptavidin- biotin interaction

Avidin and streptavidin are tetrameric large proteins (70 kDa) with four identical binding sites to which biotin can bind with very high affinity (Ka= 10^{15} M^{-1}). This value is approximately equal to a covalent bond that can only be broken under harsh conditions. Because of the high binding affinity and simplicity, avidin and streptavidin-biotin interactions are widely used in biosensors [139-143].

1.3.4.3. Covalent attachment

Covalent attachment of nucleic acid probes to the transducer surface is widely used. For example 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and sulfo-*N*-hydroxysuccinimide (NHS) are used to cross link carboxyl to amine groups forming an amide bond. This direct covalent attachment of oligonucleotide probe has been reported using amino modified probe [144] or alternatively, amino groups of the guanine bases of unmodified probe [145] to cross-link to carboxylate groups of activated glassy carbon electrodes.

Alternatively, oligonucleotide probes may be immobilised covalently with surfaces in two steps, where firstly the surface is modified with an amino or carboxyl terminated compound such as thiols [146], diazonium moieties [147-149] or conducting polymers [150]. Subsequently, in the presence of EDC and sulfo-NHS amino/carboxy modified nucleic acid probe can be covalently immobilised to the derivatised surfaces.

A new approach has been explored for direct covalent immobilisation of DNA probe on electrode surface based on diazonium chemistry. Recently, biomolecules immobilisation by grafting of diazonium salts has gained attraction because of its high stability at harsh solvent conditions, sonication, and storage in air for several months [151], and also it shows stability at high temperature [152]. Direct covalent attachment of DNA probe modified with 4-aminobenzylamine aniline was reported by Corgier et al. [153], where they covalently immobilised the modified probe via electroaddressing on a screen-printed graphite electrode. This method was used to develop a biochip for the detection of the p53 tumour suppressor gene [153].

1.3.4.4. Self Assembled Monolyers (SAM) on Au

There are many self assembled monolayer (SAM) systems, but monolayers of alkanethiolates on gold are the most studied [154]. Expanding from these reported SAMs, thiol modified oligonucleotide probes have been commonly used for direct immobilisation on gold electrodes. SAM formation occurs through chemisorption on to a Au surface through S linkages [155]:

$$RSH + Au \leftrightarrow RS\text{-}Au + e^{-} + H^{+}$$

Mixed self-assembled monolayers of oligonucleotides and short-chain alkanethiols were first reported by Tarlov [156] and take advantage of a two-step method, by exposing a gold electrode to a micromolar solution of thiolated nucleic acid probe, followed by exposure to a millimolar solution of a short-chain alkanethiol [156-162], referred to as backfiller. This system allows good packing density, and the "backfiller" can also prevent non-specific adsorption, while improving probe orientation.

Alternatively, simultaneous co-immobilisation of thiol-modified DNA with a thiolated spacer molecule to form a mixed monolayer has also been used [163-167], where the probe surface density is controlled by optimisation of the molar ratio between the thiolated probe and the alkanethiol co-immobiliser in the deposition solution.

The ionic strength of the immobilisation solution was found to play an important factor in the effect on surface density of the probe, as it has been demonstrated that high ionic strength minimises the electrostatic repulsion between the DNA probes [156].

Recently, mixed SAMs of dithiolated scaffolds were used for preparation of highly stable DNA biosensors [152, 168, 169]. These molecules contain a phenyl ring, poly(ethylene glycol) chain and two identical alkylthiols that provide two attachment points on surface. Dithiols provide more stable SAMs than monothiols and adequate probe spacing, whilst the poly (ethylene glycol) chain prevents nonspecific interactions.

1.3.4.5. Retention in a polymeric matrix

Probe immobilisation by retention in polymeric matrices has also been used in DNA biosensors [170-173], and electropolymerisation of conducting polymer is the most common approach for nucleic acid probe entrapment into polymeric matrix [170, 171, 174, 175]. This method is simple and can be carried out in one-step through application of an

appropriate potential to the working electrode immersed in an aqueous solution containing the DNA probe and an electropolymerisable monomer. During polymerisation, the DNA probes are physically incorporated within the growing polymer. This method provides immobilisation of high amount of probe, but with poor orientation and conformational mobility leading to decreased accessibility and hybridisation, and also suffers from long term stability problem.

1.3.5. DNA hybridisation detection

1.3.5.1. Optical

Several types of optical transducers have been used in DNA biosensors, such as surface plasmon resonance (SPR), fluorescence, and colorimetric as discussed below.

a) Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) is an optical technique that measures changes in the refractive index at the interface of a thin metal-coated prism and a solution (Figure 1.11). Binding of biomolecules will change the refractive index and appears as a change in the resonance angle, which is directly proportional to the mass adsorbed on the surface [176-179]. SPR is a label free real-time monitoring of biomolecular interaction and has been extensively used for detection of antigen-antibody [180, 181], RNA- protein [182, 183], DNA-protein [184, 185] and DNA-DNA interactions [186-188].

Milkani et al. [189] used SPR to study a single base mismatches at different location, and they achieved a limit of detection of 20pM. A single base mismatch study showed that a better discrimination for the middle mismatch than proximal mismatches. In their experiments, changing buffer concentration, flow rate, and temperature did not influence the sensor performance in single nucleotide mismatch detection. Rachkov et al. [190] developed a DNA biosensor based on SPR, where thiolated probes immobilised on gold surface was used to detect the normal and mutated rpoB genes of *Mycobacterium tuberculosis*. The sensor showed a clear sensogram difference between the fully complementary and single-base mismatched oligonucleotide, with a limit of detection of 10nM for a 21-mer target. Zhang et al. [191] used a SPR based DNA biosensor to detect *invA* gene of *Salmonella*. A hybridisation study with different concentrations of synthetic targets showed a linear response from 5 nM to 1000 nM

with a detection limit of 0.5 nM. Assay sensitivity was confirmed by the detection of real PCR product in a range from 10^2-10^{10} CFU ml^{-1}.

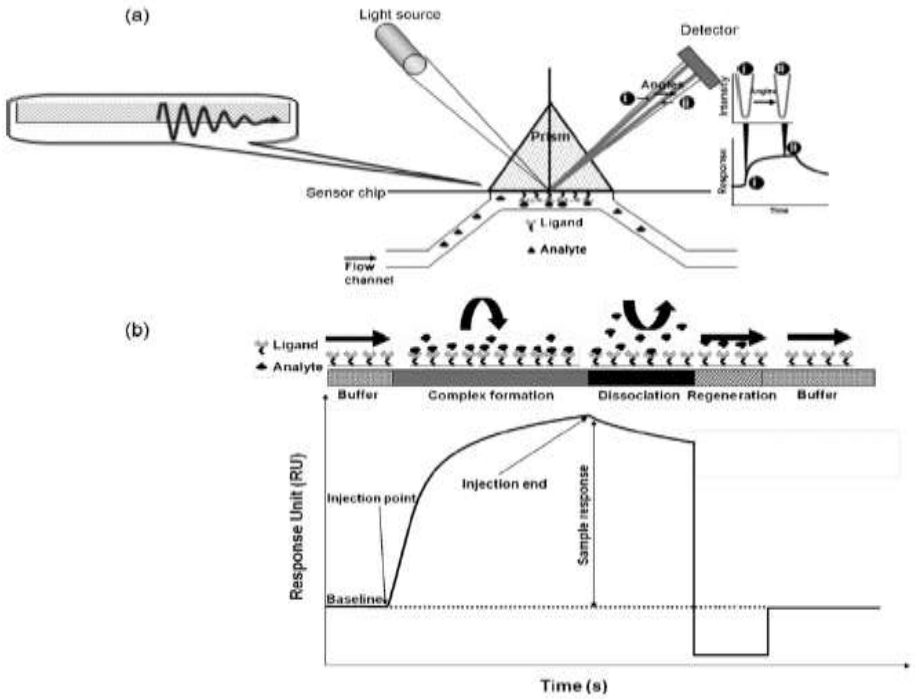

Figure1.12. Basic set-up of surface plasmon resonance (a) and typical sensogram response (b), [179].

SPR imaging (SPRi) technology is based on the same conventional SPR principle, except that the signal detection is recorded for sensogram and images using CCD camera [192-194]. The main advantages over classical SPR are the ability to visualise the whole biochip surface in real time and the possibility to detect in an array format for up to hundreds of molecular interactions simultaneously [194].

b) Fluorescence detection

The use of fluorescence has been widely applied in DNA biosensors because of its simplicity and sensitivity. Hybridisation of target DNA labelled with a fluorophore dye is detected by emission intensity which is proportional to the target concentration [195-199]. Another approach for the direct fluorescent detection of DNA hybridization has been developed using molecular beacons (MB) as a probe [200-202]. MB is a single stranded oligonucleotide, which has stem and loop hairpin structure. The loop contains a probe sequence complementary to a target, while the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A

MB is labelled with a fluorophore and a quencher on the two ends of the stem. In normal conditions, MB has a hairpin structure and the close proximity between the fluorophore and quencher prevents emission of fluorescence. With hybridisation of the complementary target, the hairpin structure opens, separating the fluorophore from the quencher and emission of fluorescent can be detected (Figure 1.12).

Beni et al. [203] used a gold nano-particle supported fluorescence molecular beacon for the detection of cystic fibrosis mutation. The sensing platform showed a good discrimination between the mutant and wild type gene with detection limit of 1 nM

To improve MB assay sensitivity, Lin et al. [204] exploited a MB with seven thymidine bases in the presence of Hg^{2+} for SNP detection. Following hybridisation with the target, the DNA duplex formed and lead to fluorescence emission, the assay detecting single base mismatched with a 0.5 nM limit of detection.

The MB approach can be used for simultaneous multiplex DNA detection by labelling it with a different fluorophores colour [205-207].

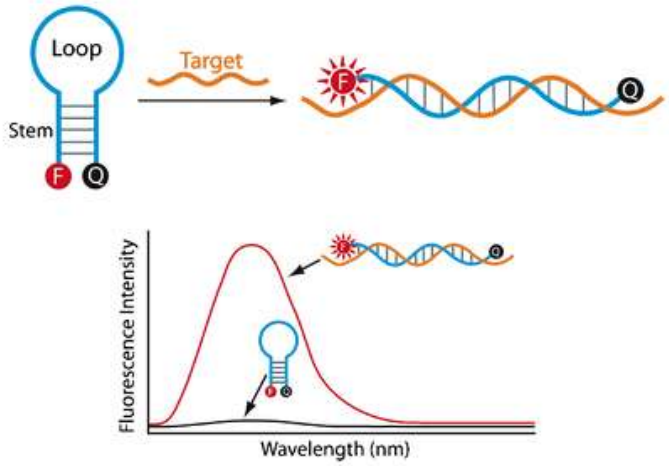

Figure 1.13. Principle of molecular beacon based fluorescent DNA biosensor [208].

c) Enzyme Linked Oligonucleotide Assay (ELONA)

An Enzyme-linked immunosorbent Assay (ELISA) a colorimetric technique, used to detect an antibody or antigen in a sample [209-214] and ELISA was introduced for the first time by Peter Perlmann and Eva Engvall at Stockholm University in the early 70s [215]. Since then ELISA has been widely used in clinical practise as a diagnostic immunoassay tool. Lately, Enzyme-linked oligonucleotide assay (ELONA) for the detection of nucleic acid hybridisation has been reported [216-221], where the target sequence is sandwiched between a short immobilised probe and an analyte labelled

reporter probe. DNA hybridization is evaluated by colour change after addition of enzyme substrate, and measured by absorbance signal which is directly proportional to the concentration of target hybridisation [220, 222]. Acero Sanchez et al. developed a colorimetric sandwich ELONA for rapid screening of five different genes related to breast cancer by detection of single-stranded MLPA-PCR product [218]. In another work, Deng et al. used this method for multiplex detection of single nucleotide polymorphisms in *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* from clinical samples [223], and recently Seo and his collages developed a colorimetric assay for the detection of hepatitis E virus [224]. The use of colorimetric assay for HLA typing using microtitre plates has been reported. Moribe and his co-workers developed a hybridisation assay for rapid low resolution typing of class I (HLA-A, B and C) [225], Allen et al. [226] and Kawai et al. [227] used a microtitre plate for typing of HLA class II DRB alleles.

Mirkin and his group introduced colorimetric DNA hybridisation detection using nanoparticles in 1997 [228]. They modifying two sets of gold nanoparticles (AuNPs) with different DNA probes, that where complementary to the 3' and 5' end of a DNA target, respectively. Upon hybridisation the functionalised AuNPs were brought in close proximity, resulting in aggregation, which was detected by a colour change in the colloidal solution from red to blue. This method has garnered a lot of interest and increasingly used for the detection of DNA hybridisation [229-234].

1.3.5.2. Electrochemical detection

Electrochemical transduction for detection of DNA hybridisation is widely used due to its high sensitivity, minimal power requirements, simple design, suitability for miniaturisation, portability and relatively low cost compared to other techniques.

Electrochemical techniques used for biosensing include amperometric, voltammetric, conductimetric, potentiometric and electrochemical impedance spectroscopy, and these can be further divided into labelled and label-free detection methods.

a) Label free electrochemical detection

Guanine oxidation

Palecek and his group discovered the electroactivity of the nucleotide bases in the early 1960s [235], and guanine has been shown to be the most easily oxidized one and has been used for indicator-free hybridization detection [236]. A reduction in guanine oxidation

signal can be used as an indication for target hybridisation, but this cannot be applied to targets containing guanine. To overcome this limitation, Wang et al, [237, 238] substituted guanine with inosine bases (guanine analogue that can form hydrogen bond with cytosine) in the sequence of the capturing probe and DNA hybridisation is detected via oxidation of guanine bases in the target [237].

Souza et al. [239] reported detection of a sequence for dengue virus gene 1, where the hybridisation between the probe and DNA target was monitored via guanine oxidation by differential pulse voltammetry (DPV). A linear detection range was achieved from 1 to 40 nM with a detection limit of 0.92 nM. Moreover, specificity was tested in the presence of non-complementary sequences of dengue virus 2 and 3, and showed good selectivity of the assay. Mascini et al. [240] developed sensors based on inosine modified (guanine-free) capture probes immobilized on screen-printed graphite electrodes for the identification of mammalian species. Extracted genomic DNA samples from bovine and porcine were subjected first to restriction enzyme treatment, then hybridisation over the captured probe without polymerase chain reaction (PCR) amplification. Square-wave voltammetry method was used to measure guanine oxidation and they were able to discriminate between different species with a detection limit of 30 μg / ml.

Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopy (EIS) is considered as a powerful tool, label-free approach for detection of DNA hybridisation [241]. The principle of EIS depends on the measurement of the current response to an applied oscillating potential as a function of the frequency by recording the resistance/capacitance changes that occur at electrode surfaces [242-244]. Faradaic EIS is normally performed in the presence a redox active species ($[Fe(CN)_6]^{3-/4-}$) that are alternately oxidized and reduced by electron transfer to and from the electrode surface [245, 246].

EIS is an attractive tool for DNA biosensor characterisation and has been used as simple labelless method by many groups [247-250], it can be used to monitor the build up of the genosensor starting from probe immobilisation up to target hybridisation [250-252]. Liu et al. [253] used PNA probe to study the kinetics of in-situ hybridisation of PNA/DNA by measuring the change of charge transfer resistance (R_{ct}) with time. For single base mismatch detection, Ito and his group [254] used a 15-mer DNA probe to investigate EIS detection of a single base mismatch. Nasef et al. [255] reported a thermally modulated electrochemical impedance spectroscopy for the discrimination of the DF508 mutation

of cystic fibrosis from the wild-type sequence. They observed an improvement in the discrimination between the mutant and wild type sequence by raising the temperature to 40 °C during hybridisation and post hybridisation washing. Moreover, several approaches have been used for EIS signal enhancement such as using polymer layers [173], carbon nanotubes [256], dendrimers [257] and nanoparticles [258, 259].

b) Labelled based electrochemical detection

Interacting Electroactive Substances

Electroactive redox markers represent one of the first methods used for development of electrochemical DNA biosenors [145, 260-262]. This method is simple as it does not require chemical modification of the DNA target as it is based on selective interaction of these substances with the double stranded DNA (dsDNA).

Cobalt complexes [$Co(bpy)_3^{3+}$ and $Co(phen)_3^{3+}$] are electroactive indicators that bind electrostatically to the minor groove of the dsDNA helix [145, 261, 263]. Hoechst 33258 also works as a minor groove binder by interacting mainly with adenine/thymine rich area of DNA helix [262, 264, 265].

Daunomycin and doxorubicin are anthracycline antibiotics for cancer therapy, which are also used for DNA hybridisation detection by intercalation G-C base pairs of DNA duplex [266-268].

Methylene blue is an organic dye that has strong affinity to DNA. Methylene blue affinity to DNA has been explained by different mechanisms [269-271], including electrostatic interaction of methylene blue to free guanine bases on ssDNA [111, 271, 272]. However, interacting electroactive materials for DNA hybridisation have the disadvantage of a high background signals due to the nonspecific binding of intercalators [135]. Ruthenium metal complexes such as $Ru(bpy)_3^{+2}$ are redox active mediators, frequently used for electrochemical DNA detection. Hybridisation of the target is detected by guanine oxidation in the presence of $Ru(bpy)_3^{+2}$, which increases the rate of electron transfer between DNA and the electrode [273, 274]. Probes without guanine base are commonly used to decrease background signal [273]. Electrostatic interaction of $[Ru(NH_3)_6]^{3+}$ with the anionic phosphate backbone of DNA has been used for facile electrochemical DNA detection [275]. Steichen et al. [276] developed a DNA biosensor for *H. pylori* detection, using PNA probes that lack anionic phosphate groups as a capture oligomer on the gold substrate. Upon hybridization, $[Ru(NH3)_6]^{3+}$ is

adsorbed on the DNA backbone, giving a clear hybridization detection signal in ac voltammetry.

Ferrocene

The use of ferrocene-labelled oligonucleotides for the direct detection of target hybridisation is not very common. Nakayama et al. [277] demonstrated a sandwich hybridisation assay using a ferrocene-conjugated DNA probe (Figure 1.13), where the electrochemical signal was proportional to the amount of hybridised target, and were able to detect single-base mutation using differential pulse voltammetry (DPV) as the electrochemical technique.

The CMS eSensor™ system of Motorola Inc. was one of the first commercial electrochemical DNA chips based on a sandwich hybridisation assay using a ferrocene label. Target DNA hybridisation is detected by sandwich hybridisation of ferrocene-modified nucleotides in close proximity to the surface [278, 279].

Mir and Katakis [280] developed a DNA biosensor by pre-hybridisation of the capture probe with a sub-optimum mutated oligonucleotide labelled with ferrocene. Hybridisation of the fully complementary target displaced the sub-optimum ferrocene-labelled DNA and the decrease in electrochemical signal observed from the ferrocene, was proportional to the target concentration.

An alternative approach exploiting a ferrocene-labelled oligonucleotide used a molecular beacon probe (MB) labelled with ferrocene and thiol at the two ends of the probe immobilised onto a gold electrode [281-283]. Initially, the ferrocene is in close proximity to the electrode surface, facilitating electron transfer. Target hybridisation induces the opening of the stem-loop, increasing the distance between the ferrocene and the electrode, and a concomitant lowering of the electrochemical signal.

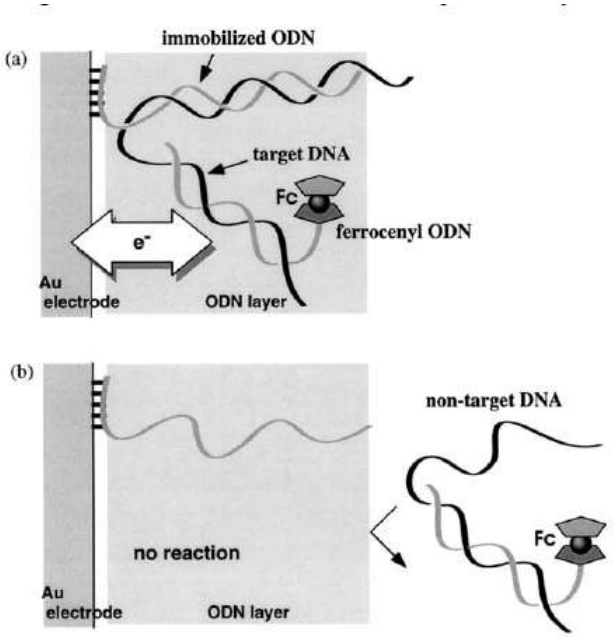

Figure1.14. Schematic of electrochemical detection based on ferrocene labelled DNA [277].

Enzymes

Detection of DNA hybridisation using an enzyme labelled reporter molecule has been widely reported [169, 284-288]. Enzyme may be linked to the target through biotin–avidin interaction or to a reporter oligonucleotide in the sandwich assay. Several enzymes can be used for oligonucleotide labelling such as horseradish peroxidase [HRP] [169, 288], alkaline phosphatase [ALP] [286], and glucose oxidase [GOx] [287]. From these, horseradish peroxidase (HRP) is the most commonly used one due to its stability, high turnover rate, and the availability of a wide variety of substrates.

The hybridisation event is detected by converting the catalytic activity of the enzyme in the presence of the substrate to an electrochemical signal, which is proportional to the target DNA concentration. This approach provides higher sensitivity and rapid DNA detection in comparison to other methods.

Wang and co-workers [289] developed an enzymatic DNA biosensor for detection of the K-ras gene. A thiol modified probe was immobilised on a gold electrode via self-assembly, and hybridisation of a complementary nucleic acid and HRP labelled oligonucleotide reporter probe was performed in a sandwich assay for 30 minutes for each step. The presence of K-ras gene was evaluated by measurement of H_2O_2 electroreduction current catalyzed by HRP in the presence of hydroquinone as mediator.

An enzymatic DNA biosensor for the detection of femtomolar DNA was reported by Liu and his colleagues [290]. The highly sensitive sensor was developed employing a

stem-loop (hairpin) structured DNA probe that was dually labelled with biotin and digoxigenin (DIG) at each end. Firstly, probe was immobilised on an avidin-modified electrode surface through the biotin-avidin interaction. Hybridisation of the complementary target induced opening of the stem-loop structure facing the DIG labelled end to the bulk solution and accessible for anti-DIG-HRP binding. Target hybridisation was performed for 30 minutes at 37 °C, and the sensor was incubated for a further 10 minutes for anti-DIG-HRP binding. Detection was performed by amperometry measuring the electrochemical reduction of hydrogen peroxide in the presence of TMB. Dong et al. [291] presented a sensitive electrochemical biosensor for sequence-specific DNA detection, based on sandwich hybridisation assay. For signal enhancement, multiple HRPs were adsorbed on Fe_3O_4 nanoparticles via layer by layer and a further gold nanopartiocles layer was used to facilitate DNA immobilisation. Following hybridisation of the target on immobilised captured probe, the sandwich hybridisation of conjugate (DNA-Au-HRP-Fe_3O_4) was carried out. The electrochemical behaviour of the prepared biosensor was investigated by the cyclic voltammetry (CV), chronoamperometry, and electrochemical impedance spectroscopy (EIS), and showed a detection limit of 0.7 fmol with an excellent discrimination of two-base mismatched DNA. Zhang et al. [292] reported an enzymatic DNA biosensor based on an electrode coated with cationic redox polymers containing osmium (4-vinylpyridine and acrylamide with some of the pyridines complexed with [Os(4,4'-bipyridine)$_2$Cl]). DNA probe was immobilised by electrodeposition on a 10-μm-diameter glassy carbon electrode and hybridisation was monitored following sandwich hybridisation with a HRP modified reporter probe, via amperometry after addition of H_2O_2, with a detection limit of 0.5 fM. For further signal enhancement, Thiruppathiraja et al. [293] developed an electrochemical DNA biosensor for *Mycobacterium* sp. genomic DNA detection in a clinical specimen. Target hybridisation was performed for 2 hours at 42 °C followed by sandwich hybridisation with a solution of AuNP modified with multi-detecting probe and ALP enzyme for 1 h at 40 °C. This approach showed high sensitivity with detection limit of 1.25 ng / ml.

Recently, Civit el al. [294] reported a genosensor array composed of 16 working electrodes for the simultaneous detection of three high-risk human papillomavirus (HPV) sequences (Figure 1.14). Detection of synthetic and PCR products was performed using sandwich hybridisation format and the genosensor exhibited high selectivity and detection limits within a pM range.

CombiMatrix (USA) [295] developed enzymatic arrays of 12,544 individual microelectrodes for genotyping of influenza H5N1 sub-types and gene expression analysis for phage lambda genome. Probes were exposed to biotin-labeled targets, and consequently, HRP-streptavidin conjugate was added to interact with biotinlyated target. Electrochemical detection was carried out in 25s by electro-reduction of the HRP reaction product in the presence of TMB using CombiMatrix ElectraSenseTM.

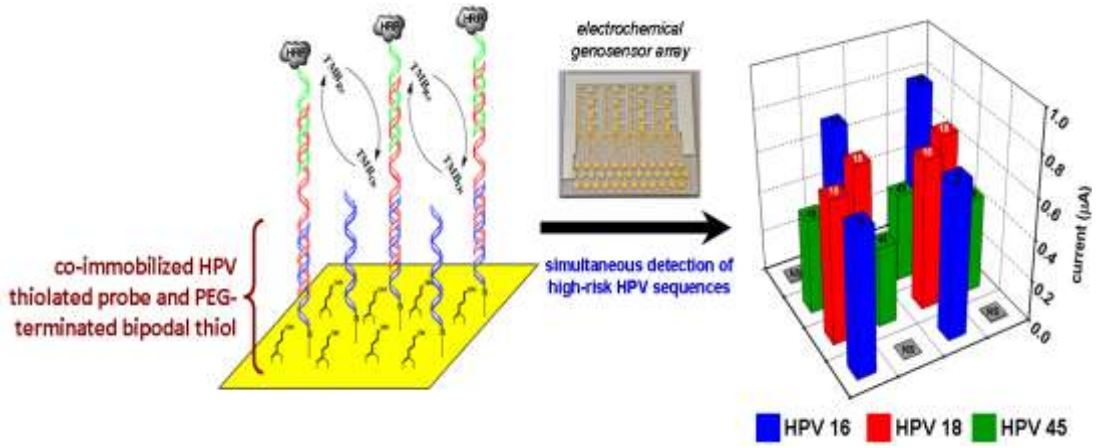

Figure 1.15. Schematic of an electrochemical genosensor array showing sandwich hybridisation assay for the simultaneous detection of three high-risk human papillomavirus (HPV) DNA sequences (HPV16, 18 and 45) [294].

Nanoparticles

Electrochemical detection of DNA hybridisation employing nanoparticle (silver or gold) as a labelling agent has been reported in various publications [296-300]. Several strategies have been used to measure the electrochemical activity of the metal nanoparticles, mainly based on potentiometric and voltammetric stripping techniques. The simplest method is the direct detection of the specific sequences via DNA hybridisation by anodic analysis of the gold nanoparticles on the electrode [298]. Moreover, nucleic acids modified with gold nanoparticles can be electrochemically detected using stripping techniques after dissolving the nanoparticles by HBr/Br_2 treatment [296]. Signal amplification can be achieved by silver deposition on gold nanoparticles [297].

1.3.6. Genosensors for PCR product analysis

Amplification of the part of the genome of interest decreases the DNA length to a few hundred base pairs (bp), whilst at the same time increasing the concentration of the sample. Conventionally, amplified PCR sample requires ssDNA generation before

hybridisation to the capturing probe, however several groups have reported PCR sample analysis without further treatment by detection of dsDNA or using triplex-forming oligonucleotides (TFOs), as discussed below.

1.3.6.1. ssDNA detection

In the majority of DNA biosensors and microarrays, post-PCR single stranded DNA (ssDNA) generation is necessary to obtain a single stranded DNA target. Several techniques have been used to generate ssDNA from double stranded PCR product, including heating, heat/alkaline treatment of avidin/biotin-dsDNA, asymmetric PCR and lambda exonuclease digestion.

The generation of ssDNA from PCR sample by heating is carried out by heating the sample at 95-100°C followed by rapid cooling on ice [114, 301, 302]. This is the most simple method but it has very poor efficiency. Another widely used method for ssDNA generation is the use of a biotin-modified primer (forward or reverse) to amplify the sample, following PCR amplification the biotinylated amplicons are immobilised on a streptavidin-coated surface (e.g. magnetic beads), and the duplex is denatured, by applying heat or alkaline treatment [303, 304], and the desired strand of DNA released. This method has better ssDNA recovery; however, this is time consuming, needs multiple steps and requires primer modification. Alternatively, asymmetric PCR is used to amplify one strand of the DNA more than the other, by using an unequal molar ratio of forward and reverse primers. Double stranded DNA is produced until one the primers is depleted and subsequently ssDNA is generated [30, 305, 306]. Asymmetric PCR does not need further treatment, but sometimes has poor yield and usually requires optimisation of PCR protocol and primers concentration.

Enzymatic digestion is an alternative method to prepare ssDNA. Lambda exonuclease is a highly processive 5′→3′ exodeoxyribonuclease that selectively digests the 5′-phosphorylated strand of dsDNA. To perform this, one primer is modified with phosphate at 5′ end, then after PCR amplification the ssDNA is generated by lambda exonuclease digestion of the phosphorylated strand [307-309].

1.3.6.2. dsDNA detection

PCR sample treatment for ssDNA generation is laborious, time consuming and needs post PCR manipulation. In addition, most of these techniques are not 100% efficient. As a result of this, some authors have proposed approaches for the detection of double stranded DNA (dsDNA) PCR product.

De Lumley-Woodyear and his co-workers [310] reported the detection of 800 bp dsDNA PCR product that had a digoxigenin label at the 3'end and multiple biotins along its chain, which were obtained using digoxigeninylated primers and biotinylated d-UTP-16-biotin as PCR reagents. The biosensor surface was prepared by coating the electrode with an electron-conducting redox hydrogel polymer, where an anti-digoxin monoclonal antibody was covalently attached. Two µL of the amplified PCR product was mixed with 700 µL of 0.1 M acetate buffer containing 0.1 M NaCl and 0.2% Tween 20, then incubated for 5 minutes to allow binding of its 3'-digoxigenin with anti-digoxin monoclonal antibody on the conducting redox hydrogel. Amperometric detection was performed after addition of horseradish peroxidase-labeled avidin for a further 5 minutes. Another approach for direct dsDNA detection was investigated by Hayashi et al. [311, 312], who demonstrated a SPR imaging biosensor for the detection of dsDNA PCR product. The forward primer was modified with a mirror-image DNA (L-DNA) tag that remained as a single strand after PCR amplification. Consequently, PCR product with the ssDNA tag was hybridised with a capturing DNA probe complementary to the tag sequence immobilised on gold and detected via surface plasmon resonance (SPR) imaging. Alternatively, Brasil de Oliveira Marques et al. [313] amplified Salmonella template using double tagging of PCR product with thiolated and digoxigeninated primers. The dsDNA PCR amplicon was immobilised through the thiolated end for 30 minutes on gold nanoparticles surrounded by nonreactive, rigid, and conducting graphite epoxy composite (nano-AuGEC) electrode. Electrochemical detection was performed after addition of anti DIG-HRP reporter and incubated for 30 minutes with a detection limit of 200 fmol.

1.3.6.3. Triplex DNA detection

Another alternative is the use of triplex-forming oligonucleotides (TFOs) for sequence-specific detection of double-stranded DNA. Triplex DNA forms either by the Hoogsteen base pairing when the third oligonucleotide strand binds to the purine base to

form T•A-T or C+•G-C triads, or through reverse Hoogsteen base pairing, when the third oligonucleotide strand binds to the purine base to form A•A-G and G•G-C triads [314-316] (Figure 1.15).

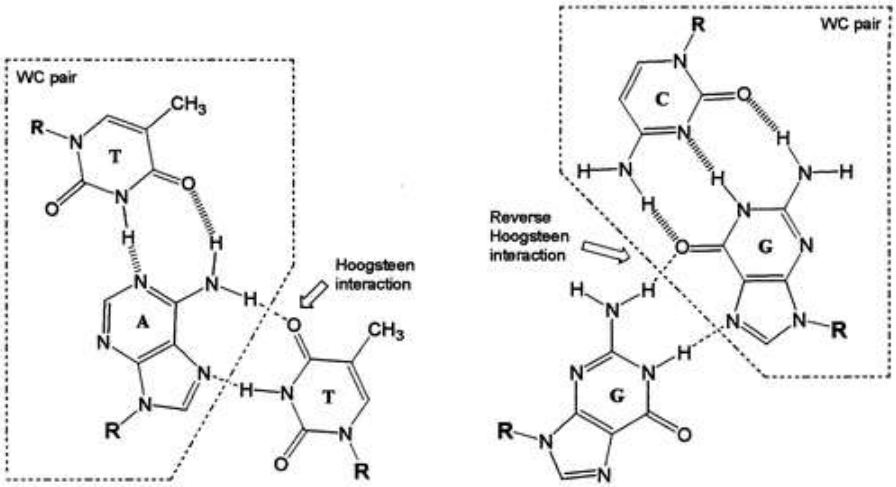

Figure 1.16. Hoogsteen and reverse Hoogsteen triplex DNA structure.

Several genosensors employing triplex-forming oligonucleotides (TFOs) as a recognition element for dsDNA, have been highlighted in the literature. Bates et al. [317] reported a plasmon resonance biosensor based experiment to study the specific triplex binding for TFO to murine c-myc gene in micromolar concentrations. They found that modification of TFO with RNA or 20-O-methyl RNA enhanced triplex formation. In another work, Patterson and co-workers [318] developed an electrochemical DNA biosensor used TFO modified at the 5′-end with a mercaptohexanol moiety and at the 3′-end with a methylene blue (MB) redox label. Hybridisation of the dsDNA HIV-1 strain was detected via square wave voltammetry (SWV), at concentrations as low as 10 nM. Pournaghi-Azar et al. [319] used a 14-mer guanine-free PNA probe to detect double-stranded oligonucleotide (dsDNA) corresponding to the hepatitis C virus genotype 3a. Hybridisation of the target was detected using differential pulse voltammetric achieving a 1.8 fM detection limit.

Triplex formation is a very elegant approach for the direct detection of dsDNA, but does require very careful design as the DNA triplex has the disadvantage of having low stability under normal physiological conditions requiring the use of stabilising agents.

1.3.7. DNA microarrays

DNA microarrays are also known as gene chips or biochips and use a set of different oligonucleotide probes ranging from tens to hundreds of thousands, immobilised on a single device [107], allowing simultaneous analysis of multiple analytes in a single experiment.

The first commercial DNA microarray was the GeneChip developed by Affymetrix in 1996 [320], using fluorescent tags to detect hybridisation, which is still the most popular label for high-throughput DNA microarrays.

Since then, research on DNA microarrays has been increased tremendously for a wide range of applications including gene expression [320-323], single nucleotide polymorphisms (SNPs) detection [324-326], pharmacogenomic research [327], HLA typing [19, 31, 328, 329] and disease diagnosis [330-332].

Microarrays are fabricated by immobilisation of oligonucleotides on a solid support, usually glass, although other materials like silicon or plastic have been used.

DNA probe immobilisation can be synthesised directly in situ on the support base by base using photolithographic techniques [333], or alternatively, the DNA probe can be presynthesized and then spotted on precise locations on chip surfaces using contact printing or noncontact printing [334, 335].

Generally, DNA microarrays may be divided into high-density DNA microarrays [295, 320, 336, 337] and low-density DNA microarrays [338-340]. High-density DNA microarrays are used for high-throughput analysis such as DNA sequencing and gene expression profiling. However, it requires long hybridisation time (16-24 hours), advanced technology for fabrication and special software for complex data analysis. Low-density DNA chips use a limited number of probes mainly for detection of specific genes or pathogens. Cannon et al. [338] reported a low density DNA microarray for detection of 16 viral and two atypical bacterial pathogens. The developed microarray showed comparable sensitivities and specificities to multiplex real-time PCR. In another work, Elsholz et al. [341] explored an electrochemical low density microarray for multiplex detection of 5 pathogens. The microarray composed of 16 individual interdigitated gold electrodes array for very sensitive detection without need of nucleic acid amplification, with a limit of detection 0.5 ng/µl.

1.3.8. Lab-on-a-chip

Micro-total analysis system (μTAS) also called "lab-on-a-chip" integrates multiple bioanalytical functions into a miniaturised device [342-344].

Development in the integrated nucleic acid based diagnostic assays was gained attention after Woolley et al. [345] reported a first work on integrated PCR and capillary electrophoresis detection in 1996. In their work, they demonstrated a high speed amplification and detection of β-globin and Salmonella in less than 20 and 45 minutes, respectively. Since then, several reports on integrated chips developed based on capillary electrophoresis detection [346-348], as it provides cheap and rapid detection platform but the main drawback is that it cannot differentiate between amplicons with the same size. An integrated microchip based on real-time PCR has been explored by several research groups [349-351]. This method has gained popularity as it provides real time detection of fluorescent signals generated in each amplification cycle, and no need for post-PCR processing, but it is an expensive technique and there are significant challenges in the miniaturisation of optical detectors. Alternatively, DNA integrated analysis using electrochemical detection has been used. Lee et al. [352] reported an integrated PCR with electrochemical detection microdevice for simultaneous DNA amplification and detection. Asymmetric PCR amplification was carried out to produce single-stranded target amplicons, followed by hybridisation on a probe-modified electrode. They employed two electrochemical based detection techniques including metal complex intercalators and nanogold particles achieve sensitive detection of target DNA analytes.

A further step in DNA analysis was the development of fully integrated and automated system with DNA extraction, purification, amplification and detection for sample-in answer-out diagnostic platform. Various detection approaches have been used including electrophoresis [353], electrochemical [354] and real-time fluorescent detection [355]. Advantages of μTAS include low cost, rapid analysis, high sensitivity, ease of use as well as low sample and reagent requirement. The growing interest in the development of lab-on-chip field has lead to the establishment of the point-of-care (POC) diagnostic test, which provides a diagnostic tool close to the patient whether in hospital, clinic, doctor's office, or at home [356].

1.4. State of the art and objectives

With the growth in genetic analysis technologies in the last two decades, genetic testing for diagnostics and disease predisposition analysis has garnered a plethora of interest.

It is well established that coeliac disease (CD) has a very strong association with HLA DQ2/DQ8 alleles, and an absence of these alleles virtually excludes the possibility of developing the disease.

The most widely used method for the detection of CD assoaciated HLA alleles is performed by sequence specific primers (SSP) usually in low resolution to produce amplicons of specific length, followed by gel electrophoresis [357, 358]. Several SSP commercial kits are available based on this method, including the Olerup SSP kit (Olerup SSP AB), HLA Ready Gene Coeliac Disease (INNO-TRAIN Diagnostik GmbH), Eu-Gen System (Eurospital) and HISTO TYPE Celiac Disease (BAG Health Care GmbH), all of which require multiple steps and several laborious reactions, requiring significant infrastructure and inherent cost.

There have been some recent advances specific for rapid and accurate DQ2/DQ8 typing such as that reported by Nils Reinton et al. [359] who developed a real-time PCR using both sequence-specific primers (SSP) and TaqMan probes for detecting CD-associated DQB1*05, DQB1*02 and DQB1*03:02 alleles. Monsuur et al. [360] explored a method to detect the CD associated HLA alleles using HLA-tagging single nucleotide polymorphisms (SNPs), using real-time PCR to detect SNPs in linkage disequilibrium with CD associated HLA alleles, reporting a very high sensitivity for the prediction of CD high risk alleles using six SNP tags. Ollikka et al. [361] developed a closed-tube PCR method for the analysis of dried blood and saliva samples using asymmetric PCR and time-resolved fluorometry for the genotyping of the HLA-DQA1*05, HLA-DQB1*02 and HLA-DQB1*03:02 alleles. Lavant et al. [362] explored a multiplex SSP method for detection of CD associated alleles based on fluorescently labelled primers. Following amplification, capillary electrophoresis was used for simultaneous visualisation and single-step typing. Profaizer and his group [363] simplified detection using SYBR Green real-time PCR with melting curve analysis for simple and one step detection of CD associated HLA-DQB*02, DQB*03:02 and DQA*05 alleles.

In this thesis, the overall objective of this work was to develop a rapid, easy to use, cost effective diagnostic genosensor array for HLA DQ2/DQ8 typing as a tool to detect potential CD predisposition.

To achieve this goal, two different and complementary approaches were explored;

- The first approach based on Sequence Specific Oligonucleotide Probes **(SSOP),** which relies on the design of sequence specific probes. Several probes were design for the CD associated DQ2/DQ8 alleles; specifically 7 probes were used for low-medium resolution typing, while 10 probes were required to achieve medium-high resolution typing. Genosensor arrays for the multiplex detection of the alleles were developed by immobilisation of these allele specific probes on electrodes followed by hybridisation of the target with detection being facilitated via hybridisation with a HRP modified secondary oligonucleotide in a sandwich format.
- The second approach, based on Sequence Specific Primers **(SSP)**, relies on the design of allele or allele group-specific PCR primers that only amplify the desired allele/s. For the typing of CD associated DQ2/DQ8 alleles, low resolution SSP method was achieved detecting DQA1*05, DQB1*02, DQB1*03:02 in addition to HGH as a positive control. A novel primer design was introduced to produce amplified double stranded PCR product with single stranded tails at each end of the amplicon, thus avoiding the need to denature the PCR product, as the ssDNA tail can hybridise directly to a capture probe on electrode surface.

Specific objectives for SSOP method are:

- To design and test probes for (medium / high) resolution genotyping of CD associated alleles.
- To evaluate assay time and temperature effect in the performance of the sensor using surface Plasmon resonance (SPR), ELONA and electrochemical genosensors.
- To apply the optimised genosensor array for high-resolution HLA typing of real samples from CD patients and healthy persons.

Specific objectives for SSP method are:

- To demonstrate the proof of concept for on surface detection of double tailed double stranded DNA (dt-dsDNA) using colorimetric method.
- To study the effect of excess un-reacted PCR primer in the detection of dt-dsDNA amplicaon, and explore possible methods to reduce it.
- To evaluate assay time and temperature in the genosensor detection of dt-dsDNA amplicon.
- To study the detection of individual DQ2/DQ8 alleles for cross hybridisation assessment
- Simultaneous genosensor detection for the low resolution genotyping of CD associated alleles from real samples amplified in a multiplex reaction.

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CHAPTER 2

Low–medium resolution HLA-DQ2/DQ8 typing for coeliac disease predisposition analysis by colorimetric assay

Analytical and Bioanalytical Chemistry, 2012, 403: 807-819

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Abstract

Coeliac disease is an inflammation of the small intestine, occurring in genetically susceptible individuals triggered by the ingestion of gluten. Human Leukocyte Antigens (HLA) DQ2 and DQ8 genes have been identified as key genetic factors in coeliac disease as they are presented in almost 100% of the patients. These genes are encoded by the combination of certain alleles in the DQA and DQB region of chromosome 6. Specifically, DQA1*05:01 and DQB1*02:01 alleles for serologically defined leukocyte antigen DQ2 cis, DQA1*05:05 and DQB1*02:02 for DQ2 trans and DQA1*03:01 and DQB1*03:02 alleles for the DQ8. Specific identification of these alleles is a challenge due to the high number of alleles that have been identified so far: 46 in the DQA region and 160 in the DQB region (as of IMGT/HLA Database 10/2011 release).

In the reported work, the development of a multiplex colorimetric assay for the low to medium HLA typing of the DQ2 and DQ8 genes is presented. The optimisation of probe design and assay conditions, performed by both surface plasmon resonance and enzyme-linked oligonucleotide assay, are reported. Finally, the performances of the developed typing platform were validated by the analysis of real patient samples and HLA typing, compared with those obtained using hospital based typing technology and an excellent correlation obtained.

Keywords: *Coeliac disease, HLA typing, sequence specific oligonucleotide probes, SPR, ELONA*

Introduction

Coeliac disease (CD) is a chronic immune-mediated enteropathy of the small intestine occurring in genetically susceptible individuals; this is characterised by bowel mucosal inflammation, crypt hyperplasia and villous atrophy [1, 2]. CD is triggered by the ingestion of dietary gluten proteins that are present in wheat and to a lesser extent in barley and rye [3]. In the past, CD was considered as a rare disorder of childhood, but more recent studies have shown that this is the most common disease in the western world affecting even up to 1% of general population [4]. However, most CD cases remain undiagnosed due to the ambiguity of its clinical presentation or to its so called clinically silent forms [3]; this lack in diagnosis is associated with a decreased quality of life and numerous long-

term complications such as osteoporosis, infertility and small intestinal malignant lymphoma [3, 5]

Currently, CD diagnosis is based on clinical presentation and serological testing for anti-tissue transglutaminase (tTG) or endomysial antibodies, confirmed by small intestinal biopsy, a rather invasive and expensive test [6, 7]. Recently, the European Society for Paediatric Gastroenterology Hepatology and Nutrition has proposed a new diagnostic criteria based on the Delphi process, where this achieved using combination of Human Leukocyte Antigen (HLA) typing and high titre levels of anti-tTG IgA antibodies, combined with detection of total IgA [8].

Several clinical studies have highlighted the relevance of HLA genes, located in chromosome 6 and CD susceptibility with ca. 95% of patients have the CD-associated HLA-DQ2 gene and almost all the remaining patients express the HLA-DQ8 gene [2, 5, 9], with individuals negative to these genotypes being excluded from developing or having CD [10]

The HLA-DQ2 antigen is an heterodimer encoded by DQA1*05:01 and DQB1*02:01 alleles in its cis form or by DQA1*05:05 and DQB1*02:02 alleles in its trans form, whilst the HLA-DQ8 antigen is encoded by DQA1*03:01 and DQB1*03:02 alleles [1].

Classically, HLA typing was performed by serological methods but these methods are only suitable for low resolution typing and do not allow the identification of the different alleles [11, 12]**.** The discovery of the polymerase chain reaction (PCR) considerably changed HLA typing leading to the development of PCR based typing methods including: sequence-specific primers (SSP)[13], sequence-specific oligonucleotide probe (SSOP) [14, 15], single strand conformation polymorphism [16], restriction fragment-length polymorphism [17] and sequencing based typing [18]. In SSOP approaches (direct [19] or reverse [20] dot blot assays), the specificity of the assay is provided by the use of probes reverse complementary to allele specific regions of amplicons obtained using generic or group specific primers.

The growing importance of PCR based typing has resulted in the development of commercial kits for low resolution HLA typing of CD associate genes [21, 22].

The enzyme-linked immunosorbent assay (ELISA) [23] is a colorimetric affinity assay based on antigen-antibody interaction, and is widely used in clinical practice as immuno diagnostic tool [24-27]. More recently “enzyme-linked oligonucleotide assay” (ELONA) [28], an affinity assay format analogue to the ELISA but based on oligonucleotide interaction

has become increasingly popular. Godfroid et al. described a colorimetric microtitre plate hybridisation assay for the detection and identification of three types of human papillomaviruses associated with cervical cancer [29]. Acero Sanchez et al. reported on a colorimetric sandwich ELONA for the rapid screening of five different genes related to breast cancer by detection of a single-stranded multiplex ligation-dependent probe amplification-PCR product [30], Deng et al. developed ELONA methods for the detection of a single point mutation in *Mycobacterium tuberculosis* genes [31] and Moribe et al. have detailed a reverse dot blot hybridisation assay for the rapid low resolution typing of class I (HLA-A, B and C) [20]. Allen et al. [32] and Kawai et al. [33] used a microtitre plate for typing of HLA class II DRB alleles and Legler et al. used the combination of multiplex polymerase chain reaction, ligase reactions and ELONA for the detection of human platelet antigen (HPA) systems HPA-1 through HPA-5 [34], highlighting the increased use of ELONA for DNA based diagnostics.

Surface plasmon resonance (SPR) is an extremely powerful tool for monitoring of biomolecular interactions and has been extensively used for the detection of antigen-antibody [35], RNA-protein[36], DNA-protein[37] and DNA-DNA [38] interactions. Several reports on the use of SPR technique for DNA biosensing and its ability for single point mutation detection can be found in the literature [39-42].

In the work reported here, the optimisation of the probe design and assay conditions, using both SPR and ELISA techniques, for the multiplex low–medium resolution HLA typing of the CD associated DQ2/DQ8 alleles and their implementation in an enzyme-linked oligonucleotide assay is presented. The SPR measurements were carried out as a support to the ELONA results for evaluation of probe, assay temperature - conditions which were then confirmed using ELONA. The SPR is a very useful system as once the probe is immobilised, the surface can be regenerated and subjected to different conditions, which is not easy to achieve with ELONA. The two techniques are complementary and also confirm each other's results.

The performances of the proposed sensing approach have been tested and validated by comparing them with those obtained at the Finnish Red Cross Blood Service (FRCBS) accredited Tissue typing laboratory using Luminex HLA-DQA1/DQB1 typing a standard technology for hospital laboratory based HLA typing.

Material and Methods

All reagents used in this work were of analytical grade: Trizma hydrochloride, sulphuric acid, phosphate-buffered saline pH 7.4 with Tween 20, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA were purchased from Sigma. Hydrochloric acid (HCl, 6M), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride (NaCl) and sodium dihydrogen phosphate (NaH_2PO_4) were obtained from Scharlau. Sodium hydroxide (NaOH) pellets were received from Panreac. Milli-Q water (18 MΩ cm) was obtained using a Simplicity water purification system (Millipore, France). Reacti-Bind™ Maleimide Activated 96-Well Plates were supplied by Pierce. DNA probes, Horseradish peroxidise (HRP) modified reporting sequences (listed in Table 2.1) and synthetic analogues of relevant PCR amplicons were supplied by biomers.net (biomers.net GmbH, Germany). Single stranded DNA samples from patients were provided from TATAA Biocenter (Sweden). Further information regarding the amplicons and their synthetic analogues can be found in the supporting information (Table 2.S1).

Surface plasmon resonance

Surface plasmon resonance experiments were carried out using the Biacore 3000 (Uppsala, Sweden) using Biacore bare gold chips (Sensor Chip Au). Following cleaning, the Au chip was docked in the instrument and flushed with PBS Tween buffer, at a flow rate of 15 µL min^{-1}, until stabilisation of the baseline was achieved. Probe immobilisation was performed by twice injecting 20 µl of 1M KH_2PO_4 containing 5 µM of specific probe at a flow rate of 5 µL min^{-1}. Subsequently, a 1 mM solution of mercaptohexanol was injected at a flow rate of 5 µL min^{-1} for 4 min to remove/displace weakly bound probes and to reduce non-specific adsorption of the DNA targets during the hybridisation. Specific and non-specific synthetic alleles were diluted to 250 nM in hybridisation buffer (10 mM Tris buffer containing 1 M NaCl pH 7.4) and injected for 5 min at a flow rate of 5 µL min^{-1}. The surface of the chip was regenerated via a 1 min wash with 15 mM NaOH to leave the chip surface still functionalised with the immobilised probe.

Table 2.1: Sequences of the probes and reporting elements.

Probes					
Probe name	**Probe sequence**	**IMGT/HLA matched alleles**	**codon**	***Tm °C***	***GC%***
DQA0201	5' SH-C6-CAAATCTAAGTCTGTGGA 3'	DQA1*02:01	58_1 – 51_2	36	38.9
DQA03	5' SH-C6- CTTCTAAATCTGCGGAACA 3'	DQA1*03:01, 03:02, 03:03	56_2 – 50_2	45	42.1
DQA05	5' TAACTCTCCTCAGCAGA-C3-SH 3'	DQA1*05:01, 05:05, 05:08, 05:09	164_2 – 159	36	47.1
DQB02A	5' CGGCAGGCAGCCCCAGCA-C3-SH 3'	DQB1*02:01, 02:02, 02:04, 02:05	58_1 – 52_2	65	77.8
DQB02gen	5' AGGCAGCCCCAGCAGCG-C3-SH 3'	DQB1*02:01, 02:02, 02:03, 02:04, 02:05	56 – 51_2	59	76.5
DQB0302A	5' CGCCCGATACACCCC-C3-SH 3'	DQB1*03:02, 03:03, 03:05, 03:06, 03:08, 03:10-12, 03:15, 03:17-18, 03:20, 04, 05:02:02, 05:03, 06:01, 06:19	49_3 – 45_1	52	73.3
DQB0302B	5' TCGGCGGCAGGCGGC-C3-SH 3'	DQB1*03:02, 03:04, 03:05, 03:07, 03:08, 03:11, 03:14, 03:18, 06:29	59_2 – 54_3	56	86.7

Reporting probes			
Reporting element name	**Reporting element sequence**	**Target exon**	**Codon**
DQAex2	5´GACAGTCTCCTTCCTCTC - HRP ´3	DQA Exon 2	45_3 - 40_1
DQAex3	5' HRP-CCCCAGTGTTTCAGAAGA 3'	DQA Exon 3	182_1 - 176_3
DQBex2	5' HRP-CTGGTAGTTGTGTCTGCA 3'	DQB Exon 2	84_3 – 79_1

ELONA hybridisation assay

Thiol functionalised probes were immobilised on maleimide activated microtitre plates following the manufacturer's instructions. Briefly, 100 µL of 500 nM of the specific probe in binding buffer (0.1 M sodium phosphate, 0.15 M sodium chloride, 10 mM EDTA; pH 7.2) was added to each well of the microtitre plate and incubated for 2 hours at 37 °C. Subsequently, 100 µL of mercaptohexanol (1 mM) was added to each well (incubation 1 hour at 37 °C) in order to inactivate excess maleimide groups. Hybridisation was performed by addition of 100 µL of 20 nM of the synthetic alleles solutions in hybridisation buffer (10 mM Tris buffer containing 1M NaCl, pH 7.4) or in case of real patients sample analysis, amplicons of the PCR product (diluted 1 to 10 in hybridisation buffer). Hybridisation with the target DNA was allowed to proceed for a pre-defined time at a controlled temperature. Subsequently 10 nM of the reporter probe in hybridisation buffer was added and left to hybridise for a given time at a controlled temperature. Between each step, the plates were washed three times with PBS-Tween using an automated microplate washer.

To detect the presence of captured DNA, TMB liquid substrate was added (100 µL) to each well and incubated for 5 min. Following incubation and colour development, the reaction was stopped by addition of 1 M H_2SO_4 and the absorbance measured at 450 nm using a microplate reader (EMax, Molecular Devices, BioNova Científica).

Multiplex PCR amplification and ssDNA generation:

Blood samples were collected from CD affected and/or suspected patients at Kuopio University Hospital (Finland). DNA isolation was performed using the Qiagen FlexiGene DNA kit, and HLA typing was performed in the accredited FRCBS Tissue typing laboratory. Typing was carried out using two different approaches: Luminex HLA-DQA1/DQB1 typing and confirmed by Olerup DQA1 or DQB1 SSP typing kits.

Multiplex PCR amplification was performed using iQ Multiplex Powermix from Bio-Rad in the presence of 20 ng of DNA template. Amplification was performed in 200 µL tube (50 µL reaction) format using an RotorGene 6000 thermocycler according to the following protocol: (a) initial denaturation at 95 °C for 10 min and (b) 40 cycles (15 sec at 95 °C and 60 sec at 60 °C).

For the multiplex amplification, three primer pairs, specifically designed for low to medium resolution DQ8-DQ2 typing, were used: DQA exon 2 (200 nM), DQA exon 3 (200 nM), DQB exon 2 (200 nM) and DQB exon 3 (100 nM). All the reverse primers

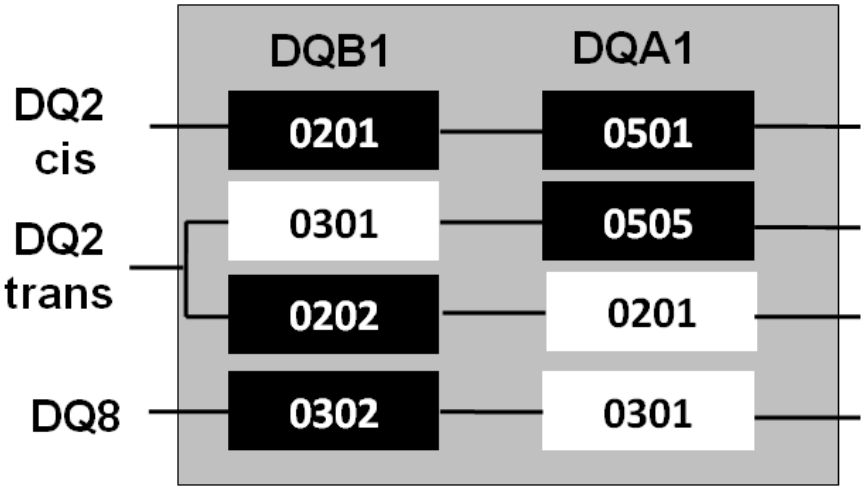
used in the reported work were phosphorylated at their 5' end in order to allow enzymatic generation of single stranded DNA via Exonuclease digestion.

ssDNA generation

In order to facilitate detection of the PCR product, generation of ssDNA is required. In the reported work generation of ssDNA was performed by means of Lambda exonuclease (Fermentas GmbH) mediated hydrolysis. Lambda exonuclease is a highly processive 5' to 3' exodeoxyribonuclease, which is able to selectively digest the phosphorylated strand of dsDNA, but it is characterised by a reduced activity on ssDNA and non-phosphorylated DNA [43, 44]. Single stranded DNA was generated by incubating the 50 µL PCR product, in the presence of 2 µL (20 U) of the exonuclease for 30 min at 37 ºC. Following the digestion, the solution was heated to 80 ºC for 10 min in order to inactivate the enzyme.

Results and discussions

In the reported work, the development of an ELONA assay, based on a SSOP hybridisation format, for the low to medium resolution typing of CD associated HLA DQ2-DQ8 antigens is presented. HLA-DQ2 is encoded by DQA1*05:01/DQB1*02:01 alleles in its cis format and by DQA1*05:05/DQB1*02:02 alleles in the trans format. HLA-DQ8 is encoded by DQA1*03:01/ DQB1*03:02 alleles (Scheme 2.1).



Scheme 2.1: Schematic description of the different possible allele combinations encoding the DQ2 and DQ8 genes.

In the reported work, low resolution assays are defined as the detection of allele groups without discrimination of specific alleles; for example, probe DQB02gen enables the detection of the whole DQB1*02 group but not the discrimination between members of the group. Medium resolution assays are defined as the detection of multiple alleles within the same group but not the identification of a single allele; for example, probe DQA05 enables the detection of both DQA1*05:01 and DQA1*05:05 alleles but it does not allow their discrimination. High resolution typing is defined as when the probe used is able to specifically identify a specific allele as in the case of the DQA0201 probe. The primary identification of the probes and of potentially interfering alleles was performed with the help of the IMGT/HLA database [45], the handbook of the 13th International Histocompatibility Working Group (IHWG) [46] and multiple sequence alignment software (Genedoc) [47]. The possible interfering alleles were chosen according to similarity and mismatch at the probe region. Alleles sharing the same sequence at the probe location were represented by one allele to be used in the assay. Finally, generic regions in the target alleles were identified for the design of different reporting sequences. Sequences of the different probes and reporting sequences, together with description of the synthetic targets/interfering alleles (sequences at probe region), are reported in Table 2.1.

Probes and interference alleles selection:

DQ2

*DQA1*05:01/05:05*

DQA05 probe was designed to recognise a sequence in the exon 3 DQA1 locus common to both DQA1*05:01 and DQA1*05:05 alleles, obtaining in this case a medium resolution typing of the DQA1*05 alleles of interest for CD. This probe was also able to detect DQA1*05:08, DQA1*05:09 and DQA1*05:10 (Table 2.1) alleles that are not associated to CD but that have a very low prevalence [48]. The specificity of the probe was tested against 3 potentially interfering alleles, DQA1*05:03, DQA1*02:01 and DQA1*03:02 presenting 1, 2 and 3 base mismatches, respectively. These alleles, described in Table 2.S1, were representative for all the known alleles in DQA1 locus at the probe region.

*DQB1*02:01/02:02*

DQB1 locus is more polymorphic than the DQA1, thus complicating the genotyping. A combination of two probes was required in order to perform the low resolution typing of DQB1*02:01 and DQB1*02:02 alleles, where the probes were designed to recognise two different regions of exon 2 of the DQB1 locus. The first probe, namely DQB02A, was designed to recognise the region between codons 52 and 58 (Table 2.1), which is a region shared by all the alleles in the DQB1*02 group with the exception of DQB1*02:03. For this probe, a total of 5 potentially interfering allele groups with 1, 2, 3, 4 and 5 base mismatches, respectively (Table 2.S1), were identified, probes that can detect some rare DQB1 alleles (DQB1*02:04, DQB1*02:05 and DQB1*02:06) that are not related to CD (Table 2.S1) but that have very low prevalence (<1% of the human population) [48].

Probe DQB02A is expected to have high cross hybridisation with the DQB1*03:02 allele, and in order to improve the specificity of the assay, the introduction of an additional probe was required. This further probe, DQB02gen, located one codon to the right side of the DQB02A probe, is able to recognise all of the DQB1*02 alleles group and has 2-3 nucleotide mismatches as compared to other DQB1 alleles. Again 3 potentially interfering allele groups, represented by the alleles DQB1*03:02, DQB1*04:01 and DQB1*05:01, were identified and used to test the specificity of the probe (Table 2.S1).

*DQA1*02:01*

In the case of this allele, the probe was designed in the exon 2 between codon 51 and 58, in a region with multiple nucleotides substitution/deletion between the perfect match (DQA1*02:01) and other DQA alleles. To test discrimination ability of the DQA0201 probe, 3 alleles: DQA1*01:01, DQA1*05:01 and DQA1*03:01, which represent all the possible interfering alleles in the DQA Exon 2 probe region, were used (Table 2.S1).

DQ8

*DQB1*03:02*

DQB1*03 allele is characterised by a large number of subgroups, making high resolution genotyping of alleles belonging to this group quite difficult. In order to achieve good resolution for the detection of the DQB1*03:02 allele, two probes (Table

2.1) were designed. Each probe was designed to exclude several nonspecific alleles, with the combination of the information recorded from these two probes facilitating identification of the DQB1*03:02 allele. The first probe (DQB0302A) is located between codon 45-49 of exon 2 of the DQB1 locus. This probe detects both the target allele as well as several alleles not related to CD (Table 2.1) including the DQB1*04 group. In order to improve typing specificity, the second probe (DQB0302B), located 5 codons to the 3' direction, was designed.

The combination of the two DQB03 probes allowed specific detection of the DQB1*03:02 allele. Again some rare alleles were also detected; these alleles, DQB1*03:05, DQB1*03:08, DQB1*03:11 and DQB1*03:18, have a very low prevalence (<1%) in the Caucasoid population [48], except for the DQB1*03:05 which is present in some population originally from Sardinia (Italy) [48, 49]. Potentially interfering alleles (Table 2.S1) were designed to allow evaluation of the specificity and selectivity of the two probes: 3 alleles were identified for the DQB0302A probe and 5 for the DQB0302B probe.

*DQA1*03*

A probe for low resolution genotyping of the DQA1*03 allele was designed in a region partially overlapping the DQA1*02:01 probe. The same potentially interfering alleles used for the DQA1*02:01 allele with the in addition of the DQA1*02:01 allele were used to test specificity of this probe.

Surface plasmon resonance

SPR experiments were carried out to evaluate in high throughput format, the specificity of the designed probes. As expected this was a function of the number of mismatches with higher nonspecific response associated with the alleles characterised by a single base mismatch.

As well established [50], increasing the assay temperature allows better discrimination between fully complementary and mismatched targets and thus the effect of the hybridisation temperature (ranging from 20 °C to 40 °C) on capture efficiency and probe specificity was tested. Figure 2.1, shows the changes in SPR responses, as a function of hybridisation temperature, obtained at the different probes for the hybridisation of the fully complementary targets. For all the SPR experiments, response was obtained for the dissociation part of the sensongrams. An example of a typical set of temperature

experiments can be found in the Supporting Information (Figure 2.S2). Interestingly, two different trends in the probe behaviour were observed. In the case of the DQA1 probes (DQA0201, DQA03 and DQA05), the SPR signal was higher with increasing temperature until a maximum was reached between 25 and 30 °C. Further increases in hybridisation temperature resulted in a significant drop in SPR response, especially for the DQA0201 and DQA03 probes.

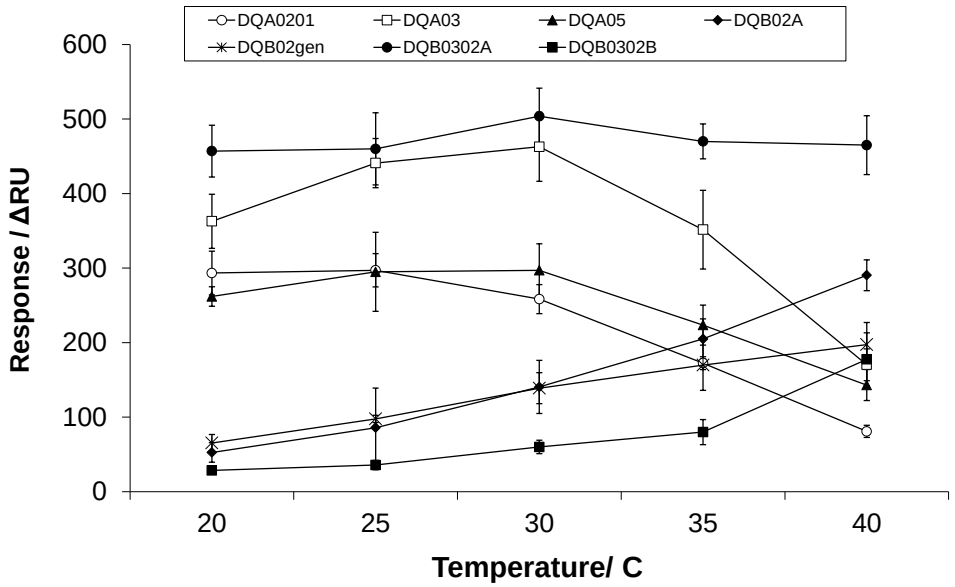


Figure 2.1: Plot of the RU variation resulting from the hybridisation of the fully complementary targets for the different probes as a function of the hybridisation temperature. Each value is the result of three sequential injection/regeneration cycles

In the case of the DQB1 probes (except DQB0302A probe), hybridisation responses increased with assay temperature. SPR signal for DQB0302A probe is almost constant throughout the different temperatures investigated.

In order to interpret the results obtained, the probe secondary structure was evaluated, as this is known to influence hybridisation [51-53]. A prediction of the presence of secondary structures was performed by calculating the melting temperature (Tm) and free energy (ΔG) of the self-complementary secondary structure of the different probes using Mfold Web Server [54], using a salt (Na^+) concentration of 1 M (those used in the hybridisation assay). From the simulation, the following thermodynamic parameters for the different probes were obtained: DQA0201; Tm = 11 °C, ΔG = 0.59 kcal/mol, DQA03; Tm = 23.1 °C, ΔG = 0.15 kcal/mol, DQA05; Tm = 25.4 °C, ΔG = -0.02 kcal/mol DQB02A; Tm = 56.7 °C, ΔG = -3.06 kcal/mol, DQB02gen; Tm = 52.7 °C, ΔG = -2.62 kcal/mol,

DQB0302B; Tm = 50.4 °C, ΔG = -1.96 kcal/mol. No secondary structure was predicted for DQB0302A probe.

In the case of the DQA1 probes no significant influence from the possible secondary structures of the probes can be expected as these are characterised by the low melting temperatures (11-25.4 °C); consequently hybridisation response can be expected to be dominated by the stability (melting temperature) of the probe/target duplex (Table 2.1). In the case of the DQB1 probes, the possible secondary structures were predicted to be very stable with melting temperature above 50 °C probably due to their high G-C content. In this case, the possible secondary structures are expected to compete with the hybridisation of the target DNA reducing the efficiency of the recognition event. When the hybridisation temperature was raised, considerable increases in SPR response were recorded, consistent with the fact that high temperature results in destabilisation of the secondary structures facilitating the hybridisation between the target and the probe.

Additionally, different probes were observed to have highly variable hybridisation efficiencies at low temperature, SPR signal for the most of probes are converged at higher temperature to cross each other in temperature between 35 and 40 °C (Figure 2.1). In Table 2.2, a summary of the normalised responses obtained for the most relevant interfering sequences is reported. For the majority of the probes, an increase in specificity was recorded with increasing temperature. This improvement was more relevant in the case of the DQA1 and DQB02gen probes where the responses recorded for the interfering alleles were considerably reduced from ca. 20%-40% (at 20 °C) to less than 10% at 40°C. In the case of the DQB1 probes, the improvement in specificity was much lower: from ca. 70%−85% to ca. 40%−70%. This reduced improvement could still be associated with the high G-C content of these probes that can provide considerable stability also for the mismatched duplex even at high temperature.

A further element that could play a role in the specificity of the assay is the position [55] and nature [56, 57] of the mismatched base. Kelley et al. demonstrated that sequences with mismatches far from the electrode surface, as in the case of DQB1*02:03, have higher stability than those with mismatches located closer to the surface, as in the case of DQA1*05:03 [55]. Furthermore, Allawi et al. showed that different base pairs possess different thermodynamic stabilities [56, 57] with the C-T pair mismatch (as in the case of DQA1*05:03) being less stable than the G-A mismatch as is present in the DQB1*02:03 allele. The surface plasmon resonance studies revealed the importance of temperature in

the optimisation of the selectivity and sensitivity of the proposed probes. As seen in Table 2.2, it is clear that hybridisation assay should be performed at elevated temperatures, and thus, it was decided to perform the ELONAs at 37 ºC despite the fact that at this temperature the responses at the DQA0201 and DQA03 probes are considerably suppressed.

Allele	**Normalised SPR response**				
	20 ºC	**25 ºC**	**30 ºC**	**35 ºC**	**40 ºC**
DQA05 Probe					
*DQA1*05:03*	38.5 %	35.3 %	26.1 %	21.3 %	14.4 %
DQB02A Probe					
*DQB1*02:03*	86.1 %	84.7 %	82.4 %	82.7 %	73.3 %
*DQB1*03:02*	86.3 %	75.1 %	65.8 %	56.2 %	36.6 %
DQB02gen Probe					
*DQB1*03:02*	44.0 %	27.0 %	15.1 %	7.9 %	6.5 %
DQB0302A Probe					
*DQB1*03:01*	31.5 %	26.9 %	14.5 %	12.2 %	9.8 %
*DQB1*05:01*	42.3 %	32.3 %	15.6 %	14.2 %	10.4 %
DQB0302B Probe					
*DQB1*02:01*	69.5 %	41.3 %	36.0 %	34.1 %	37.4 %
*DQB1*03:01*	67.7 %	41.3 %	41.1 %	36. 1%	36.1 %
DQA0201 probe					
*DQA1*03:01*	30.3 %	14.3 %	3.7 %	4.1 %	7.4 %
DQA03 probe					
*DQA1*02:01*	20.3 %	6.6 %	4.5 %	5.5 %	6.6 %

Table 2.2: SPR evaluation of the effect of temperature on the sensitivity and selectivity for all SSO probes used in this report. Temperature was investigated in the range of 20–40 °C. For each probe, % values were calculated as a ratio between RU responses obtained for the interference allele under evaluation and those obtained for the fully complementary allele.

Enzyme-linked oligonucleotide assay

Probes specificity evaluation:

Following the preliminary SPR screening, a series of ELONAs were performed to confirm the specificity of the different probes in the measurement condition. In this set of experiments, probes immobilised on maleimide activated plates were hybridised with the designed potentially interfering sequences (described in Table 2.S1).

DQ2

*DQA1*05:01/05:05*

As can be seen from Figure 2.2 A, the proposed DQA05 probe presented an excellent specificity for the DQA1*05:01 allele over the possible interfering alleles with only the DQA1*05:03 allele with a single base mismatch (G to T) giving a relatively considerable interfering signal (lower than 25% of the fully complementary allele (DQA1*0501).

*DQB1*02:01/02:02*

In the case of DQB02A probe, a good specificity over the DQB1*03:01, DQB1*04:01 and DQB1*05:01 alleles was achieved (Figure 2.2 B). As expected, the DQB1*02:03 and DQB1*03:02 alleles gave the highest interference with response associated to them being respectively the 57% and 44% of those obtained for the fully complementary target. In Figure 2.2 C, the results obtained using the DQB02gen probe are presented. The use of this probe allowed a good discrimination of the DQB1*0302 allele (response lower than 12% of the specific signal), confirming the possibility of detecting the DQB1*02:01 and DQB1*02:02 alleles with a good specificity using the combination of the two DQB probes.

*DQA1*02:01*

Probe DQA1*0201 allows high resolution typing for the DQA1*02:01 allele as this is the only allele in the DQA1*02 family. As can be observed in Figure 2.2 D, this probe was demonstrated to have a very high specificity for the fully complementary allele, due to the multiple substitutions and deletions (3-bp deletion at codon 56) between the perfect match allele and the possible interfering alleles (DQA1*01:01, DQA1*03:01 and DQA1*05:01).

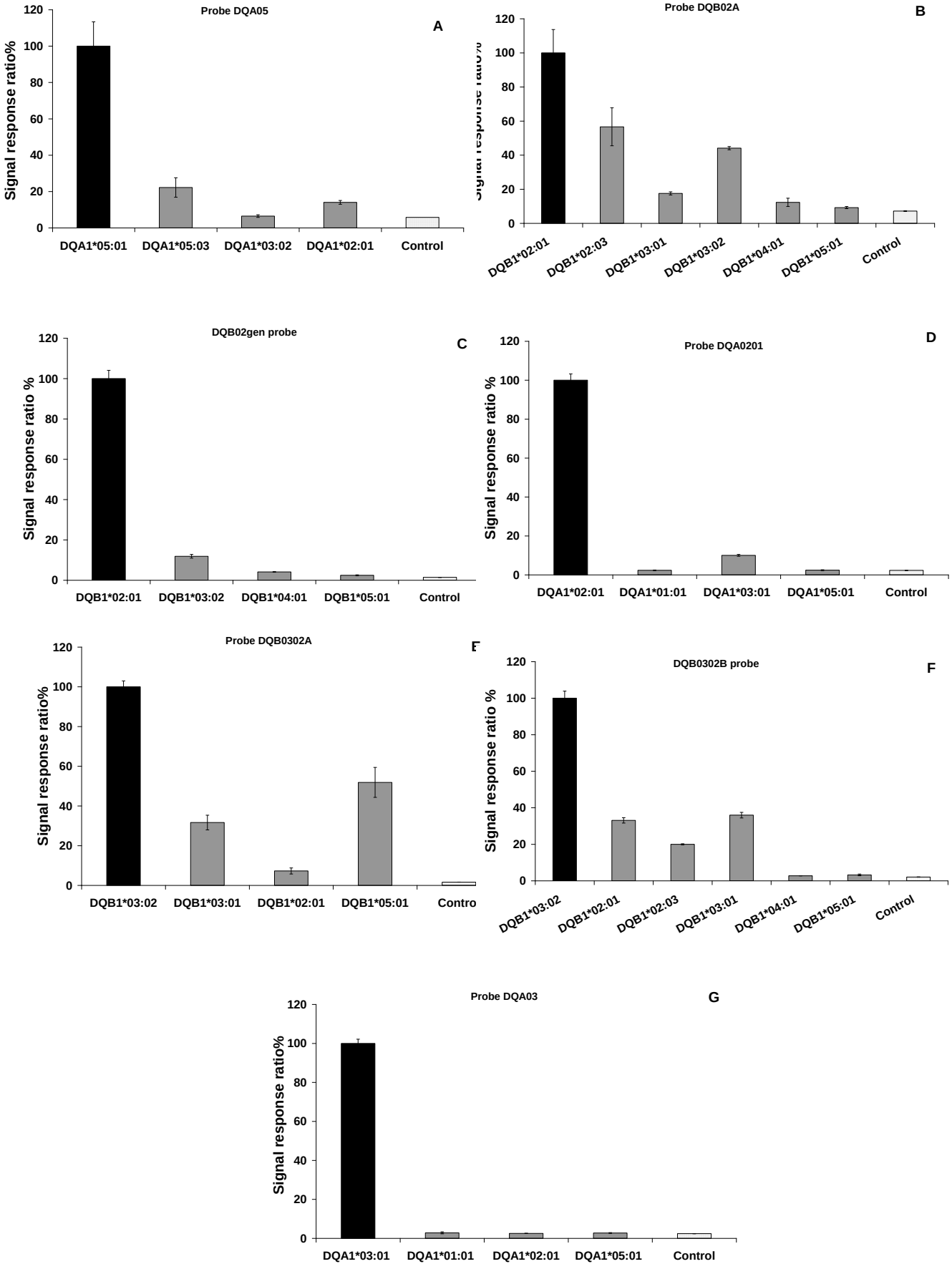

Probe DQA05
A
Signal response ratio%
DQA1*05:01
DQA1*05:03
DQA1*03:02
DQA1*02:01
Control
Probe DQB02A
B
DQB1*02:01
DQB1*02:03
DQB1*03:01
DQB1*03:02
DQB1*04:01
DQB1*05:01
Control
DQB02gen probe
C
Signal response ratio %
DQB1*02:01
DQB1*03:02
DQB1*04:01
DQB1*05:01
Control
Probe DQA0201
D
DQA1*02:01
DQA1*01:01
DQA1*03:01
DQA1*05:01
Control
Probe DQB0302A
DQB1*03:02
DQB1*03:01
DQB1*02:01
DQB1*05:01
DQB0302B probe
F
DQB1*03:02
DQB1*02:01
DQB1*02:03
DQB1*03:01
DQB1*04:01
DQB1*05:01
Control
Probe DQA03
G
DQA1*03:01
DQA1*01:01
DQA1*02:01
DQA1*05:01
Control

Figure 2.2: Evaluation of the specificity of the different probes designed for the DQ2-DQ8 typing: DQA05 (A), DQB02A (B) DQB02gen (C), DQA0201 (D), DQB0302A (E), DQB0302B (F) and DQA03 (G). Hybridisation was performed in the presence of 20 nM of the targeted DNA for 5 min at 37 °C and the capture of reporting sequence was performed from a 10 nM solution for 20 min at 37 °C. The absorbance values were normalised to the reading of the fully complementary allele.

DQ8

*DQB1*03:02*

In Figure 2.2 E, F, the results of the cross hybridisation studies for the DQB1*0302 probes (DQB0302A and DQB0302B) are presented. Probe DQB0302A allowed good specificity for the DQB1*03:02 against the interfering targets DQB1*02:01 and DQB1*03:01 as the potentially interfering sequences have between 2 and 3 nucleotide mismatches. However, a high non-specific hybridisation signal (ca. 60% of the specific response) was obtained for the DQB1*05:01 allele, characterised by one base mismatch located at the terminal end.

In order to reduce the possibility of false positives a second probe (DQB0302B) was introduced and this second probe provided a good specificity for the DQB1*03:02 allele with a lower than 35% response for the DQB1*03:01 allele (major interfering sequence). This probe allowed the discrimination of the DQB1*03:02 from the DQB1*04:01 and DQB05:01 alleles, the major potentially interfering alleles for the DQB03A probe.

*DQA1*03*

The selectivity of the DQA03 probe was tested in the presence of specific and non-specific alleles and the probe was demonstrated to be very specific with very low non-specific hybridisation, lower than 3% of the specific response (Figure 2.2 G).

Hybridisation time/temperature

Optimisation of the timing of the different steps (target hybridisation and reporting sequence hybridisation) is extremely important in affinity assays influencing as these factors affect both the response intensity and the specificity [30]. An optimisation of assay time was performed for two probes (DQA03 and DQB02A), one for each of the group identified during the SPR evaluation, at two different temperatures (22 C° and 37 C°).

In this experiment, the capture of 20 nM of the fully complementary alleles and 10 nM of the reporting element was used and hybridisation times between 5 and 60 min were evaluated.

To optimise the target hybridisation time, the capture of the reporting element was performed for 60 min, and optimisation of the hybridisation with the labelled reporter probe was carried out using an optimised 5 min target hybridisation time. In Figure 2.3A the results obtained for the target hybridisation time at the two temperatures for the DQA03 probe are presented. For this probe, no significant differences were recorded when the target hybridisation was performed at 22 C° or 37 C° and the same set of experiments indicated that 5 min is adequate for target hybridization, consistent with results reported by Acero et al. [30]. Whilst a different behaviour was recorded in the case of the DQB02A probe, where the use of higher temperature (37 C°) significantly influenced the hybridisation with the target (Figure 2.3B). These behaviours were consistent with those found with SPR measurements and discussed extensively above.

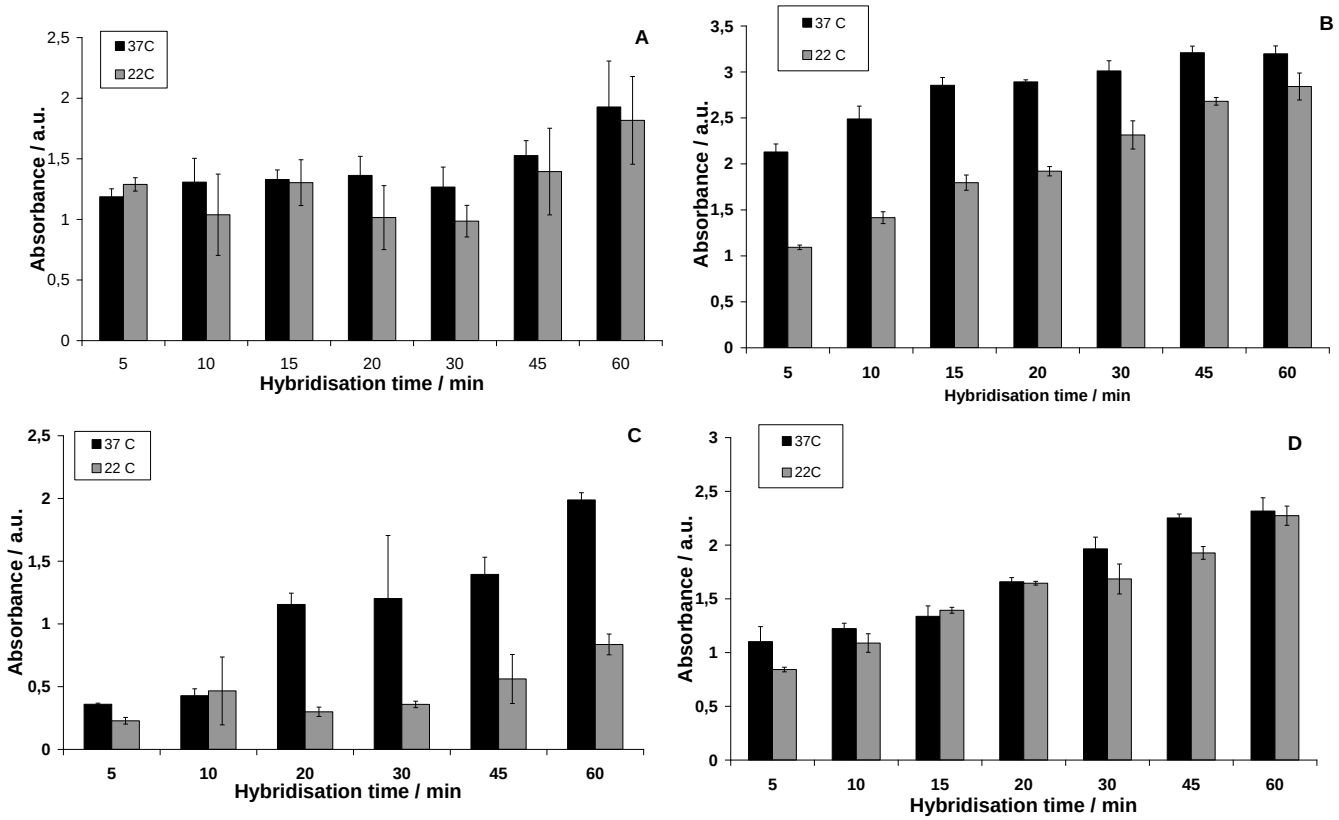


Figure 2.3: Evaluation of the target hybridisation time (A and B) and of the capture time of the reporting elements (C and D) for the DQA03 (A and C) and DQB02A (B and D) probes. ELONA assays were performed at 37 °C and 22 °C and using 20 nM of target DNA and 10 nM of reporting element.

The study of the hybridisation time of the HRP labelled reporter probe demonstrated that in the case of the DQA03 probe a significant increase in response was recorded when this step was performed at 37 C° (Figure 2.3 C), whilst no significant influence of the temperature was observed in the case of the DQB02A probe (Figure 2.3D).

As can be seen in Figure 2.3, 5 min for target hybridisation, 20 min for hybridisation with the HRP-labelled secondary probe and 37 °C can be considered a good compromise between assay time and assay performances. Figure 2.3 A, B indicates that at 37 °C hybridisation between the targets and the on-plate immobilised probes was very efficient after only 5 min. In the case of the capture of the secondary probe, for both of the probes investigated (DQA03 and DQB02A), responses increased with time but as can be seen, especially in the case of probe DQA03 (Figure 2.3C), this increase was not so relevant for times longer than 20 min. On the other hand, in the case of the DQB02A probe responses increased significantly with time (Figure 2.3D), but at 20 min of capture time similar responses were recorded for the two probes and this was thus chosen as the optimum assay time. In an attempt to further reduce the assay time, the possibility of performing the assay in a single step format, by pre-incubating the target oligonucleotides and the reporting sequence, was evaluated. As it can be seen in the supporting information this approach (see Electronic Supplementary Material Figure 2.S3) did not improved the assay performances.

Reporting label cross-reactivity

In order to perform multiplex detection of the alleles required for the low to medium resolution typing of the DQ2-DQ8 genes, use of different reporter probes, one for DQA exon 2, one for DQA exon 3 and one DQB exon 2, is required (Table 2.1). Prior to performing multiplex HLA typing the possible interaction between the different reporting elements was investigated, by performing ELONA experiments using a solution containing the specific reporter probes or a mixture of the three reporter elements at equivalent concentrations of 10 nM. The mixture of the three different reporter probes was prepared and left to incubate for 10 min at 37°C previous to use, in order to allow the possible interaction between the reporter probes to take place.

As can be seen in Figure 2.4 (results obtained for the DQA03 probe), no significant differences were recorded when the reporter probes were added as a single component or in a mixture.

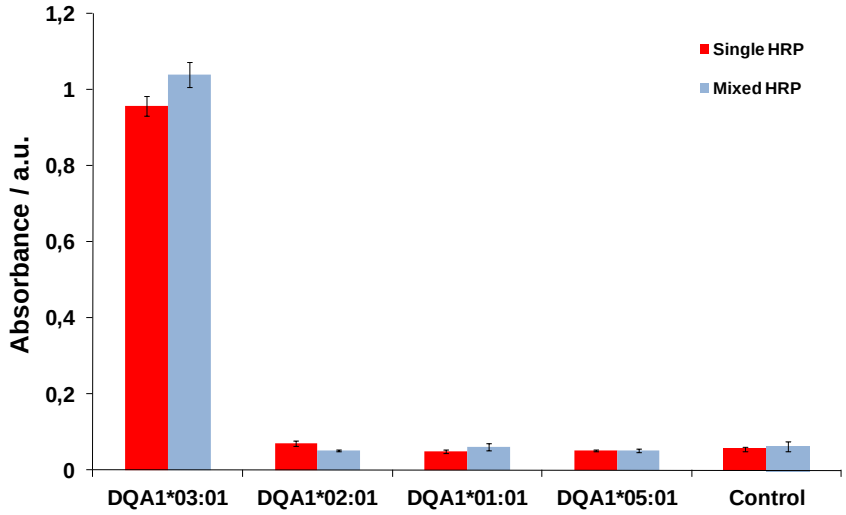


Figure 2.4: ELONA evaluation of the possible cross reactivity between the different reporting elements required for the multiplex detection according to the proposed detection platform. Study performed by using DQA03 probe and using 20 nM of target DNA and 10 nM of reporting element.

Detection of real samples

Following optimisation of the design of the probes and assay conditions with synthetic analogues of the different PCR products, an analysis of PCR product from real patients sample was carried out. In this evaluation, four previously typed patients samples and 1 cell line (LZL) from European Collection of Cell Cultures were used. The different samples were HLA typed in the accredited FRCBS Tissue typing laboratory using different methods: Luminex One Lambda Labtype® SSO HLA Class II DQA1/DQB1 Typing Test and Olerup DQA1or DQB1 SSP typing kits. Multiplex PCR amplification and ssDNA generation were performed in TATAA Biocenter .

The prepared samples were frozen and stored to -20 ºC. Sample analysis was performed, with 1 in 10 dilutions in hybridisation buffer followed by hybridisation with the 7 probes (three repetitions for each probe) and performing the detection according to the optimised protocol. In Figure 2.5, the normalised results (ration between specific response and control) are presented. The results of ELONA typing, together with those obtained using reference typing techniques (Luminex HLA-DQA1/DQB1 typing and confirmed by Olerup DQA1 or DQB1 SSP typing kits), are summarised in Table 2.3.

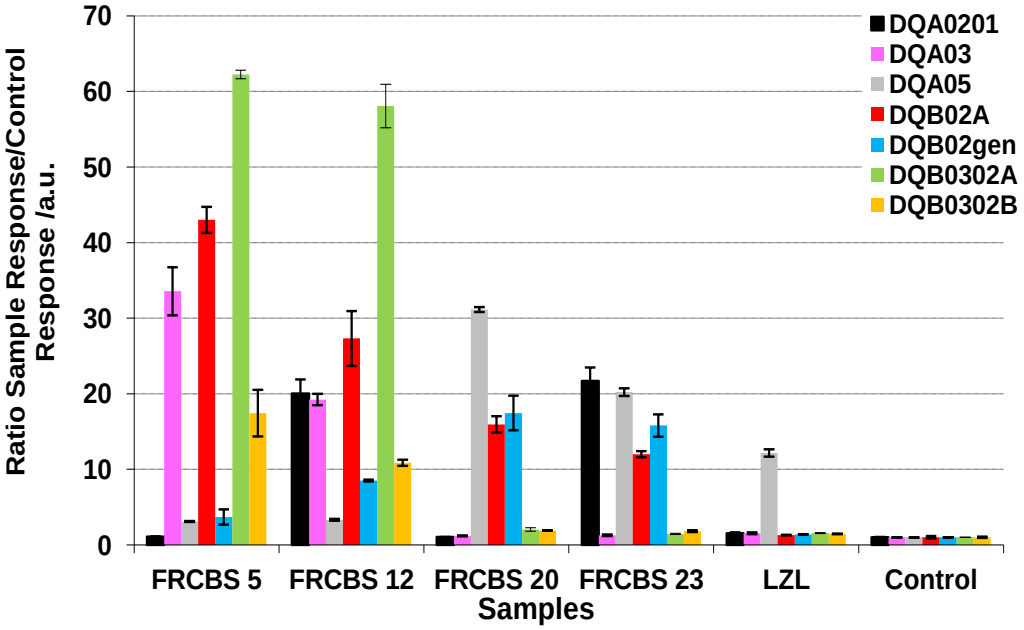


Figure 2.5: Colorimetric multiplex analysis of 4 real samples and cell line sample LZL. Hybridisation was carried out using PCR samples diluted 1:10 for 5 min. For detection, a mixture of reporter probes (10 nM each) was incubated for 20 min at 37 °C. Data presented as ration between the specific response and the control response.

In this table, the scores to the responses at the different probes were assigned according to the following criteria:

When the ratio between the response for the sample for each probe and the control experiment was lower than 5, the sample was considered negative for that probe; if the ratio was higher than 15, the sample was considered positive. All the value in between were judged accordingly to the results reported in Figure 2.2. A detailed description on how the samples' typing was obtained is presented in the supporting information.

The predicted HLA typing for the different samples was consistent with those obtained using standard HLA typing techniques (Table 2.3). Clearly, the proposed ELONA approach allowed HLA typing of the sample with a very good level of reproducibility and specificity.

Probe		Sample				
		FRCBS 5	FRCBS12	FRCBS20	FRCBS23	LZL
DQA0201		-	+	-	+	-
DQA05		-	-	+	+	+/-
DQA03		+	+	-	-	-
DQB02A		+	+	+	+	-
DQB02gen		-	+	+	+	-
DQB0302A		+	+	-	-	-
DQB0302B		+	+	-	-	-
ELONA sample typing						
DQ2		-	-	+	+	-
DQ8		+	+	-	-	-
Alleles present in the sample						
DQA region		03:01/03:01	02:01/03:01	05:01/05:01	02:01/0:501	05:03/05:03
DQB region		03:02/03:02	02:02/03:02	02:01/02:01	02:01/02:02	03:01/03:01
Reference typing						
DQ2		-	-	+	+	-
DQ8		+	+	-	-	-

Table 2.3: Summary of the HLA typing of the sample using the proposed ELONA assay and its comparison with the typing obtained with the reference approaches used in this work: Luminex HLA-DQA1/DQB1 typing and confirmed by Olerup DQA1 or DQB1 SSP typing kits.

Conclusions

In the proposed work, a set of probes for the low to medium resolution genotyping of CD associated genes has been designed and tested. The complexity of the HLA system did not allow the use of a single probe for each targeted allele making necessary the use of combination of probes to obtain the desired information. The use of the combination of several probes allowed specific identification of a specific alleles or of a restricted group of them (as in the case of the DQB1*02 alleles).

SPR and ELONA experiments were used to identify the optimal conditions, in terms of assay temperature and timing of the different steps, to perform the proposed assay.

SPR results indicated that the various probes had quite different behaviour in relation with the hybridisation temperature; this made necessary to find a suitable compromise. In the reported work assay was performed at 37 C° that allowed optimal detection of the different DQ2/DQ8 alleles. Optimisation of the timing of the different steps involved in the detection showed that detection was possible using 5 min hybridisation time and 20 min label capture time limiting the total assay to less than 30 min.

Finally, the assay was shown to be suitable for the HLA typing of real samples confirming the applicability of the proposed assay format for accurate (%RSD < 15%) CD predisposition analysis.

Acknowledgements

This work was carried out with the financial support from the Commission of the European Communities, specific RDT program "Coeliac Disease Management Monitoring and Diagnosis using Biosensors and an Integrated Chip System, CD-MEDICs, FP7-2007-ICT-1-216031". V.Beni acknowledges the European Union's Seventh Framework Program (FP7/2007-2013) under grant agreement no. PIGF-GA-2008-220928 for financial support. H. Joda thanks Universitst Rovira i Virgili for the doctoral scholarship. We thank Dr. Markku Heikkinen, MD, PhD (Kuopio University Hospital, Kuopio, Finland) for blood samples of Finnish celiac disease patients and Dr. Taina Jaatinen, PhD, Dr. Katri Haimila, PhD and Juha Peräsaari, MS (Finnish Red Cross Blood Service, Helsinki, Finland) for HLA tissue typing expertise.

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Chapter 2

Supporting information

Description of the Interfering alleles and Targets alleles identified for each of the investigated probes

Probe DQA05				
Alleles	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQA1*05:01	**TCTGCTGAGGAGAGTTA**	112 bp	147-181+3-intron 3	DQA1*05:01, 05:05, 05:08, 05:09, 05:10
DQA1*05:03	---***T***----------	112 bp	147-181+3 - intron 3	DQA1*05:03, 05:06, 05:07
DQA1*03:02	----***A***---***T***----***T***---	112 bp	147-181+3 - intron 3	DQA1*03:02, 03:03
DQA1*02:01	--------***T***----***T***---	112 bp	147-181+3 - intron 3	DQA1*02:01,all 01, 04, 06

Probe DQA0201				
Alleles	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQA1*02:01	**TCCACAGACTTAGA...TTTG**	94 bp	40 - 71	DQA1*02:01
DQA1*01:01	--***AG***--***A***-***T***--***G***--***GGT***----	97 bp	40 – 71	All DQA1*01
DQA1*03:01	---***G***----***T***-----***AGA***----	97 bp	40 – 71	DQA1*03:01,03:02, 03:03
DQA1*05:01	--***AGACA***-***T***-----...----	94 bp	40 - 71	All DQA1*05, DQA1*04, DQA1*06

Probe DQB02A				
Allele	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQB1*02:01	**TGCTGGGGCTGCCTGCCG**	137 bp	39 - 84	DQB1*02:01, 02:02, 02:04, 02:05, 02:06
DQB1*02:03	----------**A**--	137 bp	39 - 84	DQB1*02:01, 02:02, 02:04, 02:05, 02:06
DQB1*03:01	**C**--------**C**-----**A**--	137 bp	39 - 84	DQB1*03:01, 03:03, 03:06, 03:09, 03:10, 03:10, 03:12, 03:13, 03:15, 03:17, 03:19, 03:20, 03:22, 03:24
DQB1*03:02	**C**--------**C**--------	137 bp	39 - 84	DQB1*03:02, 03:04, 03:05, 03:07, 03:08, 03:11, 03:14, 03:18
DQB1*04:01	**C**--------**G**--**T**--**A**--	137 bp	39 - 84	DQB1*04:01, 04:02, 04:03
DQB1*05:01	**C**--**A**-----**G**-----**TT**-	137 bp	39 - 84	DQB1*05:01, 05:07, 05:11, , 06:04, 06:06, 06:08, 06:09, 06:12, 06:13, 06:17, 06:18, 06:21, 06:22, 06:27

Probe DQB02gen				
Allele	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQB1*02:01	**CGCTGCTGGGGCTGCCT**	137 bp	39 - 84	DQB1*02:01, 02:02, 02:03, 02:04, 02:05, 02:06
DQB1*03:02	---**C**--------**C**----	137 bp	39 - 84	All DQB1*03
DQB1*04:01	---**C**--------**G**--**T**-	137 bp	39 - 84	DQB1*04:01, 04:02, 04:03
DQB1*05:01	---**C**--**A**-----**G**----	137 bp	39 - 84	All DQB1*05, 06

Probe DQB0302A				
Allele	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQB1*03:02	**GGGGTGTATCGGGCG**	137 bp	39 - 84	DQB1*03:02, 03:03, 03:05, 03:06, 03:08, 03:10-12, 03:15, 03:17-18, 03:20, 04, 05:02:02, 05:03, 06:01, 06:19
DQB1*02:01	----***A***--***TC***------	137 bp	39 - 84	All DQB1*02 allele
DQB1*03:01	-***A***------***C***------	137 bp	39 - 84	DQB1*03:01, 03:04, 03:09, 03:13, 03:16, 03:19, 03:21, 03:22, 03:24, 03:27, 03:28, 06:35
DQB1*05:01	--------***C***-----***A***	137 bp	39 - 84	DQB1*05:01, 05:07, 05:11

Probe DQB0302B				
Allele	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQB1*03:02	**GCCGCCTGCCGCCGA**	137 bp	39 - 84	03:02, 03:04, 03:05, 03:07, 03:08, 03:11, 03:14, 03:18, 06:29
DQB1*02:01	--***T***----------	137 bp	39 – 84	DQB1*02:01, 02:02, 02:04, 02:05, 02:06
DQB1*02:03	--***T***-----***A***------	137 bp	39 - 84	DQB1*02:03
DQB1*03:01	--------***A***------	137 bp	39 - 84	DQB1*03:01, 03:03, 03:09, 03:10, 03:12, 03:13, 03:15, 03:17, 03:19, 03:20, 03:21, 03:22, 03:27, 03:28, 03:29, 03:30, 03:31, 03:33, 03:34, 03:35, 03:36
DQB1*04:01	--***G***--***T***--***A***------	137 bp	39 - 84	DQB1*04:01, 04:02, 04:03
DQB1*05:01	--***G***-----***TT***-----	137 bp	39 - 84	DQB1*05:01, 05:07, 05:11, 06:04, 06:06, 06:08, 06:09, 06:12, 06:13, 06:17, 06:18, 06:21, 06:22, 06:27, 06:34, 06:36, 06:38, 06:39, 06:42

Probe DQA03				
Alleles	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQA1*03	**TGTTCCGCAGATTTAGAAG**	94 bp	40 – 71	DQA1*03:01, 03:02, 03:03
DQA1*01:01	***A*----*A*---*A*----*G*--*G*-**	97 bp	40 – 71	All DQA1*01
DQA1*02:01	------***A***----***C***-----***TT***	97 bp	40 – 71	DQA1*02:01
DQA1*05:01	-***TC***--***A***-***ACA***-------***TT***	94 bp	40 - 71	All DQA1*05, DQA1*04, DQA1*06

Table 2.S1. Sequence alignment for the DQ2/DQ8 alleles at probes area, showing the sequences of the specific and interfering alleles used in this study. A dash (-) indicates the identity to the probe sequence.

Generation of ssDNA

Evaluation of the efficiency of the ssDNA generation was performed by comparing the melting curve of the digested sample with those obtained using sequential dilutions of the purified dsDNA. In this work, SYBR Green was used as indicator of the presence of dsDNA. As it can be seen from Figure 2.S1 efficiency in ssDNA generation was high.

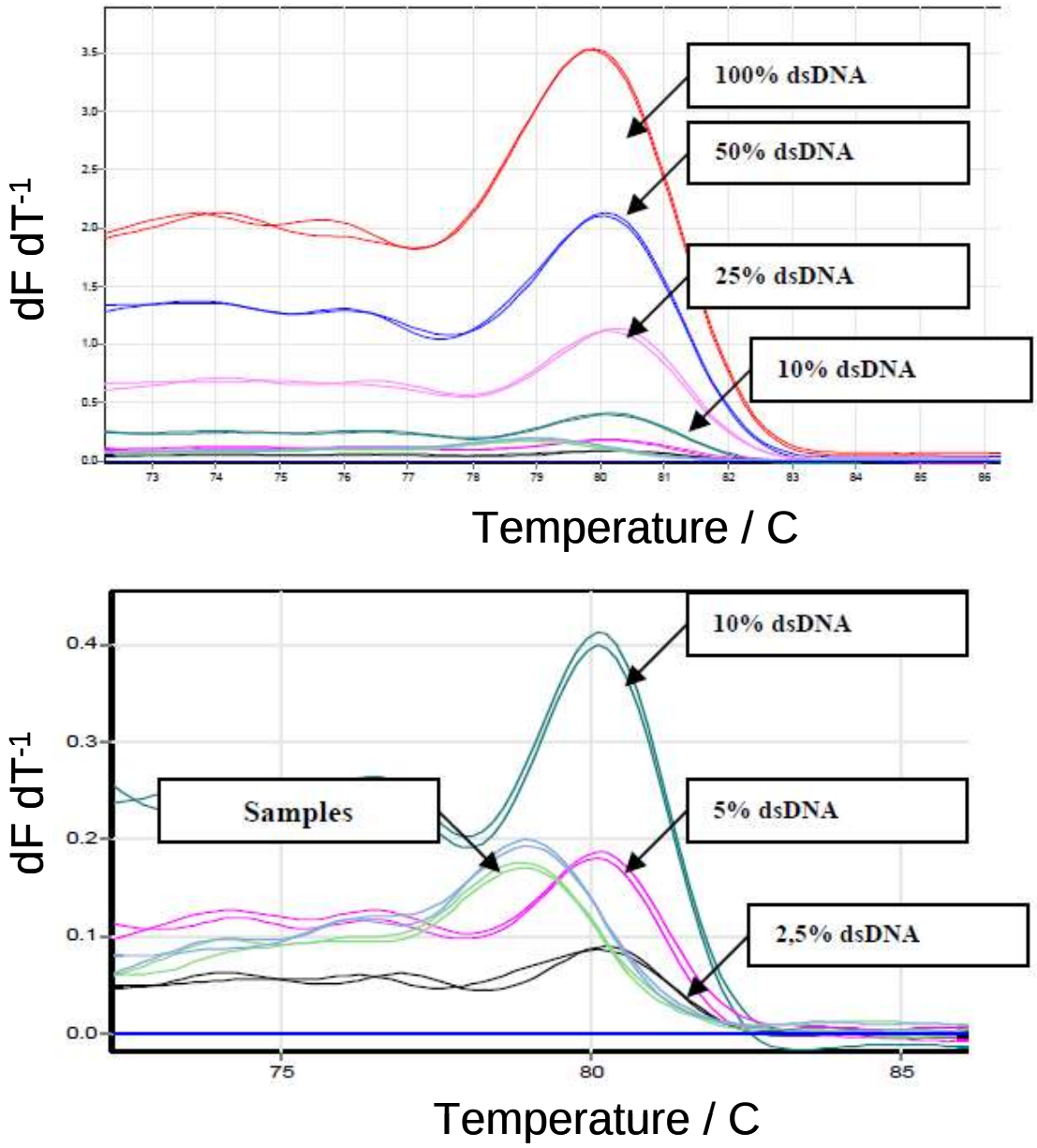


Figure 2.S1: Calibration of dsDNA using melting curve analysis.

Evaluation, via SPR measurements, of the effect of the temperature on the hybridisation efficiency of the designed probes.

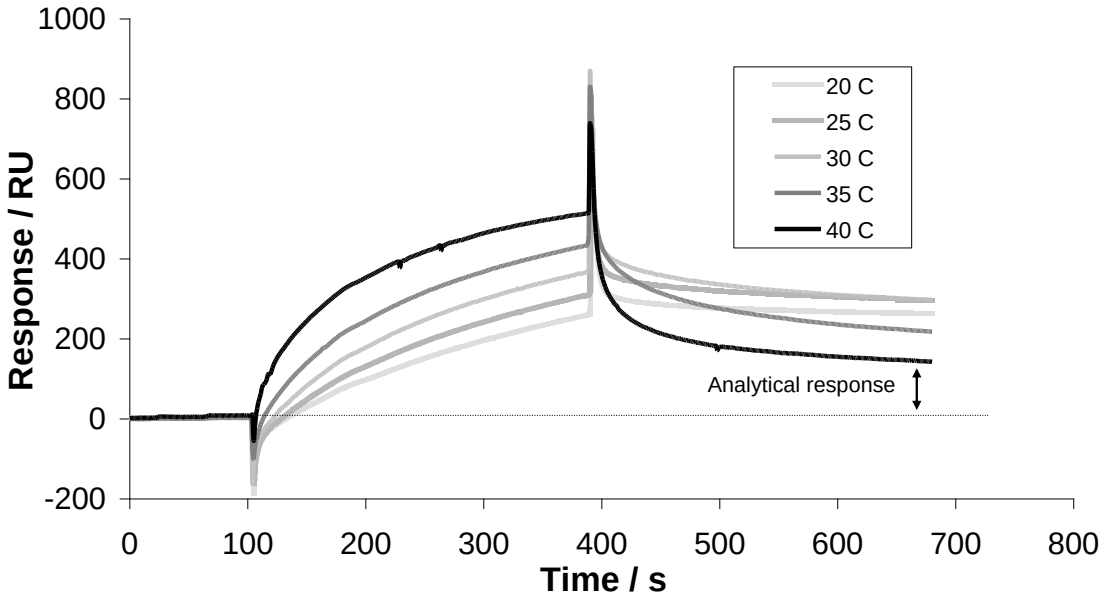
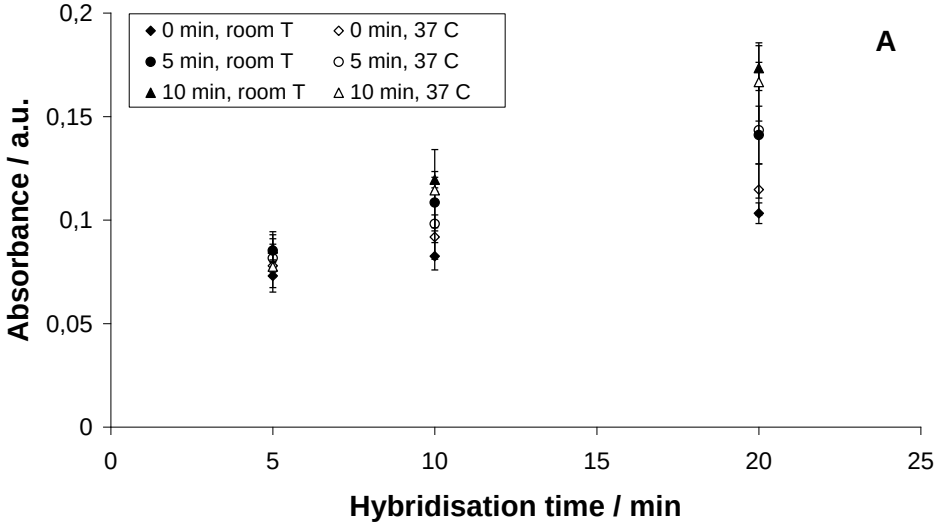


Figure 2.S2: Evaluation of the hybridisation temperature of DQA05 probe on the capture of synthetic DQA1*05:01 allele at different temperature (20, 25, 30, 35, and 40°C). Hybridisation was carried out using 250nM of DQA1*05:01in 10 mM Tris buffer containing 1M NaCl pH 7.4 at flow rate 5µl/min.

Evaluation of the effect of the sample-reporting sequence preincubation on the ELONA assay.

In these experiments, target and reporting element were added simultaneously to the immobilised probes or were mixed and left to interact (for 5 or 10 min), and then put in contact with the probes. This approach was investigated to improve the interaction between target and reporting element on the base that in-solution hybridisation is faster and more efficient than on surface one.

Only when target and reporting element were preincubated for 10 min at 37 °C and hybridised for 20 min at 37 °C, a similar response to those obtained for the previously optimised assay conditions was obtained



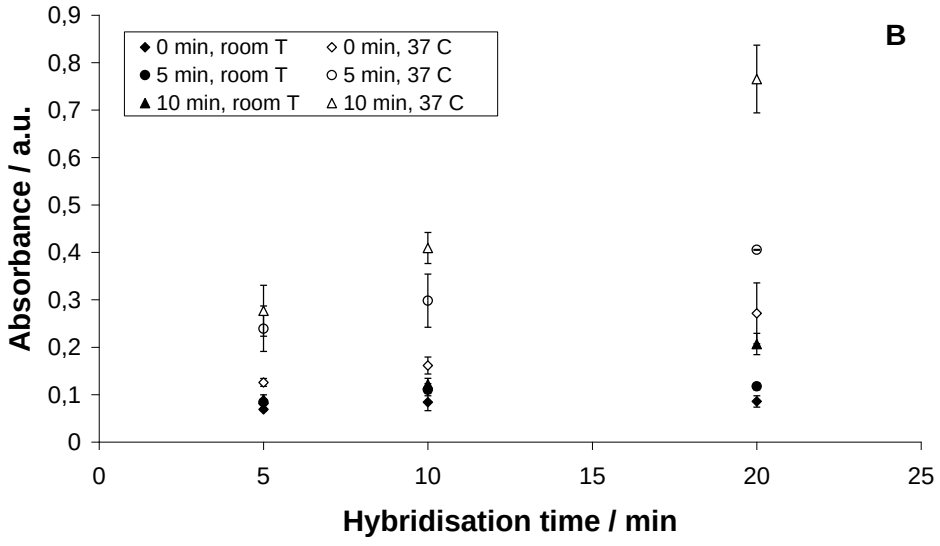


Figure 2.S3: Evaluation of the influence of the preincubation on the ELONA assay. Preincubation of the target with the reporting element was performed for various times at 22 °C and 37 °C. Assay was carried out at 22 ºC (A) and 37 °C (B). Study performed by using DQA03 probe and using 20 nM of target DNA and 10 nM of reporting element.

Description of the interpretation of the ELONA results for the real sample typing

Sample FRCBS5

For this sample positive responses were recorded for probes DQB02A, DQB0302A, DQB0302B and DQA03.

The positivity for the DQB0302A and DQB0302B probes indicate that this sample was presenting the DQB1*03:02 allele. Moreover, the fact that a negative response was recorded at the DQB02gen probe clearly indicate that the positivity of probe DQB02A was due to the presence of the DQB1*03:02 allele and not to the presence of DQB1*02 alleles.

Finally the positive response recorded at the DQA03 probe indicated the presence of the DQA1*03 alleles usually associated to the DQB1*03:02.

Summarising this sample was predicted to be DQ8 positive.

Sample FRCBS12

In the case of this sample positive responses were recorded at DQA0201, DQA03, DQB02A, DQB02gen, DQB0302A and DQB0302B.

The responses obtained clearly indicated that this sample was DQ2 positive.

Sample FRCBS20.

For this sample positive responses were recorded for the DQA05, DQB02 and DQB02gen. As a result of this, the sample was predicted to be DQ2 positive.

Sample FRCBS23.

In the case of this sample positive responses were recorded for the DQA05, DQB02A and DQB02gen probes. These results were consistent with the prediction of the DQ2 positivity for this sample.

Sample LZL

In the case of this cell line (LZL) no clear positive response were obtained: only DQA05 probe gave a positive hybridisation responses.

The absence of positive responses for all the other probes clearly indicated that the LZL sample was both DQ2 and DQ8 negative.

CHAPTER 3

Medium-high resolution electrochemical genotyping of HLA-DQ2/DQ8

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Abstract

Coeliac disease is a small intestinal disorder, induced by ingestion of gluten protein in genetically predisposed individuals. Coeliac disease has been strongly linked to human leukocyte antigens (HLA) system located on chromosome 6, as almost 100% of the coeliac disease suffers carry either a HLA-DQ2 or HLA-DQ8 heterodimer, with the majority carrying HLA-DQ2 encoded by the DQA1*05:01/05:05, DQB1*02:01/02:02 alleles, while the remaining carry HLA-DQ8 encoded by the DQA1*03:01, DQB1*03:02 alleles.

In this work, we present the development of a multiplex electrochemical genosensor array and dedicated microfluidic platform for rapid medium-high resolution HLA-DQ2/DQ8 genotyping. A total of 10 sequence specific probes were designed and immobilised on a 36 electrode array for HLA DQ2/DQ8 genotyping.

An evaluation of the selectivity of the designed probes was carried out as single base mismatch differentiations were required. Cross hybridisation studies using synthetic analogues of PCR product of all probes demonstrated good selectivity over the potentially interfering alleles. The performance of the electrochemical genosensor array was validated by the analysis of real human samples for the presence of HLA-DQ2/DQ8 alleles, and compared with those obtained using laboratory based HLA typing.

Introduction

Coeliac disease (CD) is a life-long inflammatory disorder of the small intestine, characterised by bowel mucosal inflammation and malabsorption [1, 2]. It affects genetically predisposed individuals after exposure to gluten proteins found in wheat, barley and rye [2, 3]. CD is considered the most common dietary intolerance disorder in the Western world affecting about 1% of general population [4, 5], although recent studies have shown a high prevalence in different regions and ethnic groups (Table 3.1). In spite of the high prevalence, only a few patients are diagnosed as the disease can present with atypical symptoms or can even be completely asymptomatic or silent (coeliac iceberg) [6, 7], and undiagnosed or delayed diagnosis is associated with several long-term complications [7, 8].

Table 3.1. Examples of prevalence of CD in different countries and regions.

Country	Prevalence	Reference
Turkey	0.47 %	Dalgic et al. 2011 [9]
India	1.04 %	Makharia et al. 2011 [10]
Iran	1.3 %	Farahmand et al 2011 [11]
Israel	1.1 %	Israeli et al. 2010 [12]
Brazil	1.5 %	Oliveira 2007 [13]
Tunis	0.64 %	Hariz et al. 2007 [14]
Egypt	0.53 %	Abu-Zekry et al. 2008 [15]
Sahara	5.6 %	Catassi et al. 1999 [16]

The presence of characteristic histopathological changes in the small intestinal biopsy was until recently considered to be the gold standard diagnostic test and is obviously a highly invasive and expensive procedure, often requiring patients to travel to centralized settings. CD diagnosis is supported by clinical presentation and serological testing for anti-tissue transglutaminase (tTG) and endomysial (EMA) antibodies [17, 18], and there is also evidence of the value of IgG anti-deamidated gliadin peptide antibodies [19]. To this end, in the recent revised diagnostic guidelines of the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) based on the Delphi process, diagnosis is recommended to be based on HLA typing in the presence of high titre level of anti-tTG IgA antibodies, combined with the detection of total IgA [20].

Genetic susceptibility to CD has been clearly demonstrated, as with the exception of the very rare cases, all CD sufferers share certain HLA class II genes located on chromosome 6. About 95% of CD patients carry the HLA-DQ2 heterodimer, either in the *cis* encoded by DQA1*05:01 and DQB1*02:01 or in the *trans* configuration (on one chromosome from each parent) encoded by DQA1*05:05 and DQB1* 02:02, whereas most of the remainder carry the HLA-DQ8 encoded by DQA1*03:01 and DQB1*03:02 [21] (Figure 3.1).

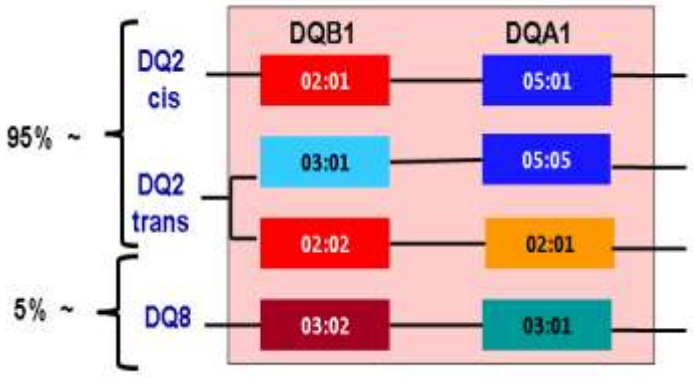


Figure 3.1. Schematic description of the different possible allele combinations encoding the DQ2 and DQ8 heterodimers. 95% of CD patients carry the HLA-DQ2 heterodimer, either in the *cis* encoded by DQA1*05:01/DQB1*02:01 or in the *trans* (on one chromosome from each parent) encoded by DQA1*05:05/DQB1*03:01 and DQA1*02:01/DQB1* 02:02 alleles. HLA-DQ8 is present in about 5% of patients and encoded by DQA1*03:01 and DQB1*03:02 alleles.

Individuals who do not carry HLA-DQ2 or HLA-DQ8 genotypes are effectively excluded from developing coeliac disease [22]. HLA typing for CD predisposition or diagnosis is thus of great importance and addresses a mature market need. HLA typing could also be useful in screening high-risk individuals [4, 20] such as first-degree relatives and individuals with autoimmune diseases.

Current HLA typing methods based on PCR include, sequence-specific primer-PCR (SSP) [23], sequence-specific oligonucleotide probe-PCR (SSOP) [24], single strand conformation polymorphism (SSCP) [25], restriction fragment-length polymorphism (RFLP) [26] and sequencing based typing (SBT) [27]. However, the majority of these techniques are expensive, labour intensive, time consuming and require intensive post PCR manipulation.

In recent years, electrochemical methods have been shown to have great potential for genetic analysis including electrochemical sensors and melting curve analysis [28, 29], as this transduction method is characterised by high sensitivity, ease of use, low cost, rapid response, low power requirements and compatibility with integration in microsystems [30]. Electrochemical genosensors have found application in several areas including detection of genetic disease [31], cancer markers [32], viruses [33], environmental contaminants [34] and food allergens [35]. Particularly, electrochemical arrays have gained attention in the last decade for

high throughout, rapid, simultaneous analysis of multiple markers requiring a low amount of sample [36, 37].

HLA typing using DNA microarrays has been reported in several works. Zhang et al. [38] used a microarray with 19 immobilised sequence specific oligonucleotide probes to the HLA-DRB1 region. A fluorescein-labelled primer was employed to amplify the HLA-DRB1 exon 2, and hybridisation was monitored using fluorescence. Using the same approach, Lee [39] and Yoon [40] and their co-workers developed a microarray for the genotyping of HLA-A / HLA-DRB1 and HLA-C respectively.

Recently, we reported a colorimetric assay for multiplex low-medium resolution typing of CD associated DQ2/DQ8 alleles [41]. Sequence specific oligonucleotide probes were designed for DQ2/DQ8 genotyping, the selectivity was evaluated and hybridisation conditions optimised using an enzyme linked oligonucleotide assay colorimetric detection. Here, we present an extension of the reported work using a disposable electrochemical genosensor array, employing the optimised probe design and hybridisation conditions. Moreover, additional probes were designed to improve DQ2/DQ8 typing to achieve medium-high resolution typing. The performance of the electrochemical genosensor array was tested using real samples and validated by comparing them with the luminex HLA typing and Olerup typing kits used in the FRCBS reference laboratory. To our knowledge, this is the first report of electrochemical HLA typing for the assessment of CD genetic predisposition.

Material and Methods:

Materials

All reagents used in this work were of analytical grade: Trizma hydrochloride, sulphuric acid, hexacyanoferrate(III) [$K_3Fe(CN)_6$], phosphate-buffered saline (pH 7.4) with 0.05% v/v Tween 20 (PBS-Tween), and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA were purchased from Sigma. Hydrochloric acid, (HCl, 6 M), potassium dihydrogen phosphate (KH_2PO_4), Sodium hydroxide (NaOH) and sodium chloride (NaCl) were obtained from Scharlau. 6-mercaptohexanol (MCH) was received from Fluka, Milli-Q

water (18 MΩ cm) was obtained using a Simplicity water purification system (Millipore, France). Dithiol (10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol (DT1) was provided by SensoPath Technologies (Bozeman, NT). StabilCoat® Plus Microarray Stabilizer was purchased from SurModics, Inc (Eden Prairie, MN, USA). DNA probes, Horseradish peroxidise (HRP) modified reporting sequences (Table 3.2) and synthetic analogues of relevant PCR amplicons were supplied by biomers.net (biomers.net GmbH, Germany). Further information regarding the amplicons and their synthetic analogues can be found in the supporting information (see Electronic Supplementary Material Table 3.S1).

Electrochemical instrumentation

All electrochemical experiments were performed using an Autolab model PGSTAT 12 potentiostat/galvanostat controlled with the General Purpose Electrochemical System software program (Eco Chemie, The Netherlands), equipped with a MUX module (Eco Chemie B.V., The Netherlands) that allowed sequential addressing of up to 64 working electrodes (Scheme 3.1).

The electrochemical cell used in the work reported here consisted of a customised electrode array chip (Scheme 3.1), which was fabricated on a glass substrate using standard photolithographic methodology [42] and consisted of 36 working electrodes (1 mm^2), each arranged with a gold counter electrode and a silver reference electrode.

In order to develop a fully operational fluidic prototype and functional evaluation tool, a set of dedicated fluidic cells (Scheme 3.1) were designed and fabricated by high-precision milling of a polycarbonate (PC) substrate. Fluidic channels (1.2 mm in width) were created by precise laser cutting of double-sided medical grade adhesive foil of 50 µm thickness, which was sandwiched between the electrode array and the fluidic cell, either in 3 channels (12 electrodes/channel), or 12 channels (3 electrodes/channel) arrangement.

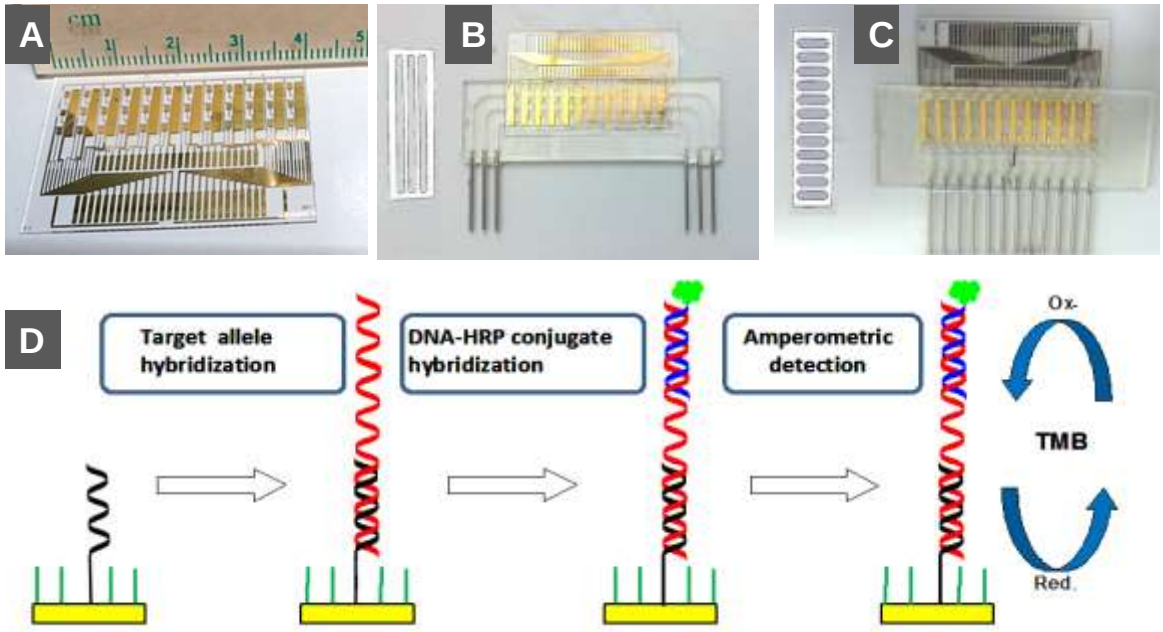
The electrode array was interfaced with a multiplexed potentiostat using a custom-designed pogopin connector. To avoid non-specific adsorption of the HRP-labelled oligonucleotides, the microfluidic channels were protected by cross-linking to a PEG-based monomer supplied by Institut für Mikrotechnik Mainz GmbH (www.imm-mainz.de). First, 0.5% (w/v) PEG-monomer solution was prepared in Milli-Q water and used to fill all channels and to cover the area where the electrode array will be mounted, and was then exposed to

UV light for 1 h to covalently crosslink the PEG monomer and finally was rinsed with Milli-Q water and blow dried.

Table 3.2. Sequences of the probes and reporting elements.

Probes			
Probe name	**Probe sequence**	**IMGT/HLA Matched alleles**	**Codon**
DQA0201	5'-SH-CAAATCTAAGTCTGTGGA-3'	DQA1*02:01	58_1 – 51_2
DQA03	5'-SH-CTTCTAAATCTGCGGAACA-3'	DQA1*03:01, 03:02, 03:03	56_2 – 50_2
DQA05	5'-TAACTCTCCTCAGCAGA-SH-3'	DQA1*05:01, 05:05, 05:08, 05:09	164_2 – 159
DQB02A	5'-CGGCAGGCAGCCCCAGCA-SH-3'	DQB1*02:01, 02:02, 02:04, 02:05	58_1 – 52_2
DQB02gen	5'-AGGCAGCCCCAGCAGCG-SH-3'	DQB1*02:01, 02:02, 02:03, 02:04, 02:05	56 – 51_2
DQB0302A	5'-CGCCCGATACACCCC-SH-3'	DQB1*03:02, 03:03, 03:05, 03:06, 03:08, 03:10-12, 03:15, 03:17-18, 03:20, 04, 05:02:02, 05:03, 06:01, 06:19	49_3 – 45_1
DQB0302B	5'-TCGGCGGCAGGCGGC-SH-3'	DQB1*03:02, 03:04, 03:05, 03:07, 03:08, 03:11, 03:14, 03:18, 06:29	59_2 – 54_3
DQA0302	5'-TAAATCTCATCATCAGA-SH-3'	DQA*03:02, 03:03	159_1 – 164_2
DQA0505A	5'-CTT***T***TCCAGTCCCCAGT-SH-3'	DQA1*05:05, 05:08, 05:09, 05:10	170_2 – 175_3
DQB0202	5'- CCTCCTGGCCATTCC-SH -3'	DQB1*02:02	159 – 164_2

Reporting probes			
Reporting element name	**Reporting element sequence**	**Target exon**	**Codon**
DQAex2	5´-GACAGTCTCCTTCCTCTC-HRP ´3	DQA1 Exon 2	45_3 - 40_1
DQAex3	5'-HRP-CCCCAGTGTTTCAGAAGA-3'	DQA1 Exon 3	182_1 - 176_3
DQBex2	5'-HRP-CTGGTAGTTGTGTCTGCA-3'	DQB1 Exon 2	84_3 – 79_1
DQBex3	5-HRP-AGTCACCATTCCTAATAA-3	DQB1 Exon 3	153_1-147_2



Scheme 3.1. Schematic of electrochemical genosensor array, showing (A) the 36 electrodes array, (B) after mounting on 3-channels fluidic cell and (C) 12-channels fluidic cell. (D) Steps for the development of electrochemical genosensor detection.

Electrode surface functionalisation

Before functionalisation, the electrode arrays were cleaned by a series of chemical washes (required to remove the protective layer coated onto the array during storage) and electrochemical steps. Briefly, electrode arrays were washed by sequential sonication for 5 min in acetone and then isopropanol, and finally in ethanol for 5 min. The electrodes were further cleaned electrochemically in 0.5 M H_2SO_4, at 1.6 V for 5 s, followed by 30 voltammetric cycles in the potential range 0 to 1.6 V at a scan rate of 0.5 V s^{-1}. Following cleaning the arrays were thoroughly rinsed with MilliQ water, dried with N_2 gas and subsequently modified with the probes.

Electrode surface functionalisation was performed following the co-immobilisation approach [43] which was achieved by spotting a drop (0.5-1 µL) of a freshly prepared solution containing 1 µM of thiolated DNA probe with different length and type of spacers (C3, C6 or C3-poly T) and 100 µM of co-immobiliser molecule (DT1 or MCH) in 1 M KH_2PO_4 onto each electrode. Self-assembling of the thiolated molecules was left to take place for 3

h at room temperature within a humidified chamber [33], and then the electrodes were rinsed with MilliQ water and dried in N_2 gas.

Multiplex PCR amplification and ssDNA generation:

Multiplex PCR amplification and ssDNA generation were performed as described previously [41]. Briefly, blood samples were collected from CD affected and/or suspected patients at Kuopio University Hospital (Finland). DNA was extracted from blood using the Qiagen FlexiGene DNA kit, followed by genotyping in the accredited Finnish Red Cross Blood Service (FRCBS) Tissue typing laboratory using Luminex One Lambda Labtype® SSO HLA Class II HLA-DQA1/DQB1 typing kits and confirmed using Olerup DQA1 or DQB1 SSP typing kits. Multiplex PCR amplification was performed using iQ Multiplex Powermix from Bio-Rad in the presence of 20 ng of DNA template. For multiplex amplification, four primer pairs specifically designed to amplify exon 2 and 3 of both DQA1 and DQB1 regions were used. All primer pairs were used at a concentration of 200 nM except those for the amplification of the DQB1 exon 3 (100 nM). Amplification was performed in a 200 µL tube (50 µL reaction) format using an RotorGene 6000 thermocycler according to the following protocol: (a) initial denaturation at 95 °C for 10 min and (b) 40 cycles (15 s at 95 °C and 60 s at 60 °C). All the reverse primers used in the reported work were phosphorylated at their 5′ end in order to allow enzymatic generation of single stranded DNA via Exonuclease digestion. ssDNA generation was performed by means of Lambda exonuclease (Fermentas GmbH) mediated hydrolysis by incubating 50 µL PCR product, in the presence of 2 µL (20 U) of the exonuclease for 30 min at 37 °C. Following digestion, the solution was heated to 80 °C for 10 min in order to inactivate the enzyme.

Electrochemical hybridisation assay

Electrochemical hybridisation assays were performed using a conventional sandwich assay approach [33]. First, the functionalised electrode array was assembled with the dedicated fluidic platform using the double-sided adhesive gasket, and prior to the assay, the electrodes were rehydrated with 300 µL PBS-Tween.

In the case of hybridisation time optimisation and sensing surface characterisation, target alleles were diluted to 20nM in hybridisation buffer (10 mM Tris buffer containing 1 M

NaCl pH 7.4). Subsequently the prepared solution was injected into the desired channel and incubated to allow hybridisation to take place for a given time at 37 °C. Following washing of the channels with 1 mL PBS-Tween, 10 nM of the labelled reporter probe (horseradish peroxidiase enzyme (HRP) functionalised probe) in hybridisation buffer was injected into the channels and incubated for a given time at 37 °C. A further washing step was performed with 1 mL PBS-Tween.

For probe selectivity experiments, similar experimental steps were performed, but in this case, for each probe the fully complementary and all the potentially interfering alleles PCR product analogues were used. Hybridisation with target alleles and reporter probe was performed for 10 min at 37 °C for each consecutive step.

For analysis of real samples, ssDNA amplicons of the PCR products were generated and diluted 1:3 in hybridisation buffer and the same experimental conditions as described above, with the exception that a mixture of four different HRP oligonucleotides probes were used as reporter molecules.

Electrochemical measurements were performed by addition of TMB substrate and monitoring electroreduction of the product formed via the HRP label by fast pulse amperometry (at -0.2 V vs Ag built-in reference).

Stability study

To perform the stability study, a short oligonucleotide probe with the sequence TGT AAA ACG ACG GCC AGT–SH (biomers.net) was co-immobilised with DT1 on all 36 electrodes of a clean chip according to the protocol previously described. Following probe immobilisation, the electrodes were coated with 1 µL of StabilCoat Plus stabiliser, or PBS and dried under vacuum, and then mounted with a 12-channel gasket, sealed and stored dry at 4 °C or at 37 °C. The stability of immobilised probes was assessed on a weekly basis by hybridisation of one of the 12 rows (containing 3 electrodes) with 10 nM of HRP modified complementary oligonucleotide probe for 2 min at room temperature. The channel was then washed with 1 mL PBS-Tween to remove the nonspecifically adsorbed oligonucleotides and electrochemical measurement was carried out as detailed previously.

Results and discussions

Optimisation of surface chemistry for probe immobilisation

Optimisation of surface chemistry for the immobilisation of DNA is essential in order to achieve maximum signal and minimal non-specific adsorption. In the reported work, DQA0201 and DQA03 probes were modified with thiol at the 5'end, while the rest of probes were modified with thiol at the 3'end. Due to commercial availability, thiol at 5'end was linked via a C6 spacer, while the C3 spacer was linked to thiol at the 3' end and to further increase the C3 spacer, an extra six poly-thymine (poly T) was added.

In the reported study, co-immobilisation of thiolated DNA probes with various spacers (C3, C6, C3-polyT) using different diluting alkanethiols (MCH and DT1) at a 1:100 ratio was investigated [43, 44]. As can be seen in Figure 3.2A, no significant differences in current intensity were recorded as a function of the composition of the SAM assembled onto the sensor surface, except for the case of the DT1/C3-spacer. This low current response, can be attributed to the fact that the DT1 co-immobiliser having a longer chain length, gave rise to steric hindrance, thus affecting the target hybridisation. In order to better appreciate the effect of the composition of the different surfaces on the sensor performance, the signal to noise ratio between the responses recorded for the hybridisation of immobilised probe with 20 nM complementary positive target and that recorded in the absence of target. The use of DT1 as co-immobiliser independently of the spacer used in the DNA probe, always resulted in a better specific/nonspecific ratio (Figure 3.2B). No significant differences were observed for C6 or C3-polyT spacers, whilst when the C3 spacer was used a lower ratio was obtained. On the other hand, the use of MCH as co-immobiliser always resulted in a low specific/nonspecific ratio, which was due to the non-specific signal recorded in the absence of the target. From this result, the combination of DNA probe with C6/DT1 was chosen for probes modified with thiol at the 5'end, while C3-polyT/DT1 was used for thiolated probes at the 3'end.

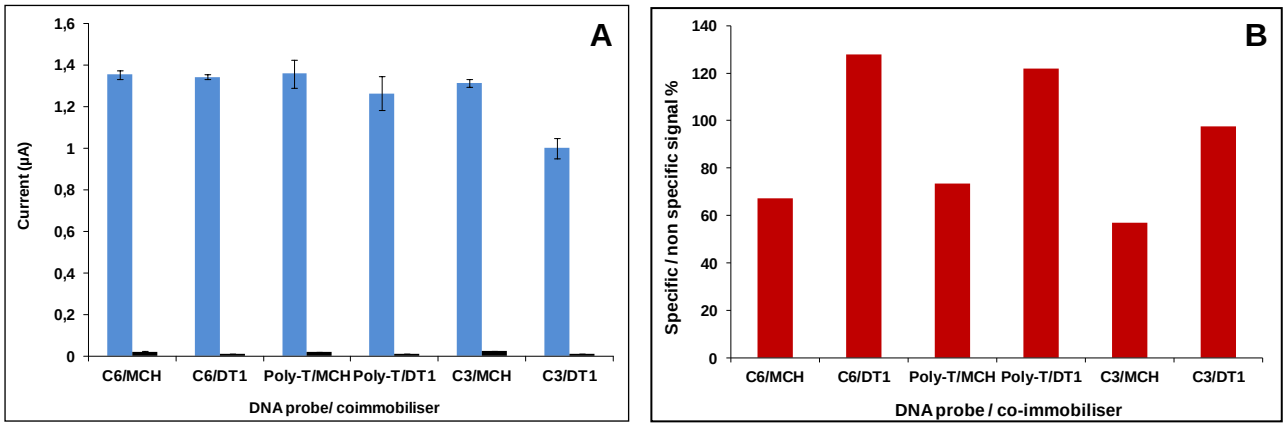


Figure 3.2. Surface chemistry evaluation of different probes co-immobilised with MCH and DT1; (A) Showing the actual current response of the complementary target (20nM) and non-specific background signal. (B) Showings the ratio between the current response of the complementary target (20nM) and non-specific background signal.

Hybridisation time

Using the previously optimised hybridisation temperature of 37 °C, we studied different hybridisation times for the target allele (20nM) and reporter probe (10nM) for DQA03 and DQB02A probes, within the fluidic set-up. An electrode array modified with the probe and co-immobiliser was mounted on the cell, then heated to 37 °C, and the target and reporter probe were incubated between 2 and 30 min (Figure 3.3). The hybridisation signal increased gradually with increasing hybridisation time for the target and reporter in both probes, and the optimum time was chosen to be 10 min+ 10 min.

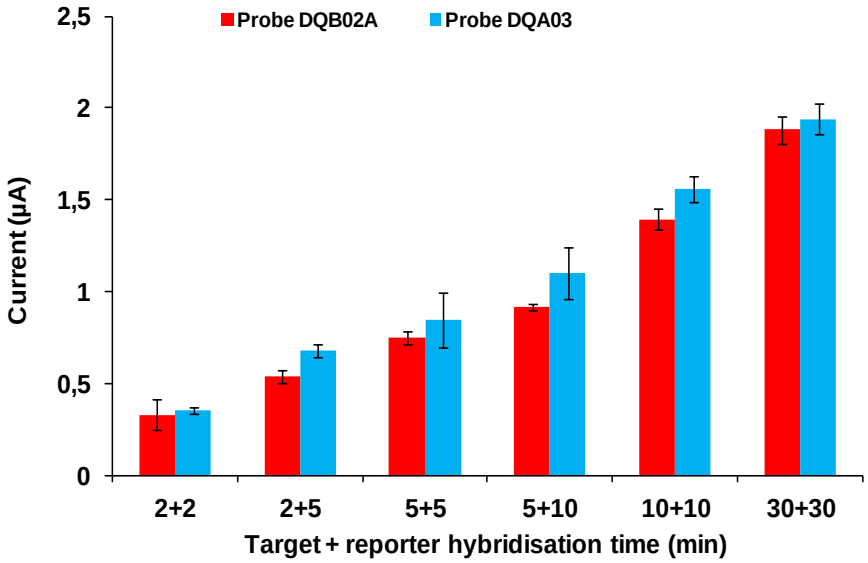


Figure 3.3: Evaluation of the target/reporter probe hybridisation time for the DQA03 (■) and DQB02A (■) probes. Hybridisations were carried out at 37 ºC and with 20 nM of target DNA and 10 nM reporter probe.

DQ2/DQ8 probes and identification of interference alleles

The primary identification of the probes and of potentially interfering alleles was performed with the help of the IMGT/HLA database [45], the handbook of the 13th International Histocompatibility Working Group (IHWG) [46] and multiple sequence alignment software (Genedoc) [47]. A detailed description of the optimised design of the probes for the low - medium resolution HLA typing of DQ2 and DQ8 genes, together with the description of relevant interfering alleles have been detailed previously [41]. The sequences of the different probes and reporting sequences are presented in Table 3.2.

In this work, in order to achieve medium-high resolution typing, additional probes were needed and an expanded set of probe and potentially interfering alleles were designed and tested (Electronic Supplementary Material, Table 3.S1). The additional probes were: (i) probe DQA0505A at DQA1 exon 3 to differentiate between DQA1*05:01 and DQA1*05:05 allele; (ii) probe DQA0302 at DQA1 exon 3 to rule out the non-CD associated alleles DQA1*03:02 and DQA1*03:03 alleles from DQA1*03:01 allele; (iii) probe DQB0202 at DQB1 exon 3 to differentiate between DQB1*02:01 and DQB1*02:02 alleles.

*DQA1*05:05*

In combination with probe DQA05, which is designed to detect both DQA1*05:01 and DQA1*05:05 alleles, probe DQA0505A has the ability to recognise a sequence in the exon 3 DQA1 locus belonging to allele DQA1*05:05 but not the DQA1*05:01 allele, thus allowing discrimination between the two. This probe also detects DQA1*05:08, DQA1*05:09, and DQA1*05:10 alleles (Electronic Supplementary Material, Table 3.S1) that are not associated to CD, but they have a very low prevalence in the Caucasian population [48]. Evaluation of probe selectivity was performed by cross hybridisation in the presence of 4 potentially interfering alleles; DQA1*05:01, DQA1*02:01, DQA1*03:02 and

DQA1*06:01 having 1, 2 and 3 base mismatches, representing all the known alleles in the DQA1 locus at the probe region (Electronic Supplementary Material, Table 3.S1).

*DQA1*03:02/ 03:03*

For high resolution detection of CD associated DQA1*03:01 allele, probe DQA0302 was designed at DQA1 exon 3 to detect DQA1*03:02/ 03:03 alleles. The need to design a further probe for this allele was due to the fact that the DQA03 probe is used to detect all the DQA1*03 family, and in order to achieve specific detection of the DQA1*03:01 allele, a probe to rule out the non-CD alleles (DQA1*03:02 and DQA1*03:03) was required. The selectivity of the designed probe was tested against DQA1*03:01, DQA1*05:01 and DQA1*05:03 (see Electronic Supplementary Material Table 3.S1) as interfering alleles.

*DQB1*02:02*

A further probe was designed in the exon 3 DQB1 region to differentiate between DQB1*02:01 and DQB1*02:02 alleles, with only one nucleotide mismatch. Probe selectivity was tested in the presence of 2 allele groups (DQB1*02:01 and DQB1*05:01) with 1 and 2 base mismatches, representing all potentially interfering alleles in the region of the probe (see Electronic Supplementary Material Table 3.S1).

Evaluation of probe selectivity

The selectivity of the probes required for the medium-high resolution typing of the DQ2/DQ8 was evaluated using synthetic oligonucleotides analogous to the real PCR amplicons. In Table 3.3, the normalized responses obtained for the selectivity of the different probes for medium-high resolution typing are reported.

*DQA1*05:01/05:05*

In Table 3.3, the normalised responses recorded for the DQA05 probe in the cross hybridization experiments are reported. A clear difference between the response obtained for the fully matched allele DQA1*05:01 and the single point mismatched allele DQA1*05:03 was observed. and even lower responses were recorded in the case of the other potentially interfering alleles, DQA1*02:01 and DQA1*03:02 (respectively 12.8% and 6.8% of the fully matched response).

*DQA1*05:05*

As detailed in the Table 3.S2 in the supporting information, several probes were designed varying in length and sequence and were tested for their ability to discriminate between the DQA1*05:01 and DQA1*05:05 alleles, without achieving good discrimination between the two alleles.

In order to overcome this selectivity problem the use of probes according to the reported amplification refractory mutation system (ARMS) approach [49, 50] was attempted by introducing a (C → T) mismatch at 4th nucleotide position of the probe (*italic underlined base in* Table 3.1).

The ARMS approach was originally used for PCR based genotyping to increase the specificity of the sequence specific primers by introducing a deliberate base mismatch in a primer to improve the detection of a single nucleotide mismatch. The proposed probe (DQA0505A) design showed an excellent selectivity in the cross hybridisation experiment, as the signal recorded for the single base mismatched DQA1*05:01 allele dropped from 80.7 % to only 20.1 % of the signal obtained with the fully complementary DQA1*05:05 allele. Moreover, other potentially interfering alleles with 2 and 3 base mismatches showed less than 10% of cross hybridisation signal. In the final multiplex assay, the presence of DQA1*05:05 allele will thus be indicated by a positive signal from both the DQA05 and DQA0505A probes, while the presence of the DQA1*05:01 alleles is will be indicated by a positive hybridisation signal with the DQA05 probe and no hybridisation signal with the DQA0505A probe.

DQB1*02:01/02:02

To achieve low-medium resolution typing for both DQB1*02:01 and DQB1*02:02 alleles, two probes were designed as described previously [41].

As can be seen in Table 3.3, the single base mismatched alleles DQB1*02:03 and DQB1*03:02 gave a signal approximately 55% of that obtained with the fully complementary target, and the other alleles had signal of less than 30% of the fully complementary allele.

Using the second probe (DQB02gen), discrimination against the possibly interfering alleles was significantly improved showing only 21.5%, 18% and 14.3% of the fully complementary signal for DQB1*03:02, DQB1*04:01 and DQB1*05:01 respectively.

*DQB1*02:02*

The DQB0202 probe was used to discriminate between the CD associated DQB1*02:01 and DQB1*02:02 alleles. The probe was designed to recognise a sequence at the DQB1 exon 3 region, specific DQB1*02:02 allele and having a single point mismatch with the DQB1*02:01 allele. As can be seen in Table 3.3, this probe had a high discrimination ability even for the single base mismatched DQB1*02:01 allele (a signal of 17.7 % of the fully complementary DQB1*02:02 allele), much lower responses (6%) was obtained for the other tested potentially interfering sequence (DQB1*05:01).

DQA1*02:01

The amperometric signal ratio between the fully complementary DQA1*02:01 allele and the possible interfering alleles at DQA0201 probe region on DQA1exon 2 is detailed in Table 3.3, and as can be seen the DQA0201 probe provides a high resolution typing for the DQA1*02:01 allele with low signal from the possible interfering alleles ($\leq$ 6%).

*DQB1*03:02*

Whilst there are tens of sub DQB1*03 alleles, only the DQB1*03:02 allele is associated with CD and high-resolution typing for this allele is thus very complicated and requires the use of several probes. Two probes (DQB0302A and DQB0302B) were used to minimise numerous cross hybridisations with non CD related alleles, even though most of them are very rare [48].

As can be seen in Table 3.3, Probe DQB0302A demonstrated good selectivity over the possibly interfering alleles with the highest cross hybridisation signal observed being from the DQB1*05:01 allele (about 51,4% of fully complementary) which has 2 base mismatches, one at the centre and one at the 5'terminal of the probe. The DQB1*03:01 allele also has 2 base mismatches, one at the centre while the other one is at the second

nucleotide position from the 3' end of the probe and has about 19.5 % of the fully complementary signal. The DQB1*02:01 which has separate 3 point mutations demonstrated minimal cross reactivity (4.4 %).

The DQB0302B probe has potentially more interfering alleles than the DQB0302A probe see (Electronic Supplementary Material Table 3.S1). It has a single base mismatch from the DQB1*02:01 and DQB1*03:01 alleles, 2 base mismatches from DQB1*02:03 and 3 base mismatches from the DQB1*04:01 and DQB1*05:01 alleles. High interfering signals from alleles with single and two base mismatches (DQB1*02:01, DQB1*02:03 and DQB1*03:01 alleles), of 46.5 %, 34.1% and 43.3%, respectively were observed, whilst the cross hybridisation signal from the 3 base mismatched alleles (DQB1*04:01 and DQB1*05:01) were only about 6 % of fully complementary signal.

*DQA1*03*

Probe DQA03 was designed for low resolution typing of the DQA1*03 allele group. The alignment of DQA1 alleles with the DQA03 probe revealed that there are multiple nucleotide mismatches between the DQ1*03 and other DQA1 alleles. As a result of this, the cross hybridisation signals from possibly interfering alleles representing DQA1 exon 2 at DQA03 probe region, recorded less than 10 % of fully complementary DQA1*0301 alleles (Table 3.3).

*DQA1*03:02/03:03*

In Table 3.3, the normalised electrochemical responses obtained during the cross hybridisation study for the DQA0302 probe in the presence of the potentially interfering alleles at DQA1 exon 3 region are presented. Clearly, the probe showed good selectivity over the single base mismatched DQA1*03:01 allele (about 40.2 % of the response for the fully complementary allele). Furthermore, responses lower than 5% were recorded with other interfering sequences.

In the final multiplex assay, positive hybridisation signals from both DQA03 and DQA0302 probes will indicate the presence of either the DQA1*03:02 or the DQA1*03:03allele, while, the presence of the CD associated DQA1*03:01 allele in the sample will result in a positive signal with the DQA03 probe and no signal with the DQA0302 probe.

DQ2 probes selectivity

DQA1*05:01/05:05	
Probe DQA05	
Allele	%
DQA1*05:01	100 ± 7,8
DQA1*05:03	38,5 ± 5,8
DQA1*02:01	12,8 ± 2,5
DQA1*03:02	6,8 ± 1,2

DQA1*02:01	
Probe DQA0201	
Allele	%
DQA1*02:01	100 ± 5,7
DQA1*03:01	5,7 ± 0,8
DQA1*01:01	1 ± 0,3
DQA1*05:01	6 ± 1,8

DQB1*02:01/02:02	
Probe DQB02A	
Allele	%
DQB1*02:01	100 ± 12
DQB1*02:03	56,4 ± 4,6
DQB1*03:01	26,9 ± 9
DQB1*03:02	55,6 ± 9,8
DQB1*04:01	18,5 ± 0,5
DQB1*05:01	12,7 ± 4

DQB1*02:02	
Probe DQB0202	
Allele	%
DQB1*02:02	100 ± 2
DQB1*02:01	17,7 ± 5
DQB1*05:01	6 ± 0,75

DQB1*02:01/02:02	
Probe DQB02gen	
Allele	%
DQB1*02:01	100 ± 9
DQB1*03:02	21,5 ± 0,3
DQB1*04:01	18 ± 4
DQB1*05:01	14 ± 3

DQA1*05:05	
Probe DQA0505A	
Allele	%
DQA1*05:05	100 ± 4,8
DQA1*05:01	20,1 ± 5
DQA1*01:01	4,5 ± 1,4
DQA1*02:01	5,4 ± 0,6
DQA1*06:01	7,3 ± 0,8

DQ8 probes selectivity

DQB1*03:02	
Probe DQB0302A	
Allele	%
DQB1*03:02	100 ± 10,7
DQB1*02:01	4,4 ± 0,4
DQB1*03:01	19,5 ± 2
DQB1*05:01	51,4 ± 2

DQB1*03:02	
Probe DQB0302B	
Allele	%
DQB1*03:02	100 ± 7
DQB1*02:01	46,5 ± 1,6
DQB1*02:03	34,1 ± 1,4
DQB1*03:01	43,3 ± 3,7
DQB1*04:01	6,4 ± 0,7
DQB1*05:01	6,2 ± 0,5

DQA1*03	
Probe DQA03	
Allele	%
DQA1*03:01	100 ± 3,5
DQA1*02:01	9,3 ± 2
DQA1*01:01	1,1 ± 0,1
DQA1*05:01	0,9 ± 0,2

DQA1*03:02/03:03	
Probe DQA0302	
Allele	%
DQA1*03:02	100 ± 5,4
DQA1*03:01	40,2 ± 5,4
DQA1*05:01	4,8 ± 1
DQA1*05:03	4,1 ± 0,1

Table 3.3: Normalised electrochemical signal for selectivity evaluation of SSO probes with potentially interfering alleles. For each probe, (%) values were calculated as a ratio between amperometric responses obtained for the potentially interfering allele under evaluation and those obtained for the fully complementary allele. Hybridisation was performed with 20 nM of the target allele for 10 min at 37 °C, followed by hybridisation with an enzyme labelled reporter (10 nM) for 10 min at 37 °C.

Multiplex PCR samples dilution effect

The effect of the post multiplex PCR buffer components (e.g. Taq, ddNTP, primers, lambda exonuclease) on the sensor response was evaluated using a real patient sample (FRCBS 12), which had homozygous DQA1*05:01 alleles

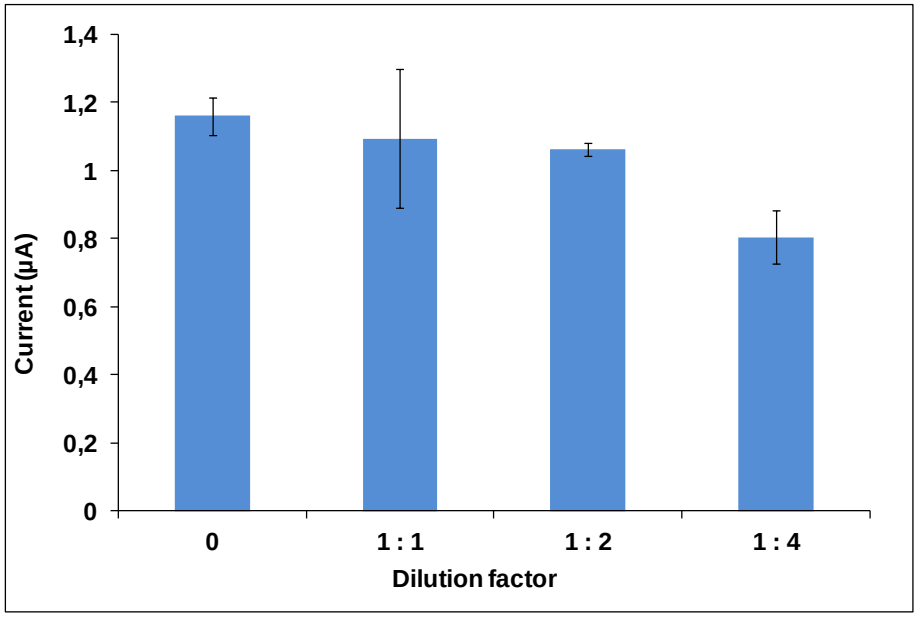


Figure 3.4: Electrochemical evaluation of real sample amplified using multiplex PCR. The study was performed using probe DQA05 and serial dilutions of real sample containing DQA1*05:01 amplicon and 10 nM of reporter probe.

An electrode array was modified with the DQA05 probe and mounted in the fluidic set-up, which was heated to 37 °C. Undiluted and different dilutions (1:1, 1:2 and 1:4) of the sample following ssDNA generation were introduced to the individual channels and left to hybridise for 10 min. Subsequently, HRP modified reporter oligonucleotide probe (10nM) was injected to each channel and hybridised for a further 10 min. As can be seen in Figure 3.4, the PCR matrix has no influence on hybridisation and electrochemical detection, with the highest signal being obtained from the sample without dilution.

Multiplex detection real sample analysis

Using the optimised surface chemistry, hybridisation time, temperature and probe design genosensor array was used for the analysis of real patient samples. The array of 36

electrodes was functionalised in triplicate with the ten probes for medium-high resolution DQ2/DQ8 typing.

Five real patient samples and LZL cell line from the European Collection of Cell Cultures were used to validate the genosensor array.

Multiplex PCR was carried out using a mixture of four primer pairs, as explained in materials and methods. Following amplification, single stranded DNA was generated via exonuclease digestion. The amperometric signals obtained are shown in Figure 3.5, and the comparison of the results obtained with the developed genosensor array and the standard techniques of Luminex One Lambda Labtype® SSO HLA Class II DQA1/DQB1 Typing Test, and Olerup DQA1or DQB1 SSP typing kits shown in Table 3.4, demonstrating an excellent degree of correlation between the techniques.

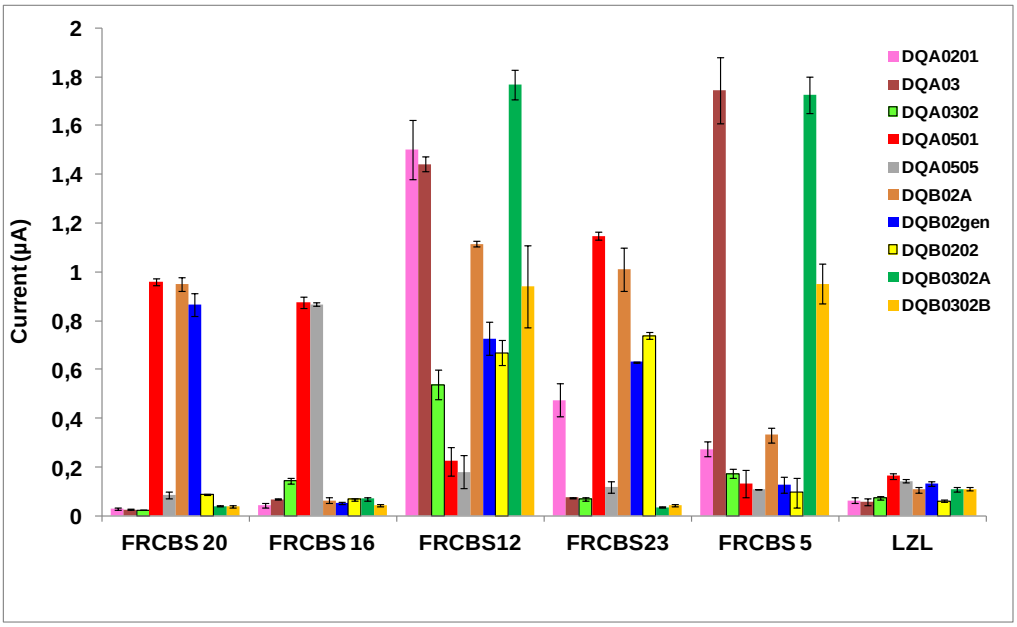


Figure 3.5. Multiplex detection of 5 real patient samples and cell line sample (LZL). Hybridisation was carried out with 1:3 dilution of the single stranded DNA generated from multiplex PCR product for 10 min. For detection, a mixture of four reporter probes (10 nM each) was added and again incubated for further 10 min at 37 °C.

Probe		Sample					
		FRCBS 5	FRCBS12	FRCBS20	FRCBS23	FRCBS16	LZL
DQA0201		-	+	-	±	-	-
DQA05		-	-	+	+	+	-
DQA0505A		-	-	-	-	+	-
DQA03		+	+	-	-	-	-
DQA0302		-	±	-	-	-	-
DQB02A		-	+	+	+	-	-
DQB02gen		-	+	+	+	-	-
DQB0202		-	+	-	+	-	-
DQB0302A		+	+	-	-	-	-
DQB0302B		+	+	-	-	-	-
DQA region		03:01/03:01	02:01/03:01	05:01/05:01	02:01/0:501	01:02/05:05	05:03/05:03
DQB region		03:02/03:02	02:02/03:02	02:01/02:01	02:01/02:02	03:01/06:02	03:01/03:01

Table 3.4. Summary of the HLA typing of the sample using the proposed electrochemical genosensor array and its comparison with the typing obtained with the reference approaches used in this work: Luminex HLA-DQA1/DQB1 typing and confirmed by Olerup DQA1 or DQB1 SSP typing kits.

(+) assigned for amperometric signal higher than 500 nA and considered and positive signal.

(-) assigned for amperometric signal lower than 500 nA and considered and negative signal.

Stability of DNA probe on gold electrode

One of the main parameters that plays an important role in real world application and commercialisation of biosensors is the shelf life stability. Poor probe stability due to desorption of the probe from the electrode surface affects the sensitivity of the genosensor array. In the literature, it has been reported that stability of a SAM is between 3 and 7 weeks for both dried and solution phase storage at room temperature [51, 52].

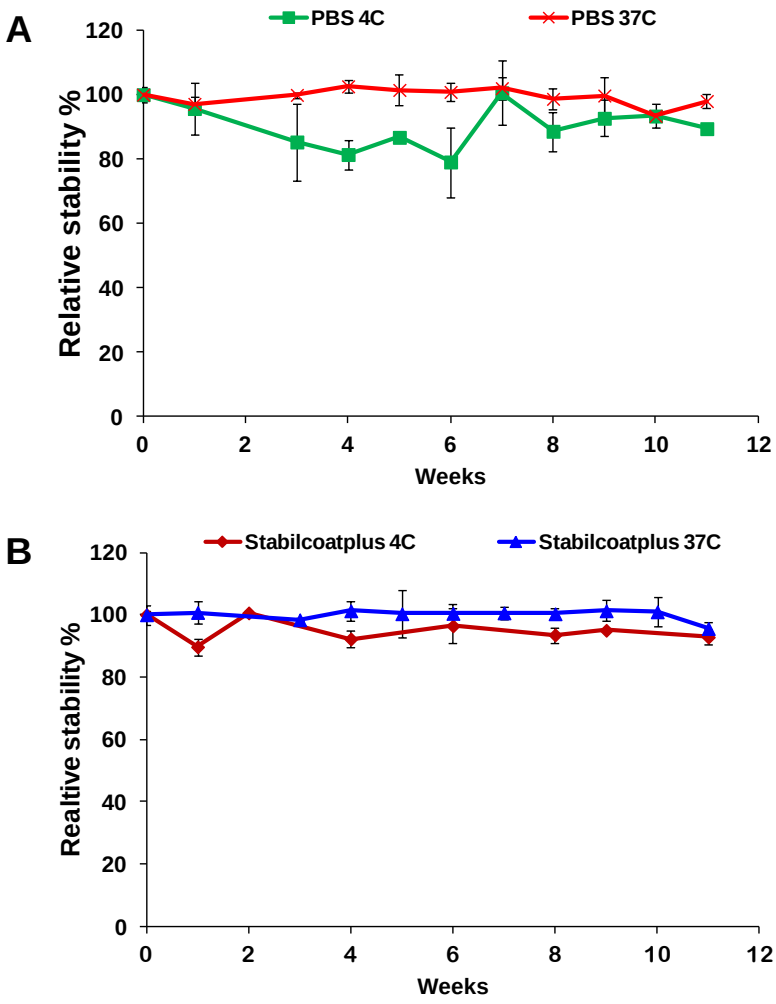


Figure 3.6: Probe stability evaluation by recording the electrochemical responses for 11 weeks. Electrode array were coated with either (A) PBS buffer or (B) stabilCoat Plus solution and stored dry at 37 and 4 °C.

High temperature is used to study the accelerated stability test [53, 54] and to simulate the longer real time storage conditions, and allows prediction of the storage stability. In this work, we tested modified electrode arrays for 11 weeks that were stored at 4 °C for real time stability and 37 °C for accelerated stability. In both cases, electrodes were covered with PBS buffer as a control stabiliser, or StabilCoat Plus stabiliser (http://www.surmodics.com/). Results showed a good stability of DNA probe (Figure 3.6A and B), at both storage conditions (4 °C and 37 °C) in the presence of either of PBS or stabilCoat plus revealing almost similar trends and only lost less than 10% of the initial

signal. From the accelerated study and Arrhenius equation, we can predict a real time (4 °C) storage stability of at least 2 years of the modified electrode array [54].

Conclusions

An electrochemical genosensor array for detection of CD genetic risk has been developed. Because of the high numbers of HLA alleles and the complexity of the HLA system, more than one probe was required for some alleles to achieve the desired resolution. Electrochemical selectivity for ten sequence specific probes designed for medium to high resolution DQ2/DQ8 genotyping was performed using synthetic alleles analogues to PCR products, and demonstrated a good discrimination capacity of the proposed probes. Finally, the proposed genosensor array was used for the analysis of real clinical samples for the presence of DQ2/DQ8 genes, demonstrating the usefulness of the developed genosensor array for the facile, rapid and precise screening of coeliac disease predisposition.

The immobilised DNA probe on the gold electrode surface exhibits good stability over time in both real time (4 °C) and accelerated stability studies (37 °C), demonstrating the suitability of the developed genosensor array as a point of care diagnostic tool.

Acknowledgments

This work was carried out with the financial support from the Commission of the European Communities, specific RDT program “Coeliac Disease Management Monitoring and Diagnosis using Biosensors and an Integrated Chip System, CD-MEDICs, FP7-2007-ICT-1-216031”. V.Beni acknowledges the European Union’s Seventh Framework Program (FP7/2007-2013) under grant agreement no. PIGF-GA-2008-220928 for financial support. H. Joda thanks Universitst Rovira i Virgili for the doctoral scholarship. We thank Dr.Markku Heikkinen, MD, PhD (Kuopio University Hospital, Kuopio, Finland) for blood samples of Finnish celiac disease patients and Dr. Taina Jaatinen, PhD, Dr. Katri Haimila, PhD and Juha Peräsaari, MS (Finnish Red Cross Blood Service, Helsinki, Finland) for HLA tissue typing expertise.

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Chapter 3

Supporting information

Description of the potentially Interfering alleles and target alleles identified for each of the investigated probes.

Table 3.S1. Sequence alignment for the DQ2/DQ8 alleles at probe area, showing the sequences of the specific and interfering alleles used in this study. A dash (-) indicates the identity to the probe sequence.

Probe DQA05				
Alleles	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQA1*05:01	**TCTGCTGAGGAGAGTTA**	112 bp	147-181+3-intron 3	DQA1*05:01, 05:05, 05:08, 05:09, 05:10
DQA1*05:03	---***T***----------	112 bp	147-181+3 -intron 3	DQA1*05:03, 05:06, 05:07
DQA1*03:02	----***A***---***T***----***T***---	112 bp	147-181+3 -intron 3	DQA1*03:02, 03:03
DQA1*02:01	--------***T***----***T***---	112 bp	147-181+3 -intron 3	DQA1*02:01,all 01, 04, 06

Probe DQA0505				
Alleles	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQA1*05:05	**ACTGGGGACTGGACAAG**	112 bp	147-181+3-intron 3	DQA1*05:05, 05:08, 05:09, 05:10
DQA1*05:01	-------***C***---------	112 bp	147-181+3-intron 3	DQA1*05:01, 05:03, 05:06, 05:07
DQA1*01:01	-------***C***------***C***--	112 bp	147-181+3-intron 3	All DQA1*01
DQA1*02:01	-------***C***-----***TG***--	112 bp	147-181+3-intron 3	DQA1*02:01, and all 03
DQA1*06:01	-------***C***------***G***--	112 bp	147-181+3-intron 3	All DQA1*06 and DQA1*04

Probe DQA0201				
Alleles	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQA1*02:01	**TCCACAGACTTAGA...TTTG**	94 bp	40 - 71	DQA1*02:01
DQA1*01:01	**--AG--A-T--G--GGT----**	97 bp	40 – 71	All DQA1*01
DQA1*03:01	**---G----T-----AGA----**	97 bp	40 – 71	DQA1*03:01,03:02, 03:03
DQA1*05:01	**--AGACA-T-----...----**	94 bp	40 - 71	All DQA1*05, DQA1*04, DQA1*06

Probe DQB02A				
Allele	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQB1*02:01	**TGCTGGGGCTGCCTGCCG**	137 bp	39 - 84	DQB1*02:01, 02:02, 02:04, 02:05, 02:06
DQB1*02:03	**----------A--**	137 bp	39 - 84	DQB1*02:01, 02:02, 02:04, 02:05, 02:06
DQB1*03:01	**C--------C-----A--**	137 bp	39 - 84	DQB1*03:01, 03:03, 03:06, 03:09, 03:10, 03:10, 03:12, 03:13, 03:15, 03:17, 03:19, 03:20, 03:22, 03:24
DQB1*03:02	**C--------C--------**	137 bp	39 - 84	DQB1*03:02, 03:04, 03:05, 03:07, 03:08, 03:11, 03:14, 03:18
DQB1*04:01	**C--------G--T--A--**	137 bp	39 - 84	DQB1*04:01, 04:02, 04:03
DQB1*05:01	**C--A-----G-----TT-**	137 bp	39 - 84	DQB1*05:01, 05:07, 05:11, , 06:04, 06:06, 06:08, 06:09, 06:12, 06:13, 06:17, 06:18, 06:21, 06:22, 06:27

Probe DQB02gen				
Allele	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQB1*02:01	**CGCTGCTGGGGCTGCCT**	137 bp	39 - 84	DQB1*02:01, 02:02, 02:03, 02:04, 02:05, 02:06
DQB1*03:02	---**C**--------**C**----	137 bp	39 - 84	All DQB1*03
DQB1*04:01	---**C**--------**G**--**T**-	137 bp	39 - 84	DQB1*04:01, 04:02, 04:03
DQB1*05:01	---**C**--**A**-----**G**----	137 bp	39 - 84	All DQB1*05, 06

Probe DQB0202				
Allele	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQB1*02:02	**GGAATGGCCAGGAGG**	96 bp	122- 154	DQB1*02:02
DQB1*02:01	------**A**--------	96 bp	122- 154	All DQB1*02:01, 06:01, all DQB1*03 and DQB1*04
DQB1*05:01	------**AT**-------	96 bp	122- 154	DQB1*05:01, 05:02, 05:03, 06 :02, 06:03, 06:04, 06:09, 06:12

Probe DQB0302A				
Allele	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQB1*03:02	**GGGGTGTATCGGGCG**	137 bp	39 - 84	DQB1*03:02, 03:03, 03:05, 03:06, 03:08, 03:10-12, 03:15, 03:17-18, 03:20, 04, 05:02:02, 05:03, 06:01, 06:19
DQB1*02:01	----**A**--**TC**------	137 bp	39 - 84	All DQB1*02 allele
DQB1*03:01	-**A**------**C**------	137 bp	39 - 84	DQB1*03:01, 03:04, 03:09, 03:13, 03:16, 03:19, 03:21, 03:22, 03:24, 03:27, 03:28, 06:35
DQB1*05:01	--------**C**-----**A**	137 bp	39 - 84	DQB1*05:01, 05:07, 05:11

Probe DQB0302B				
Allele	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQB1*03:02	**GCCGCCTGCCGCCGA**	137 bp	39 - 84	03:02, 03:04, 03:05, 03:07, 03:08, 03:11, 03:14, 03:18, 06:29
DQB1*02:01	**--*T*----------**	137 bp	39 - 84	DQB1*02:01, 02:02, 02:04, 02:05, 02:06
DQB1*02:03	**--*T*-----*A*------**	137 bp	39 - 84	DQB1*0203
DQB1*03:01	**--------*A*------**	137 bp	39 - 84	DQB1*03:01, 03:03, 03:09, 03:10, 03:12, 03:13, 03:15, 03:17, 03:19, 03:20, 03:21, 03:22, 03:27, 03:28, 03:29, 03:30, 03:31, 03:33, 03:34, 03:35, 03:36
DQB1*04:01	**--*G*--*T*--*A*------**	137 bp	39 - 84	DQB1*04:01, 04:02, 04:03
DQB1*05:01	**--*G*-----*TT*-----**	137 bp	39 - 84	DQB1*05:01, 05:07, 05:11, 06:04, 06:06, 06:08, 06:09, 06:12, 06:13, 06:17, 06:18, 06:21, 06:22, 06:27, 06:34, 06:36, 06:38, 06:39, 06:42

Probe DQA03				
Alleles	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQA1*03	**TGTTCCGCAGATTTAGAAG**	94 bp	40 – 71	DQA1*03:01, 03:02, 03:03
DQA1*01:01	***A*----*A*---*A*----*G*--*G*-**	97 bp	40 – 71	All DQA1*01
DQA1*02:01	**------*A*----*C*-----*TT***	97 bp	40 – 71	DQA1*02:01
DQA1*05:01	**-*TC*--*A*-*ACA*-------*TT***	94 bp	40 - 71	All DQA1*05, DQA1*04, DQA1*06

Probe DQA0302				
Alleles	Sequence at probe region	Amplicon size	Codon	Representative alleles
DQA1*03:02	**TCTGATGATGAGATTTA**	112 bp	147-181+3-intron 3	DQA1*03:02, 03:03
DQA1*03:01	----***C***----------	112 bp	147-181+3-intron 3	DQA1*03:01, 02:01, 06:01, 06:02 all DQA1*01/ DQA1*04,
DQA1*05:01	----***C***---***G***----***G***---	112 bp	147-181+3-intron 3	DQA1*05:01, 05:05, 05:08, 05:09
DQA1*05:03	---***TC***---***G***----***G***---	112 bp	147-181+3-intron 3	DQA1*05:03, 05:06, 05:07

Table 3.S2: Sequence of probes used to study for detection of DQA1*05:05 allele and single base mismatched DQA1*05:01 allele. Base in bold and underlined indicates the artificial mismatch.

Probes	Sequence
DQA0505	5'-CTTGTCCAGTCCCCAGT –SH-3'
DQA0505sh1	5'-TTGTCCAGTCCCCAG-SH3'
DQA0505sh2	5'-CTTGTCCAGTCCCCA–SH-3'
DQA0505A	5'-CTT***T***TCCAGTCCCCAGT-SH-3'
DQA0505AR2	5'-CTTGTCCAGTCCC**A**AGT–SH-3'

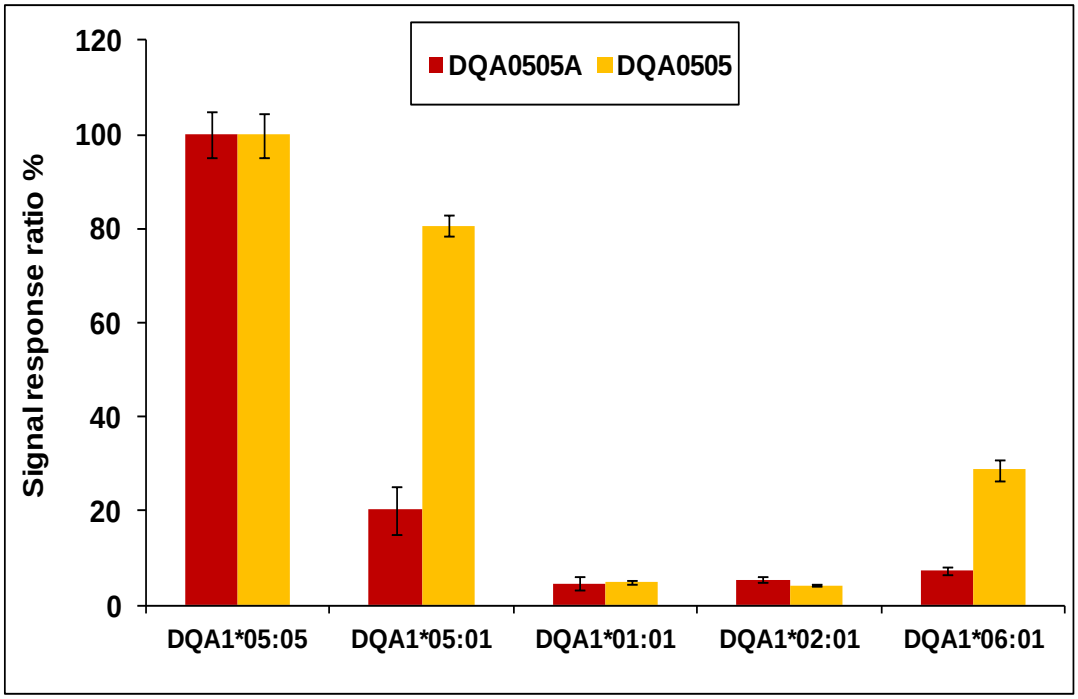

Figure 3.S1: Normalised electrochemical response showing the comparison of cross hybridisation study of DQA exon 3 alleles for (■) DQA0505 and (■) DQA0505A probes. Electrochemical signal were normalised to the reading of the fully complementary allele.

Description of the interpretation of the electrochemical genotyping for the real sample typing

Sample FRCBS5

Amperometric responses higher than the cut-off point (500 nA) were recorded over probes: DQA03, DQB0302A, and DQB0302B.

High electrochemical signal over DQA03 probe combined with low response over probe DQA0302 indicates the presence of DQA1*03:01 alleles and the absence of both DQA1*03:02 and/or DQA1*03:03 alleles. This finding was confirmed by a positive response for the DQB0302A and DQB0302B probes that genotype DQB1*03:02 allele, as they have strong linkage disequilibrium [1].

The overall genotyping of this sample was predicted to be DQ8 positive.

Sample FRCBS12

Electrochemical analysis of this sample showed signal over 500 nA from all probes except DQA0501 and DQA0505A probes. On the other hand, positive signal over DQB02A, DQB02gen and DQB0202 probes indicate the presence of DQB1*02:02 allele, confirmed by the positive response of DQA1*02:01 allele, as both alleles are in strong linkage disequilibrium and usually inherited together [2]. Although low positive response was recorded for the DQA0302 probe (538,8 ± 60,7 nA), the very high signal for probe DQA03 probe (1442,4 ± 29 nA) with positive response for DQB1*03:02 allele indicates the presence of DQA1*03:01, and the absence of DQA1*03:02 and DQA1*03:03 alleles.

The responses obtained clearly indicated that this sample has one chain DQ2 heterodimer and is DQ8 positive.

Sample FRCBS 16

An amperometric signal was recorded only for DQA05 and DQA0505A probes, indicating the presence of DQA1*05:05 allele with or without DQA1*05:01 allele. The presence of the DQA1*05 allele in the absence of the DQB1*02 allele was considered as DQ2 negative and is not associated with a risk to develop CD [3].

Sample FRCBS20.

For this sample, positive responses were recorded for the DQA05, DQB02 and DQB02gen probes. Thus, this sample was predicted to have at least one copy of DQA1*05:01 and DQB1*02:01 alleles and considered as DQ2 positive.

Sample FRCBS23.

Analysis of this sample recorded positive responses for the DQA05, DQB02A, DQB02gen and DQB0202 probes, whilst a low positive signal was recorded for probe DQA0201 (475,8 ± 68 NA), but was considered as positive because it is usually inherited with the DQB1*02:02 allele [4].

Electrochemical analysis of this sample predicts the presence of DQ2 (in both cis and trans haplotypes).

Sample LZL

A low amperometric response was recorded from all probes, with the highest signal for the DQA05 probe. The absence of positive responses for all other probes clearly indicated that the LZL sample was both DQ2 and DQ8 negative.

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CHAPTER 4

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Modified primers for direct detection of double stranded PCR

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Abstract

Since the completion of the sequencing of the human genome, there have been huge advances in the application of genetic analysis for clinical diagnostics However, detection of PCR product via hybridisation is time consuming requiring post amplification treatments such as the generation of single stranded DNA. Direct detection of double stranded PCR product would facilitate the development of rapid diagnostic tools for genetic analysis. In this work, a specifically PCR primer designed having the ability to generate PCR amplicons suitable for direct detection is presented. The use of the proposed novel primer design results in a PCR amplicon tagged with double-tailed single stranded DNA that can be used for direct hybridisation with a surface immobilised probe and an enzyme labelled reporter probe. The production of amplicons with the above mentioned characteristic was made possible by the different functional elements implemented in the primer design: a DNA tail for amplicon capture on surface or labelling; a PCR stopper for polymerase reaction restriction; DNA sequence for sample purification (primers fishing) and finally a DNA sequence having the functionality of conventional PCR primers. Primarily, the presence of un-reacted primers was shown to decrease the colorimetric signal. An approach for the removal of un-reacted primers “fishing” using magnetic beads functionalised with a DNA sequence complementary to a sequence in the primer was evaluated. However, changing PCR protocol to a two-temperature protocol and the use of a higher concentration of PCR product for hybridisation showed that the fishing step can be omitted and hybridisation can be performed directly using the un-purified PCR product. Furthermore, different hybridisation times for direct PCR detection were assessed demonstrating the ability to decrease the assay time to less than 5 minutes.

In the work reported here, as a proof of concept the HLA-DQB1*02 allele was amplified using modified sequence specific primers and the PCR product was directly detected via enzyme linked oligonucleotide assay.

Introduction

The development of the Polymerase Chain Reaction (PCR) in 1984 [1] revolutionised molecular biology, facilitating enormous advances in genetic analysis and leading to the sequencing of human genome, resulting in the identification of a plethora of disease associated genes. Furthermore, addressing the future paradigm of individualised theranostics, the possibility to monitor expression patterns to assist in deciding upon appropriate medical intervention is being investigated. Whilst qualitative detection of PCR products can be achieved using gel electrophoresis, there is increasing interest in hybridisation based detection approaches that can provide both qualitative and quantitative information. However, the use of hybridisation based detection generally requires processing of the PCR product to generate single stranded DNA (ssDNA) for hybridisation to a complementary probe. Addressing this requirement, several methods for ssDNA generation have been reported, the most simple method is the thermal denaturation method, in which ssDNA is generated by heating the sample to 95-100°C followed by rapid cooling on ice [2, 3]. Another method relies on the immobilisation of biotinylated PCR product (biotin is normally introduced via the use of a biotinylated primer) on a streptavidin-coated surface (e.g. magnetic beads), followed by either thermal or alkaline denaturation resulting in release of the unmodified strand [4, 5]. Alternatively, asymmetric PCR can be used to obtain amplified ssDNA, where unequal molar ratios of forward and reverse primers are used for sample amplification. Thus, an excess of ssDNA is produced in each cycle from the higher primer concentration [6, 7] and eventually the primer of lower concentration becomes exhausted, followed by linear amplification of the single stranded product. Finally, the use of the lambda exonuclease enzyme for the selective digestion of a phosphorylated DNA strand has been used as a simple method for ssDNA generation, where one primer is modified with phosphate at the 5´ end, and following PCR amplification, ssDNA is generated by digestion of this phosphorylated strand [8, 9]. Whilst these techniques are widely used, they do however suffer from many drawbacks, they are inherently time consuming and add a cost and complexity factor to the analysis of PCR products, and most importantly, in some cases they are poorly reproducible and in all cases they suffer from a lack of efficiency.

To avoid the need for post-amplification sample treatment, significant effort has been made to achieve direct detection of double stranded DNA (dsDNA) PCR products.

De Lumley-Woodyear et al. [10] reported the detection of a dsDNA PCR product labelled with digoxigenin at the 3'end with multiple biotins along its chain. The resulting PCR product was captured through binding of the 3'-digoxigenin with anti-digoxin monoclonal antibody which was covalently attached to an electrode surface modified with a conducting redox hydrogel, followed by addition of avidin modified horseradish peroxidase and amperometric measurement.

Further alternative approaches include the use of molecular interactions and self-assembling for the direct electrochemical detection of a dsDNA product [11-13], exploiting the amplification of DNA templates using double modified primer pairs either with thiol and digoxigenin [12] or biotin and digoxigenin [11, 13]. The amplified double tagged PCR product was then immobilised through either the thiolated or the biotinylated end, while the digoxigenin label was used for amperometric detection after binding of an anti DIG-HRP reporter [12, 13] or impedimetric detection following binding of an anti DIG-gold nanoparticle or anti DIG-Protein G [11], with the assays being completed in less than 60 minutes.

Hayashi et al. [14, 15] reported a surface plasmon resonance (SPR) imaging biosensor for the detection of a dsDNA PCR product, where one of the primers was modified with a mirror-image DNA (L-DNA) tag that remained as a single strand after PCR amplification. Subsequently, PCR product with L-DNA tag was hybridised with single-stranded DNA probes immobilised on a gold chip for 10 minutes at a flow rate of 100 µl / min and the recognition event was imaged using SPR.

Another approach proposed for the direct detection of dsDNA is based on the use of triplex-forming oligonucleotides (TFOs) as recognition probes. Triplex nucleic acids structures are formed through sequence-specific Hoogsteen, or reverse Hoogsteen interactions in the major groove of DNA [16, 17]. One of the first applications of the formation of triplex structures for the detection of DNA used immobilised biotinylated oligonucleotides and studied triplex formation in real time using surface Plasmon resonance (SPR) [18]. The direct detection of double-stranded oligonucleotide (dsDNA) corresponding to the hepatitis C virus genotype 3a, using a gold electrode functionalised with a self assembled monolayer of a 14-mer guanine-free PNA probe has also been reported [19]. Following addition of methylene blue, which selectively interacts with the guanine residues of the duplex [20, 21], hybridisation with the dsDNA target was measured electrochemically via differential pulse voltammetry. In another report, a triplex

forming oligonucleotide probe was modified at the 5′-end with a mercaptohexanol moiety and at the 3′-end with a methylene blue redox label and hybridisation of the dsDNA target was detected via square wave voltammetry (SWV). Triplex formation is a very elegant approach for the direct detection of dsDNA, but does require very careful design as the DNA triplex has the disadvantage of having low stability under normal physiological conditions requiring the use of stabilising agents.

In this work, we present a novel primer design that results in a PCR product flanked by two single stranded DNA tails. These "tails" are used for hybridisation with a surface immobilised probe and an enzyme labelled reporter probe and detected colorimetrically. The use of modified primers for the production of dsDNA that can be directly detected is cost effective, efficient, robust and easily multiplexed. We demonstrate this proof-of-concept using sequence specific primers designed to detect the DQB1*02 allele associated with a predisposition to coeliac disease. The influence of un-reacted primers using different PCR protocols on the detection efficiency was also investigated and an approach for the removal (fishing) of excess primer from the PCR product was evaluated.

Material and Methods

Materials

All reagents used in this work were of analytical grade: Trizma hydrochloride, sulphuric acid (H_2SO_4), phosphate-buffered saline (pH 7.4) with 0.05% v/v Tween 20 (PBS-Tween), Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA were purchased from Sigma Aldrich (Sigma Aldrich, Spain). 6 M hydrochloric acid (HCl), sodium chloride (NaCl) and sodium dihydrogen phosphate (NaH_2PO_4) were obtained from Scharlau. Sodium hydroxide (NaOH) pellets was received from Panreac (Panreac, Spain). Milli-Q water (18 MΩ cm) was obtained using a Simplicity water purification system (Millipore, France). Reacti-Bind™ Maleimide Activated 96-Well Plates and Streptavidin magnetic beads were received from Thermo Scientific Pierce (Cultek SLU. Spain). Double tailed–double stranded DNA (dt-dsDNA) capture, HRP labelled reporter and fishing DNA probes were supplied by biomers.net GmbH (biomers.net GmbH, Germany). Details of the sequences used in this work can be seen in Table 4.1.

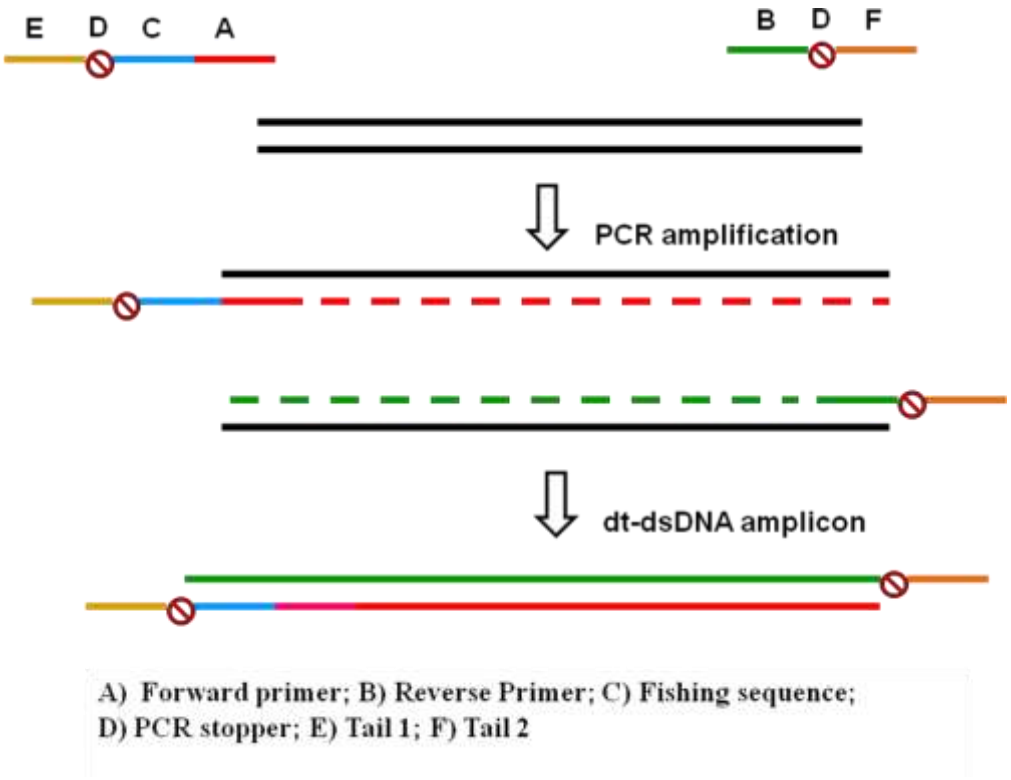
Table 4.1: Sequence of the oligonucleotides used in this work.

Sequence Name	Sequence
Forward Primer (3bF)	5´GTTTTCCCAGTCACGAC-Spacer-CAGGAAACAGCT ATGAC CCGTGCGTCTCGTGAGCAGAAG3´
Reverse Primer (3bR)	5´TGTAAAACGACGGCCAGT-Spacer-TGCAAGGTCGTGC GGAGCT3´
Probe (3b Probe)	5'GTCGTGACTGGGAAAAC–TEG-SH3'
Reporting sequence	5'ACTGGCCGTCGTTTTACA–HRP-3'
Fishing probe (FP)	Biotin -5' GGTCATAGCTGTTTCCTG '3

Primer design

Sequence specific primers (SSP) are designed to amplify a particular allele or group of alleles, where amplification only takes place in the presence of a fully complementary sequence, resulting in the generation of a product of a specific size [22, 23], whilst the absence of amplification product indicates the existence of one or more mismatches between the sequence and the primers.

In Scheme 4.1, a pictorial description of the primer design herein is presented. Four main functional elements can be identified in the proposed design: i) a short oligonucleotide chain located at the 3' end of each primer that corresponds to active primers (segments A and B respectively in the forward and reverse primers), ii) a short oligonucleotide sequence, obtained from a viral M13 sequence, used to remove un-reacted forward primers (segment C). This is present only in the forward primer because this is the only primer that could interfere with the detection of the dt-dsDNA product, as the ssDNA tail associated with this primer can compete with dt-dsDNA for hybridisation with a capture probe, iii) a PCR stopper that has the function of stopping the polymerase reaction (element D in both primers) and finally iv) two specific short oligonucleotide tails used for the capture and/or labelling of the dt-dsDNA PCR product (segments E and F respectively) [24]. For any further information regarding the primers please contact INNO-TRAIN Diagnostik GmbH (http://www.inno-train.de/)



Scheme 4.1: Pictorial representation of PCR amplification using the proposed primers design.

PCR amplification

The composition of the amplification mixture was the following: 400 nM of each primer, 0.8µL DNA Polymerase "AxiTaq" and 30 µL PCR buffer "ready PCR" (Inno-Train Diagnostik GmbH Germany), 150 ng DNA template (DQB1*02:01/ *02 :01, DQA1*02:01/ *05:01), and the volume was brought to 100 µL using MilliQ water. Amplification was performed on a Bio-Rad icycler thermal cycler according to the following protocol: initial denaturation at 96 ºC for 2 min, followed by 10 cycles at 96 ºC for 15 sec, and then 65 ºC for 60 sec, followed by 20 cycles at 96 ºC for 15 sec, 61 ºC for 50 sec and 72 ºC for 30 sec. Two-temperature PCR amplification was performed via an initial denaturation 96°C for 1 min, 30 cycles of 96°C for 15 sec and at 61°C for 50 sec.

Gel based Primer extraction was performed according to the supplier's instruction. Briefly, purification of PCR product from un-reacted primers was achieved using a PureLink Quick Gel Extraction Kit. The portion of the gel containing the amplified DNA was carefully cut and placed into a microcentrifuge tube, and the gel was subsequently dissolved with gel solubilisation buffer. The obtained solution was then loaded onto the Quick Gel Extraction column and centrifuged, after which washing

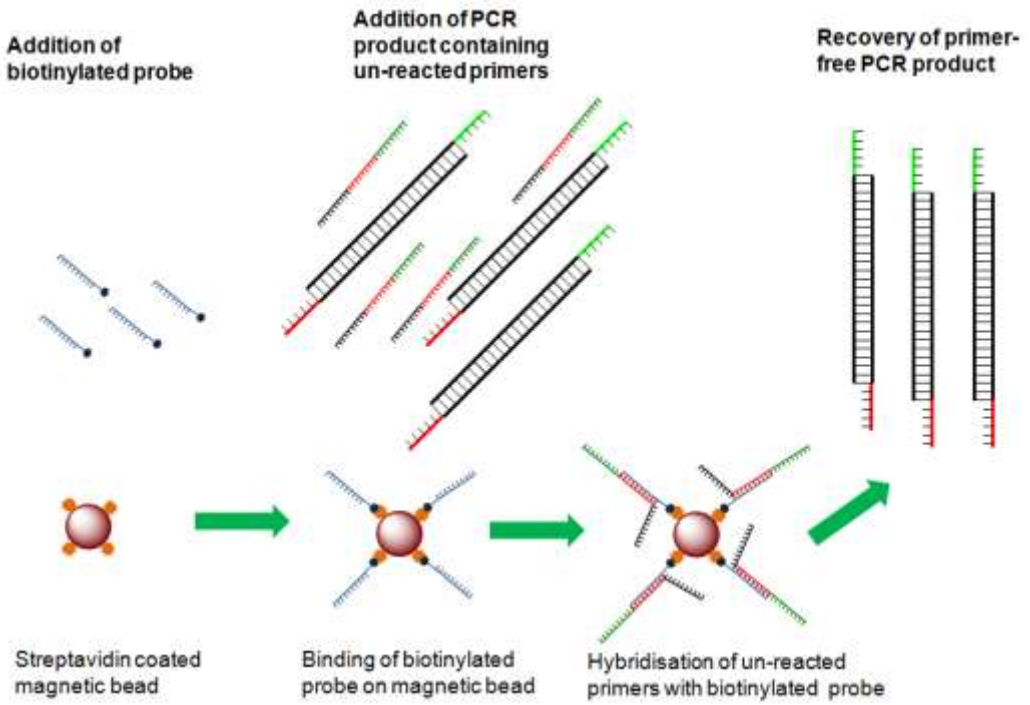
buffer was added and again centrifuged. Finally, elution buffer was added followed by a final centrifugation to recover purified DNA.

Magnetic beads primer fishing

Fifty microliters of the streptavidin coated magnetic beads (10 mg/mL) solution were placed in a microcentrifuge tube and washed 3 times with binding buffer (10 mM Tris pH 7.4 + 1 M NaCl + 1 mM EDTA). Following washing, 200 µL of biotinylated oligonucleotide "fishing probe" (1 µM) in binding buffer were added to the magnetic beads and incubated at room temperature for 60 min under gentle shaking. The "fishing probe" is the reverse complementary of the "fishing sequence" (segment C in Scheme 4.1) incorporated in the forward primer and designed to allow elimination of any un-reacted primers if necessary. The tube was placed on a magnetic rack and the supernatant discarded. The obtained DNA modified magnetic beads were then washed 3 times with the binding buffer to remove non-specifically adsorbed DNA.

In order to perform primer fishing, 100 µL of 10-fold diluted un-purified PCR product were mixed with the desired concentration of the modified beads and "primer fishing" left to take place for 1 h under gentle shaking (Scheme 4.2).

After termination, the magnetic beads were collected by magnetic field and the supernatant containing clean PCR product (primer free) was collected and ready for use.



Scheme 4.2: Steps of magnetic beads based primer removal.

Enzyme Linked Oligonucleotide Assay (ELONA) hybridisation assay

ELONA is a colorimetric technique analogue to ELISA used for the detection of DNA [25, 26]. Thiolated DNA probe immobilisation on maleimide activated microtitre plates was performed by adding 250 nM of the probe diluted in binding buffer (0.1 M NaH_2PO_4, 0.15 M NaCl, 10 mM EDTA; pH 7.2) to each well and incubating for 2 h at 37 °C. Subsequently, 200 µl of 0.1 mM of MCH were added to each well and incubated for 1 h at 37 °C in order to block the remaining maleimide groups.

PCR products were diluted in hybridisation buffer (10 mM Tris buffer containing 1M NaCl pH 7.4), added to each well and incubated for 1 h at 37 °C. Subsequently, the HRP labelled reporter probe (5 nM in hybridisation buffer) was added and left to hybridise for 1 h at 37 °C. Between each consecutive step, the wells were washed three times with PBS-Tween. Finally, to detect the captured reporter probe, TMB liquid substrate was added (100 µL) to each well, incubated for 15 min and then the enzymatic reaction stopped by addition of an equal amount of 1 M H_2SO_4 and the absorbance was read at 450 nm using a multiplate reader Wallac Victor2 1420 Multilabel counter (Perkin Elmer).

Results and discussion

PCR Amplification using modified primers

The gel electrophoresis of the PCR product using modified primers for positive sample and negative template control (NTC), can be seen in Figure 4.1, where in the lane corresponding to the positive sample there is an intense band with the expected size for the dt-ds DNA PCR product [225 bp + 2 single stranded tails (17 and 18 mer)], whilst, no band appears in the lane corresponding to the no template negative sample (NTC). The successful amplification of the desired allele using the modified primers is clearly observed, demonstrating that the presence of the PCR stopper and of the different tails did not inhibit amplification. Two faint bands corresponding to un-reacted forward primer (top band) and reverse primer (low band) can also be observed.

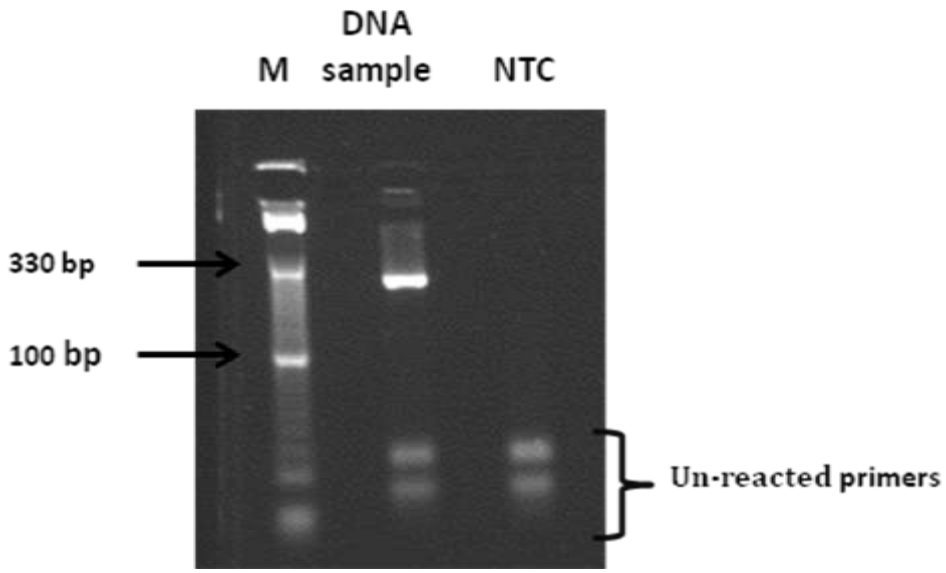


Figure 4.1: Agarose gel electrophoresis of the amplified PCR product: (M) 10bp PCR marker; DNA positive sample; (NTC) negative non template control sample.

Detection of dt-dsDNA

Once the use of the modified primers in PCR amplification had been demonstrated, the ability to detect the resulting PCR product in the presence and absence of un-reacted primers was evaluated.

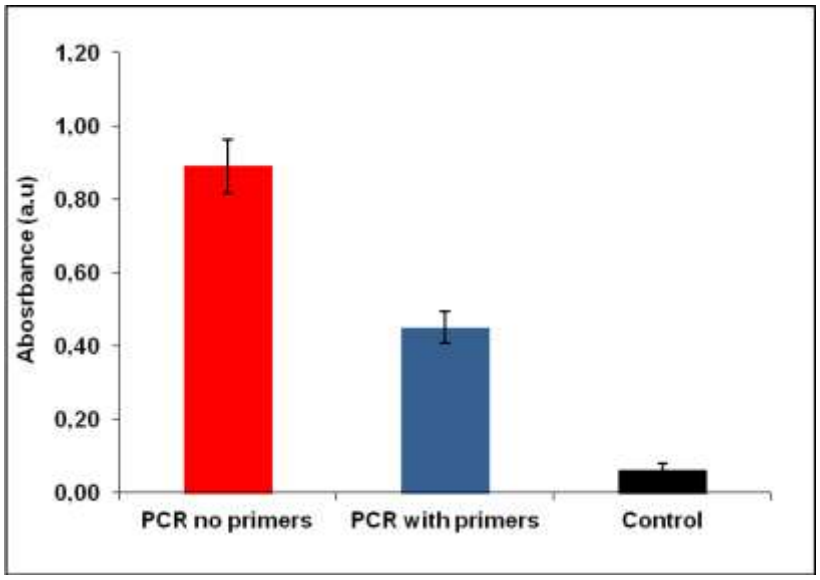


Figure 4.2: Proof of concept of the detection of dt-dsDNA by ELONA assay. **PCR with primers:** Response obtained using un-purified PCR product; **PCR no primer**: Response obtained using dt-dsDNA extracted from PCR product using PureLink Quick Gel Extraction Kit. **Control:** Response of the unspecific adsorption of enzyme labelled reporter probe.

In Figure 4. 2, the result of an ELONA assay performed before and after primer removal using the PureLink Quick Gel Extraction Kit is reported, and as can be seen, a clear response for the capture of the dt-dsDNA was recorded. Using the primer concentration of 400nM, the presence of un-reacted primers influenced the efficiency of hybridisation with the dt-dsDNA target, as the un-reacted primers "compete" with the target PCR product for binding to the immobilised capture probe, whilst the gel extraction approach is very efficient for removal the un-reacted primers. Thus two approaches were investigated to eliminate this problem, offering generic and PCR specific solutions. The first method involves a post-PCR processing step, using functionalised magnetic beads to selectively bind to un-reacted primer. The advantage of this method is that it is universally applicable, however it is preferable to be able to directly detect the PCR product without further processing and to address this, an improved PCR protocol by switching to a two-temperature PCR protocol was performed.

Elimination of un-reacted primers (primers fishing) using magnetic beads

In the first approach, a protocol exploiting the specifically designed primers and taking the advantage of the incorporated "fishing" sequence (segment C in Scheme 4.1), functionalised magnetic beads for removal of un-reacted primers was investigated. This protocol has the disadvantage of being time consuming and not being particularly suitable for integration in automated protocols.

Un-reacted primer can be captured using a sequence complementary to the fishing sequence, while upon amplification the "fishing" sequence is protected as it is incorporated between the PCR stopper and the active primer sequence, and will be present as a duplex in the dt-dsDNA, thus facilitating selective fishing of only the un-reacted primers. As can be seen in Figure 4.3, the "primer fishing" using magnetic beads effectively reduced the interference of the un-reacted primers resulting in a significant improvement of the response (PCR after fishing versus PCR no fishing).

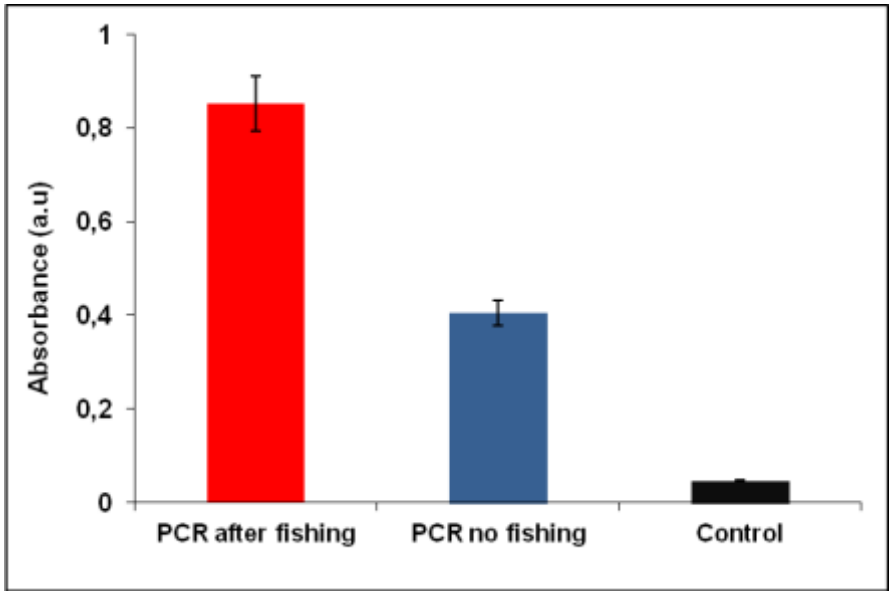


Figure 4.3: Proof-of-concept of the feasibility of primer elimination by magnetic bead fishing. **Control:** Response due to unspecific adsorption of enzymatic reporting tag;
PCR no fishing: Response obtained in the presence of primers. **PCR after fishing**: Response obtained using PCR product after primer "fishing" using "fishing" sequences immobilised onto streptavidin modified magnetic beads (fishing performed for 60 minutes). PCR product diluted 1 to 10 before fishing; sample furthermore diluted 1 to 6 before detection.

The concentration of magnetic beads was optimised using aliquots containing a constant concentration of un-purified PCR product (100 µL of 1 to 10 diluted PCR products in hybridisation buffer) placed in eppendorf tubes with increasing amounts of magnetic beads modified with the fishing probe. Following incubation for 1 h under gentle shaking the supernatant was collected from the tubes and a further 6 fold dilution with hybridisation buffer was made. As can be seen in Figure 4.4, the colorimetric responses increased as a function of the concentration of the magnetic beads used for primer removal reaching a plateau at 150 µg /mL.

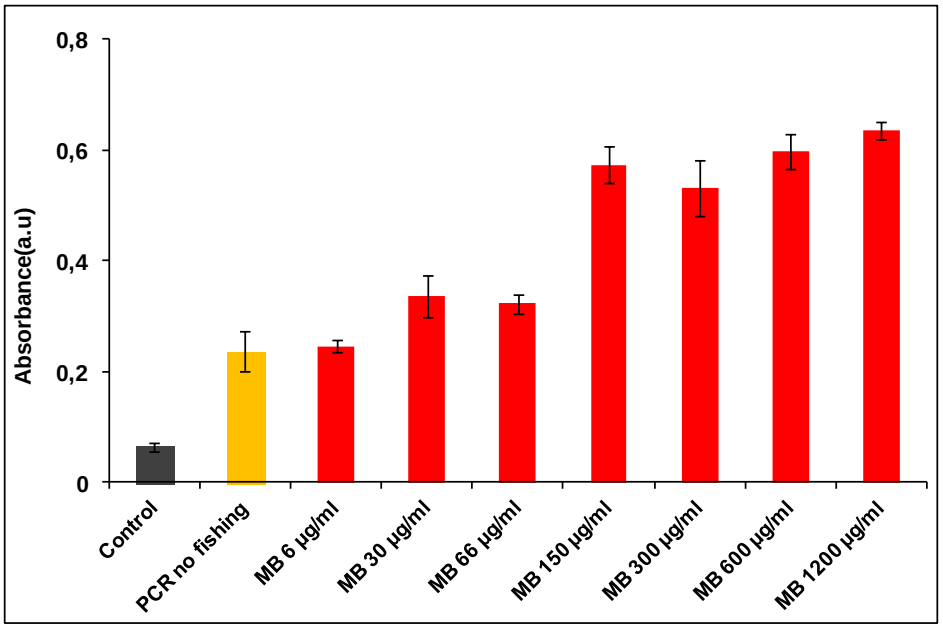


Figure 4.4: Evaluation of the use of increasing concentrations of oligonucleotide modified magnetic beads in primer "fishing". **Control:** Response associated with unspecific adsorption of enzymatic reporting tag; **PCR no fishing:** Response obtained in the presence of primers. **MB x µg/ml**: Response obtained using increasing concentrations (6, 30, 66, 150, 300, 600 and 1200 µg/ml) of modified MB during primer "fishing". No previous purification of the PCR product was performed. PCR product diluted 1 to 10 before fishing; sample furthermore diluted 1 to 6 before detection. "Fishing" performed for 60 minutes.

dt-dsDNA detection without primer fishing

To eliminate the need for post-PCR treatment, the possibility to achieve direct detection of PCR product was investigated. To achieve this, a two-temperature PCR protocol for more efficient amplification was carried out. In this experiment, DNA template was amplified using the following protocol; initial denaturation 96°C for 1 min, followed by 30 cycles of 96°C for 15sec and at 61°C for 50sec. Microplate colorimetric detection using two different dilutions of PCR product (1 to 60 and 1 to 10) in hybridisation buffer was performed.

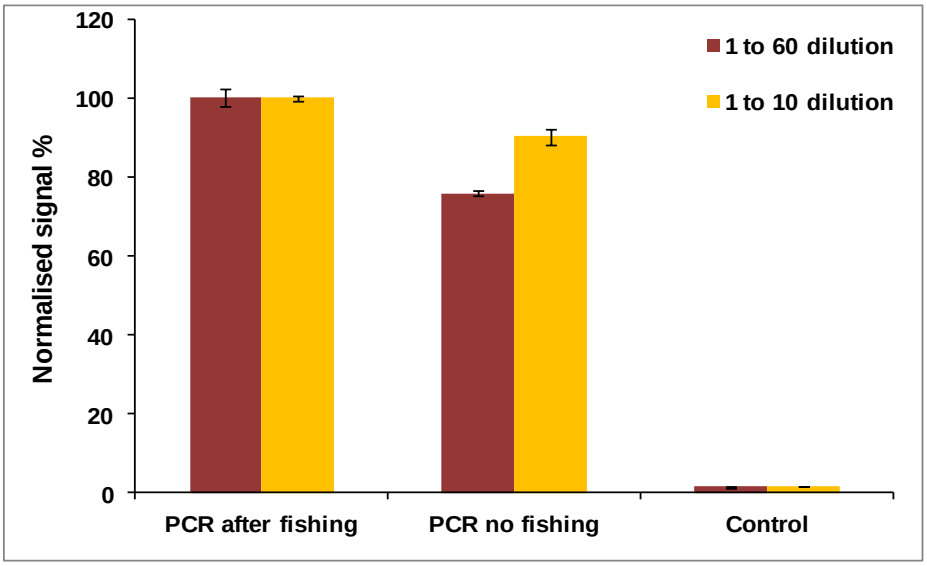


Figure 4.5: Normalised colorimetric signal for the detection of dt-dsDNA PCR product amplified using two-temperature PCR protocol (before and after primer fishing). Two different dilutions was used, 1 to 60 and 1 to 10 in hybridisation buffer. Hybridisation was performed for 60 min for PCR target and HRP reporter probe at 37 °C.

In comparison to the hybridisation signal recorded for the PCR amplicons before and after primer fishing that presented in the previous sections, the use of modified PCR protocol showed a considerable improvement in the hybridisation signal of (PCR no fishing versus PCR after fishing) as can be seen in Figure 4.5. This result indicates that using an optimised PCR protocol reduce the influence of un-reacted primers on hybridisation of dt-dsDNA PCR product. Moreover, using more concentrated PCR product (1 to 10) resulted in a further improvement of hybridisation signal of un-treated PCR product (PCR no fishing) to about 90% of the signal obtained with PCR product after fishing.

These results demonstrated the possibility of direct detection of the proposed dt-dsDNA PCR product without any further post-PCR processing.

Hybridisation time study

Hybridisation of the untreated PCR product at different time interval ranging between 4 to 60 min was investigated (Figure 4.6), indicating minimal change in hybridisation signal between 2+2 min (2 min for each PCR product and reporter probe) and 5+5 min (5 min for each PCR product and reporter probe), demonstrating that the hybridisation time can be decreased to as low as 2 min for each hybridisation step, where the

hybridisation signal showed a clear differentiation between the target signal and background control signal.

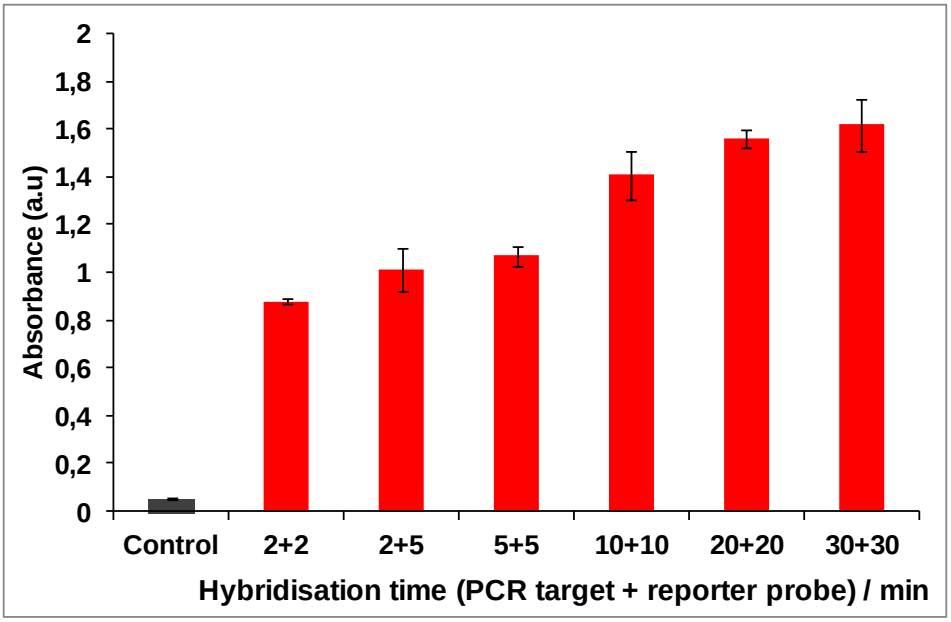


Figure 4.6: Evaluation of the effect of PCR product and HRP reporter probe hybridisation time on the colorimetric signal.

Conclusions

In the work reported, a novel design of PCR primers enabling the amplification of a PCR product suitable for direct hybridisation-based detection is detailed.

Amplification of DNA template was shown to be possible using the developed modified primer design. Furthermore, the ELONA measurement confirmed the feasibility of the direct detection of the obtained PCR product without a need for further sample treatment.

However, the presence of un-reacted primers in the PCR product was also observed to be present and in order to suppress this potential interference, a method based on the use of oligonucleotide modified magnetic beads was proposed and demonstrated. To avoid this post-PCR processing step, a two-temperature PCR protocol was observed to improve the signal of un-purified PCR product and further signal enhancement was observed upon hybridisation with a higher PCR product concentration (1 to 10). Using the optimised PCR protocol a significant decrease of the assay time was achieved.

Acknowledgments

This work was carried out with the financial support from the Commission of the European Communities, specific RDT program "Coeliac Disease Management Monitoring and Diagnosis using Biosensors and an Integrated Chip System, CD-MEDICs, FP7-2007-ICT-1-216031". V.Beni acknowledges the European Union's Seventh Framework Program (FP7/2007-2013) under grant agreement no. PIGF-GA-2008-220928 for financial support. H. Joda thanks Universitst Rovira i Virgili for the doctoral scholarship.

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CHAPTER 5

Rapid electrochemical HLA typing of coeliac disease predisposition

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Abstract

Genetic analysis for coeliac disease analysis is gaining importance. Sequence specific primer (SSP) kits are the most widely used genetic test for coeliac disease predisposition analysis, but they are expensive, laborious and time consuming. In this work, we use specifically designed sequence specific primers for the synthesis of PCR products with double single stranded tails, enabling direct electrochemical detection. Hybridisation time and temperature were optimised demonstrating the possibility to perform the analysis in less than 5 minutes at 37 °C. To achieve low-resolution typing three different primer-pairs were designed for selective amplification of coeliac disease associated alleles namely: DQA1*05, DQB1*02 and DQB1*03:02 alleles, in addition to the primer for human growth hormone as a positive control. Samples analysis was carried out using the optimised assay for the multiplex PCR product of the four primer-pairs using an electrochemical genosensor array and an excellent correlation with standard laboratory techniques was achieved.

Introduction

The human leukocyte antigen (HLA) is the human equivalent of the major histocompatibilty complex (MHC), controlled by genes on the short arm of chromosome six (6p21.3) [1, 2]. This HLA is known to be highly polymorphic region in the human genome and to have an essential role in the immune system. HLA is associated with a susceptibility to several autoimmune diseases such as coeliac disease [3], insulin dependent diabetes mellitus [4], rheumatoid arthritis [5] and IgA deficiency [6].

Coeliac disease (CD) is a common immune-mediated disorder of the small intestine that affects genetically susceptible individuals after exposure to gluten and related proteins [7, 8]. The genetic risk of CD is known to be very strongly associated with certain alleles in HLA-DQ region, as about 95% of coeliac patients carry DQ2 variant, while DQ8 variant is carried by the remainder [9, 10]. However, it has been reported that about 20-30% of Caucasian carry DQ2/DQ8 [11, 12], therefore whilst the presence of DQ2 or DQ8 is essential for developing CD, the presence of DQ2/DQ8 does not necessary result in onset of the disease. The HLA-DQ2 heterodimer is encoded by DQA1*05:01 and DQB1*02:01alleles either in the cis or can also be encoded in trans by DQA1*05:05 and DQB1*02:02 alleles DQ2 haplotype. The HLA-DQ8 hereodimer is encoded by DQA1*03:01 and DQB1*03:02 in cis haplotype [13, 14]. Thus, HLA-DQ2/DQ8 typing is increasingly used in CD diagnosis and is used for the screening of high-risk individuals [15] such as first-degree relatives, individuals with autoimmune diseases, IgA deficiency, with Down, Turner or Williams syndromes [16].

The typical methods for HLA typing include sequence-specific primers (SSP) [17], sequence-specific oligonucleotide probe (SSOP) [18] and sequencing based typing (SBT) [19]. SSP HLA typing is based on the use of primers specifically designed to amplify a specific allele or alleles group where the presence of a product of a specific size is detected using gel electrophoresis [17, 20].

There are several commercial kits for DQ2/DQ8 mainly based on SSP amplification and sizing by gel electrophoresis such as: Olerup SSP kit (Olerup SSP AB- Sweden), HLA Ready Gene Coeliac Disease (INNO-TRAIN Diagnostik GmbH - Germany), Eu-Gen System (Eurospital - Italy) and HISTO TYPE Celiac Disease (BAG Health Care GmbH- Germany). These kits are laborious as they need multiple steps, several reactions, require post-PCR manipulation, requiring expertised personnel and are

inherently expensive. However, several approaches have been reported mainly based on real-time PCR. Nils Reinton et al. [21] used real-time PCR for sequence-specific primers (SSP) and TaqMan detection, Profaizer et al. [22] developed a SYBR Green real-time PCR with melting curve analysis, Monsuur et al. [23] used real-time PCR to detect SNPs that are in linkage disequilibrium with CD associated HLA alleles using only six SNPs tags and finally, Ollikka et al. [24] developed a closed-tube PCR analysis of dried blood and saliva samples based on time-resolved fluorometry.

In conventional DNA hybridisation analysis, usually several post-PCR steps are required before sequence specific hybridisation such as gel electrophoresis, purification, ssDNA generation, which all lead to an additional load of work, cost and time. Several research groups tried to overcome these drawbacks by developing methods which enable direct detection of PCR product. One of the reported methods is the use of modified primers to produce double labelled PCR amplicons. Bonanni et al. [25] used primers modified with biotin and digoxigenin to develop an electrochemical impedimetric genosensor for *Salmonella spp*. The amplification resulted in an amplicon with biotin and digoxigenin moieties at each end. The double-labelled DNA was then immobilised using its biotin moiety, while the digoxigenin was used for signal amplification. Using this principle, Brasil de Oliveira Marques et al. [26] reported an electrochemical amperometric genosensor using a primer pair modified with a thiol group and digoxigenin, where the thiolated end allowed the immobilisation of the obtained PCR amplicon onto a gold electrode surface while the digoxigenin was used for hybridisation detection following addition of anti-digoxigenin HRP reporter. This approach provided a method for the direct detection of PCR product, but they are not sequence specific and are not practical for the simultaneous detection of multiplex PCR product and usually require longer assay times.

Direct detection of PCR amplicon modified with a ssDNA tag was reported by Hayashi et al. [27, 28] who detailed a PCR primer, which was modified with a mirror-image DNA (L-DNA) tag that prevents extension of DNA polymerase and remain as a single stranded tag. Hybridisation of the (L-DNA) tag with complementary DNA probe was detected using surface plasmon resonance (SPR) imaging.

The use of triplex-forming oligonucleotides (TFOs) as recognition probes has been reported as an alternative approach for the direct detection of PCR product [29, 30]. Triplex nucleic acids structures are formed through sequence-specific Hoogsteen, or reverse

Hoogsteen interactions in the major groove of DNA [31, 32]. This approach provides the direct detection of dsDNA, but usually requires very careful design of the TFO as the DNA triplex has low stability under normal physiological conditions and requires the use of stabilising agents.

The use of electrochemical transducers for DNA detection has gained attraction because of its simplicity, low cost, sensitivity, rapid and compatible with miniaturization [33, 34]. Moreover, the use of electrochemical microarrays provides rapid simultaneous analysis of multiple samples / genes, requiring a low amount of sample and can by fully integrated with sample preparation within a device for point-of-care applications [35, 36].

In this report, we used SSP primers for low resolution genotyping of coeliac disease related alleles. These primers were specifically modified with PCR stopper and ssDNA tails for direct detection of the amplified products on the immobilised amplicon specific probes. Primarily the proof of concept of electrochemical detection and cross hybridisation was performed individually for each allele amplified with a single primer pair. Finally, five amplicons resulted from a mixture of four primer-pairs (multiplex PCR) were analysed electrochemically and 100% correlation with hospital laboratory technique was achieved.

Material and Methods

Materials

All reagents used in this work were of analytical grade: Trizma hydrochloride, potassium hexacyanoferrate(III) [$K_3Fe(CN)_6$], sulphuric acid (H_2SO_4), phosphate-buffered saline pH 7.4 with Tween 20 (PBS-T), Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA were purchased from Sigma Aldrich (Sigma Aldrich, Spain). Hydrochloric acid (HCl, 6M), potassium dihydrogen phosphate (KH_2PO_4) sodium chloride (NaCl) and sodium dihydrogen phosphate (NaH_2PO_4) were obtained from Scharlau. Sodium hydroxide (NaOH) pellets was received from Panreac (Panreac, Spain). Potassium hexacyanoferrate (II) [$K_4Fe(CN)_6$] was purchased from Fluka (Sigma Aldrich, Germany). Dithiol 1, DT1 [10-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9-trioxadecanol] was provided by SensoPath Technologies (Bozeman, NT). Milli-Q water (18 MΩ cm) was obtained using a Simplicity water purification system (Millipore,

France). Double tailed-double stranded DNA (dt-dsDNA) CD associated probes and reporting HRP labelled DNA probes were supplied by biomers.net GmbH (biomers.net GmbH, Germany) Table 5.1.

Electrochemical instrumentation

The electrochemical cell used in this work consisted of a customised electrode array chip of 16 independent gold working electrodes (1 mm^2), arranged in a four by four configuration with a common gold counter electrode and a common silver reference electrode [37]. Electrochemical experiments were carried out using an Autolab model PGSTAT 12 potentiostat/galvanostat controlled with the General Purpose Electrochemical System (GPES) software program (Eco Chemie, The Netherlands), equipped with a MUX module (Eco Chemie B.V., The Netherlands) that allowed sequential addressing of the 16 working electrodes (Scheme 1).

Cleaning of the electrode array was performed by a combination of chemical washes and electrochemical treatment as previously described [37]. Once the chip was cleaned it was thoroughly rinsed in Milli-Q water, blow dried under nitrogen gas and subsequently modified with the DNA probes listed in Table 5.1.

Probe immobilisation

Following cleaning the electrodes were functionalised, by co-self-assembling of the DNA thiolated probes (1 µM) and the co-immobiliser DT1 (100 µM) in 1 M KH_2PO_4 solution for 3 h at room temperature within a humidified chamber. Subsequently, the electrode array was rinsed with Milli-Q water and dried with nitrogen.

Primers design

For the multiplex PCR reaction, four sequence specific primer (SSP) pairs were designed; (1) 3b primer for amplification of DQB1*02:01-05 alleles; (2) 4y primer for amplification of DQB1*02:01,02,04,05, DQB*03:02,07,08,11,18 and DQB1*06:29 alleles; (3) 5z primer for amplification of DQA1*05:01,05,08,09 alleles, and finally (4) primer for the amplification of human growth hormone (HGH) as a target for the positive control. Table 5.1 details the probe for the each CD associated alleles, with the sequence and length of the product. Primer pairs were designed to have two different oligonucleotide sequences separated by a PCR stopper, one part is the active sequence specific primer while the second part is an extra oligonucleotide sequences that will

remain as single stranded DNA (ssDNA) tail after amplification. For the purpose of multiplex electrochemical detection, this ssDNA tail in one primer (usually the forward) is designed to be amplicon specific, and is complementary to the surface immobilised probe. The ssDNA tail of the other primers (usually the reverse) has a common sequence used for hybridisation to the horse radish peroxidise labelled reporter probe (INNO-TRAIN). For further information regarding the primers please contact INNO-TRAIN Diagnostik GmbH (http://www.inno-train.de).

PCR amplification

Singleplex PCR was performed using 50 ng DNA template, 0.08 µL Axi Taq polymerase "AxiTaq" and 3 µL PCR buffer "ready PCR" (Inno-Train Diagnostik GmbH Germany) and volume adjusted to 10 µL by MilliQ water.

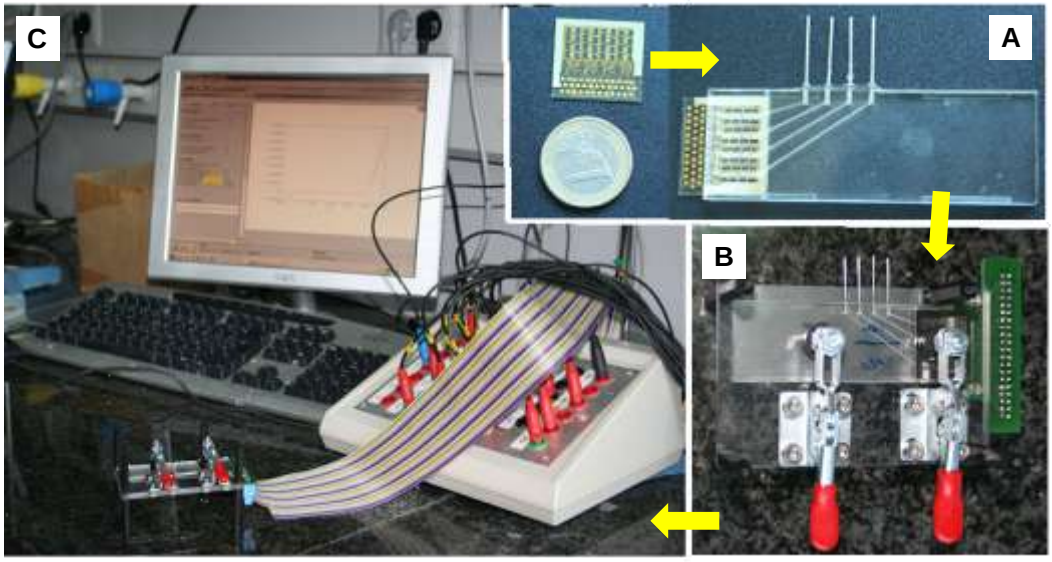
Multiplex PCR amplification was carried using 3b primer pair (1000 nM); 4y forward primer (300 nM), the same 3b reverse primer was used as the 4y reverse primer; 5z primer pair (400 nM) and finally HGH primer pair (300 nM). The primer pairs were mixed with 10 ng DNA template, 0.16 µL KAPA 2G Robust polymerase and 4 µL KAPA robust bufferA (KAPA biosystems), 400 µM dNTPs and 10 µL MilliQ water in total PCR volume 20 µL. Multiplex amplification was carried out according to the following protocol: initial denaturation at 96 ºC for 1 min, followed by 30 cycles of 96 ºC for 15 sec, 61 ºC for 50 sec. Primer specificities, amplicon length, capturing probe and HRP reporter probe are detailed in Table 5.1.

Table 5.1: primer pairs, probes, amplicons length for the CD associated alleles.

Name	Specificity	Amplicon length [bp]	Probes
Primer pair "3b"	**DQB1*02:**01-05	157	5'GTCGTGACTGGGAAAAC–TEG- SH 3'
Primer pair "4y"	**DQB1*02:**01,02, 04,05,***03:02**,07, 08,11,18,*06:29	181	5'TCCTGTGTGAAATTGTTATCCGCT-TEG-SH '3
Primer pair "5z"	**DQA1*05:**01,05 ,08,09	234	5'- GGGCATAAGTCGGACAC –TEG-SH-3
Primer pair "HGH"	**HGH (Internal Control)**	225	5'TAGCGGTGAGTCGATTCTGCCT–TEG-SH '3
HRP-reporter	-	-	5'ACTGGCCGTCGTTTTACA–HRP-3'

Description of fluidic device

First, for prevention of non-specific adsorption, particularly of the HRP-labelled oligonucleotides probe, the microfluidic device channels were protected by a PEG-based monomer supplied by the Institut für Mikrotechnik Mainz GmbH (http://www.imm-mainz.de/). 0.5% v/v PEG-monomer solution was prepared in Milli-Q water and then used to fill the channels of the fluidic device and to cover the area where the electrode array will be mounted. To covalently cross-link the PEG monomer, the device was exposed to UV light for 1 h, finally rinsed with Milli-Q water and blow dried in a stream of compressed air.



Scheme 5.1: Schematic of electrochemical microfluidic set-up used in this study; (A) mounting of electrode array on fluidic device, then (B) assembly on supporter with pogpin connector, and finally (C) interfaced with electrochemical instrument.

A functionalised electrode array was then assembled with the fluidic device using double-sided adhesive foil of 50 µm thickness, resulting in microchannel structures 1mm in width (Scheme 1). Following a rinsing of the housed electrode array, 20 µL of un-purified PCR product (diluted or undiluted) was then added and left to hybridise for a defined time followed by injection of a solution containing 10 nM of HRP labelled reporter probe in hybridisation buffer (10 mM Tris buffer containing 1 M NaCl, pH 7.4) again for defined length time. Between each step, the channels were washed with 1ml of PBS Tween. The influence of temperature on the hybridisation efficiency was investigated by performing the hybridisation at room temperature 22 °C and at 37 °C.

Finally, 20 µL of TMB substrate was added and electrochemical reduction of the HRP mediated product was measured using fast pulse amperometry (0 V for 10 ms followed by - 0.2 V for 500 ms vs Ag built-in reference electrode).

Results and discussions

Hybridisation time / temperature

The hybridisation time study was performed at 22 °C and 37 °C for the detection of amplified double tailed-double stranded PCR target (dt-dsDNA). The PCR product was diluted 1 to 10 in hybridisation buffer and injected into the corresponding channel and left to hybridise for either 2 or 5 min. The channels were then washed with PBS-Tween and finally the reporter HRP probe was passed into the channels and incubated for various times (2, 5 or 10 min). The amperometric signal obtained at 22 °C and 37 °C can be seen in Figure 5.1 and it is clear that a higher level of hybridisation can be achieved at 37 °C. Very interestingly, the time required for hybridisation was just 2 min for each of the target and the reporter probe. This can be attributed to the fact that only short tails are available and there is not a prolonged hopping required before DNA zipping occurs. In previously reports of direct PCR product detection, the typical hybridisation time required ranged between 10 to 60 min [25, 26, 38].

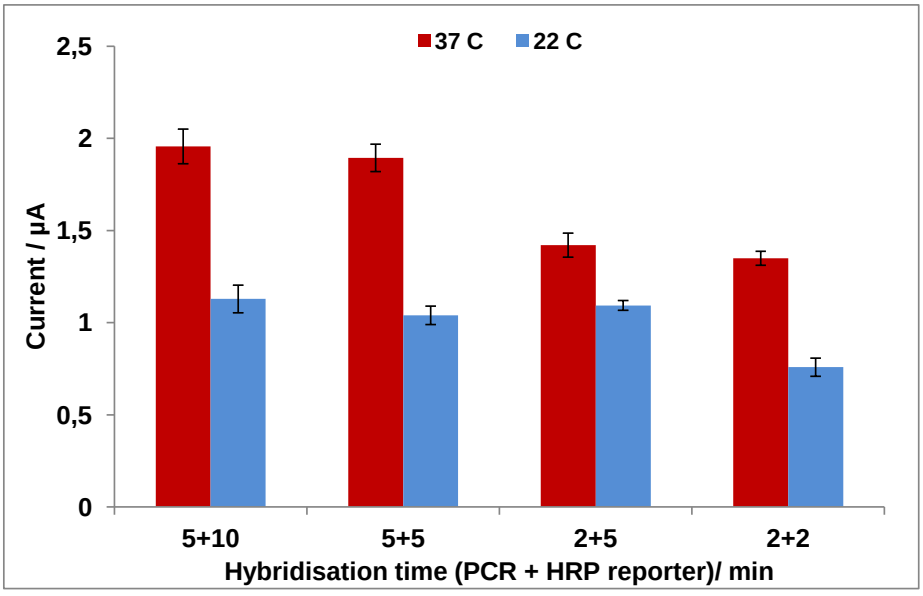
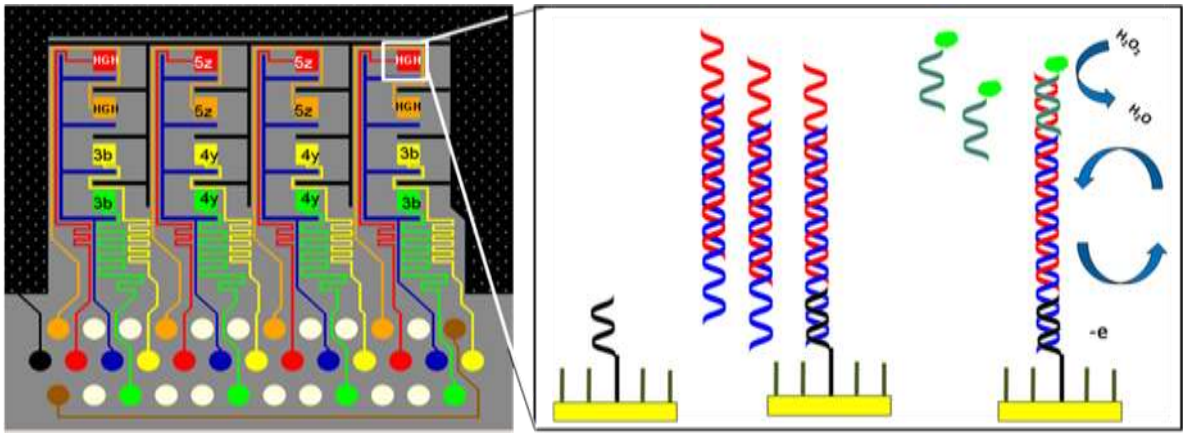


Figure 5.1: Evaluation of the PCR sample hybridisation time and the reporting label capturing time, at 37 °C and 22 °C on the electrochemical assay performance.

Singleplex PCR sample detection

Following optimisation of the hybridisation time, electrochemical detection of double tailed PCR amplicons of CD associated alleles was carried out. Probes specific for the CD associated alleles were immobilised on a 4 X 4 electrode array as depicted in Scheme 5.2. This experiment was carried out to study the feasibility of direct electrochemical detection of the amplicons generated by the proposed sequence specific primers and at the same time to study the selectivity of the particular probe in the presence of different PCR products.



Scheme 5.2. Schematic of the electrode array and electrochemical detection principles of the double stranded PCR product.

All PCR samples were amplified using a single primer-pair modified with double ssDNA tails as described above. Each of the singleplex PCR products were then added to an array functionalised with all four probes (Scheme 5.2), with the objective of observing selective detection only at the electrode functionalised with the corresponding complementary probe. Samples were diluted 1 to 10 in hybridisation buffer and hybridisation was performed for 2 min at 37 °C. As can be seen in Figure 5.2, the genosensor array demonstrated an excellent specificity of each probe, and negligible cross hybridisation was observed, highlighting the successful amplification of the desired allele using the developed primer design and the possibility of simultaneous detection of multiplex PCR products.

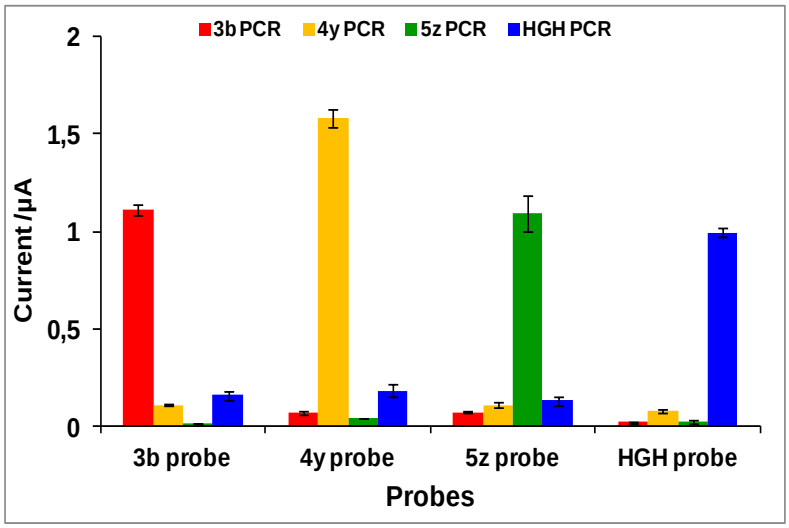


Figure 5.2. Evaluation of probes cross hybridisation for the different targets (PCR product diluted 1 to 10). Hybridisation was performed for 2 min for both target and the reporter probe at 37 ºC.

Multiplex PCR sample detection using multiplex electrode assay

Following the cross hybridisation study and demonstration of the electrochemical detection of the proposed CD associated targets amplified by singleplex PCR, the detection of multiplex PCR product was carried out using samples that had been genotyped in the accredited Finnish Red Cross Blood Service (FRCBS) Tissue typing laboratory (Table 5.2).

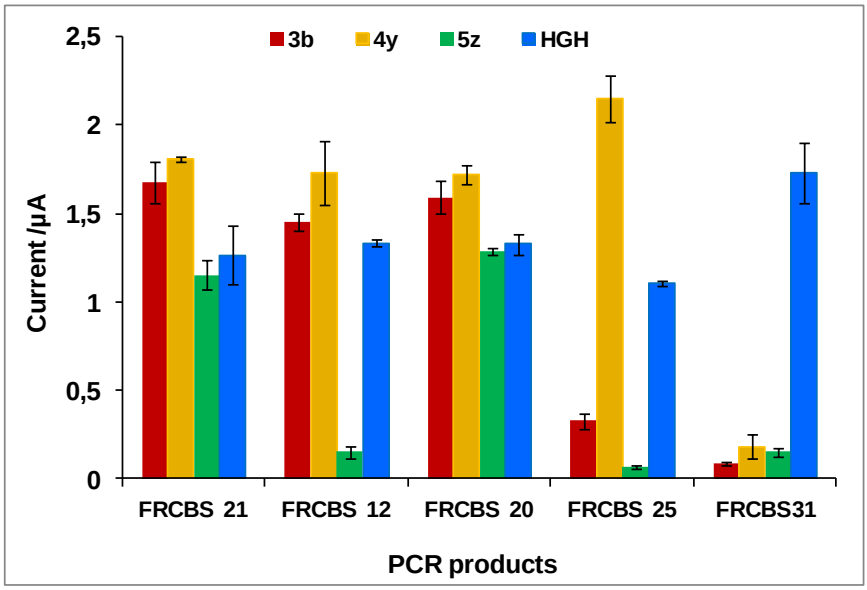


Figure 5.3. Electrochemical detection of multiplex PCR products.. Hybridisation was performed for 2 min for both target and the reporting label at 37 ºC.

Table 5.2. Clinical samples used in this study, showing the exact genotyping and predicted positivity on the developed genosensor array.

Sample	DQA1*		DQB1*		Positivity
FRCBS12	02:01	03:01	02:02	03:02	3b, 4y, HGH
FRCBS21	04:01	05:01	02:01	04:02	All
FRCBS20	05:01	05:01	02:01	02:01	All
FRCBS25	03:01	03:02	03:02	03:03	4y, HGH
FRCBS31	01:01	01:04	05:01	05:03	HGH

For the electrochemical genotyping, un-purified - undiluted PCR was added to the electrode array mounted in the fluidic device and left to hybridise for 2 min at 37 °C. Following washing, 10nM of HRP reporter probe – common to all alleles – was added and left to hybridise for 2 min at 37 °C. As can be seen in Figure 5.3, the results showed an excellent correlation with the hospital laboratory HLA typing technique (Table 5.2), whilst the reported electrochemical approach is far cheaper, faster, reliable, and possible to integrate within a microsystem that integrates DNA extraction, on-chip amplification and detection. Design of the primers and multiplex PCR conditions should be optimised carefully, as the presence of primer-dimers will give a false positive result, but in the work reported here, the results obtained indicate the absence of primer-dimers.

Conclusions

Genetic testing for coeliac disease is conventionally performed using commercial SSP kits for low resolution DQ2/DQ8 typing, by inspecting the presence of correct DNA size using gel electrophoresis. Here, we proposed an electrochemical genosensor array for the direct and rapid detection of DQ2/DQ8 alleles which was achieved by designing a specifically designed primer to produce PCR amplicons with a double ssDNA tail, which facilitated analysis without further treatment of PCR samples. Optimisation of hybridisation time demonstrated that that the whole assay can be performed in less than 5 min at 37 °C. Analysis of real clinical samples showed an excellent correlation with the standard HLA typing methods, showing the applicability of the developed electrochemical genosensor array as a point-of-care diagnostic tool for screening of CD predisposition.

Acknowledgments

Monitoring and Diagnosis using Biosensors and an Integrated Chip System, CD-MEDICs, FP7-2007-ICT-1-216031". V.Beni acknowledges the European Union's Seventh Framework Program (FP7/2007-2013) under grant agreement no. PIGF-GA-2008-220928 for financial support. H. Joda thanks Universitst Rovira i Virgili for the doctoral scholarship. Finnish Red Cross Blood Service is kindly acknowledged for providing purified DNA template.

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CHAPTER 6

Conclusions and Future Work

Coeliac disease is the most common dietary disorder, affecting the small intestine in genetically susceptible individuals on exposure to gluten protein. HLA-DQ2/DQ8 alleles have been demonstrated to be strongly associated with coeliac disease being carried by almost 100% of the patients.

Several commercial kits are currently available for HLA-typing mainly exploiting SSP typing but are expensive, require several reactions and are inherently time-consuming. Recent advances in genosensor technology have revealed an alternative possibility for rapid, low cost simple genetic analysis.

The reported thesis is focused on the development of genosensor array exploiting different amplification technologies for the genetic analysis of DQ2/DQ8 genes for screening coeliac disease predisposition. To achieve this goal, two different approaches were investigated independently and in parallel based on the well known HLA typing techniques, namely sequence specific oligonucleotide probes (SSOP) and sequence specific primers (SSP).

A genosensor array based on the SSOP approach was investigated by designing a set of probes to achieve low-medium resolution genotyping of DQ2/DQ8 genes. Due to the high numbers of alleles in the HLA DQB1 loci, combinations of probes were needed for the detection of DQB1*02:01/02:01 and DQB1*03:02 alleles. In the developed assay, single base mismatch detection was required for the most of the probes. In order to achieve this selectivity, optimisation of assay conditions was performed in terms of assay temperature and timing of the different steps using SPR and ELONA techniques. SPR results indicated that 37 °C is the optimal temperature for the maximum selectivity and sensitivity of the assay. ELONA experiments confirmed the SPR results and a study of the hybridisation timing for the different steps involved in the detection showed that detection was possible using 5 minutes for allele hybridisation time and 20 minutes for the reporter probe at 37 C°, limiting the total assay to less than 30 minutes.
Following assay optimisation, real samples were analysed for the presence of HLA DQ2/DQ8 using colorimetric detection, and an excellent correlation with the standard

HLA typing laboratory techniques was observed confirming the applicability of the proposed assay format for accurate analysis of CD predisposition.

Extending the low-medium resolution colorimetric based DQ2/DQ8 typing, an electrochemical genosensor array was developed using an electrode array of 36 working electrodes, integrated in a fluidic cell, which was used to perform simultaneous multiplex detection of CD associated alleles. Probe immobilisation onto each of the electrodes of the array was performed exploiting thiol-gold chemisorption. Additional probes were designed to achieve medium-high resolution DQ2/DQ8 genotyping. Short hybridisation times for both target allele and the reporter probe was shown to be sufficient to obtain a good hybridisation signal, and an incubation of 10 minutes at 37 °C for each step was chosen as optimum taking both sensitivity and selectivity into consideration. The specificity of the process was evaluated using cross hybridisation studies with synthetic analogues of real PCR amplicons and good selectivity over potentially interfering alleles was observed. Validation of the electrochemical genosensor array was carried out by analysing real human samples, demonstrating an excellent correlation with laboratory based HLA typing technology.

In the second approach, a genosensor array for DQ2/DQ8 genotyping was developed for the direct detection of PCR amplicons (dt-dsDNA), which was achieved by modifying primers with a PCR stopper resulting in single-stranded "tails".

Using the HLA-DQB1*02 allele as a model system, ELONA experiments demonstrated a possible interference from the presence of un-reacted primers in hybridisation efficiency. In order to avoid this, PCR product was subjected to a step for removing the un-reacted primers by incubating PCR product with magnetic beads modified with a specific oligonucleotide sequenec. Optimisation of the magnetic bead concentration showed that 150 µg/ml beads were sufficient to remove the influence of excess un-reacted primer on dt-sDNA hybridisation. Following the proof of concept of the dt-dsDNA detection, several experiments were performed to remove this extra step for removing un-reacted primers to shorten the assay time and avoiding any post-PCR processing. Changing the PCR protocol to a two-temperature protocol showed an improvement in the signal of un-purified sample, and further signal enhancement of un-purified PCR product was observed with using a higher concentration of PCR product during hybridisation. These promising results, demonstrated the possibility of direct

analysis of double ssDNA tailed PCR product without any further treatment. Assay time was further assessed by studying the colorimetric signal with various hybridisation times of un-purified PCR product and showed the possibility of detection within a few minutes.

Three different primer-pairs for the selective amplification of the DQA1*05, DQB1*02 and DQB1*03:02 alleles, in addition to the primer for human growth hormone (HGH) as positive control were designed for low resolution genotyping of DQ2/DQ8. And hybridisation of the un-purified PCR product with immobilised probe and enzyme-labelled reporter probe was achieved in less than 5 minutes at 37 °C.

Electrochemical detection and cross hybridisation were performed individually for each allele amplified with each single primer pair revealing excellent probe selectivity. Finally, five amplicons resulting from a mixture of four primer-pairs in multiplex PCR were electrochemically analysed and a 100% correlation with the hospital laboratory technique was achieved.

Furthermore, analysis of real clinical samples showed an excellent correlation with the standard HLA typing methods, showing the applicability of the developed electrochemical genosensor array as a rapid diagnostic tool for the screening of CD predisposition. With the advances in the miniaturisation and development in lab-on-a-chip, the proposed genosensor array could be integrated in a fully automated DNA analysis system and provide a ‘sample-to-answer’ point-of-care diagnostic tool.

The development of the genosensor arrays reported in this thesis for the detection of both SSOP and SSP products was accomplished and validated using real patient samples.

Comparing both approaches, the genosensor array for the detection of SSOP products required 10 different probes to achieve medium-high resolution genotyping whilst the genosensor array for detection of SSP products 4 different probes were required for low-resolution DQ2/DQ8 genotyping. The specificity of the SSOP assay relies on the probe design, and for the most of probes, single base mismatch discrimination was required, adding considerable complexity and furthermore requires ssDNA generation. The specificity of the SSP assay on the other hand, depends on the primer design and the developed SSP assay provided direct analysis of the PCR product without any further treatment, omitting the step of ssDNA generation with hybridisation occurring in

a very short time, combining to contribute to a tremendous improvement in the overall assay time.

Finally, the thesis reports the development of a generic sensing platform for the multiplexed HLA typing of alleles associated with coeliac disease. The proposed platform allows genotyping of DQ2/DQ8 at a resolution higher or comparable with commercially available kits, but is more cost effective, rapid and easy to use. The developed sensing platform could be applied to all HLA associated diseases such as diabetes mellitus and rheumatoid arthritis.

Future works.

This work can be extended in the future by integration in a microsystem with DNA extraction, PCR and electrochemical sensing modules for fully automated DNA analysis. This platform provides a ‘sample to answer’ and would be very useful as point-of-care diagnostic tool at doctor office avoiding the long time required for centralised laboratory analysis. The genosensor provides a platform for developing a genosensor array for other HLA associated diseases such as diabetes mellitus and rheumatoid arthritis.

ANNEX 1

Anal Bioanal Chem
DOI 10.1007/s00216-011-5493-2

ORIGINAL PAPER

Gold nanoparticle fluorescent molecular beacon for low-resolution DQ2 gene HLA typing

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Received: 5 August 2011 / Revised: 5 October 2011 / Accepted: 10 October 2011

Abstract Coeliac disease is an inflammation of the small intestine triggered by gluten ingestion. We present a fluorescent genosensor, exploiting molecular-beacon-functionalized gold nanoparticles, for the identification of human leukocyte antigen (HLA) DQ2 gene, a key genetic factor in coeliac disease. Optimization of sensor performance was achieved by tuning the composition of the oligonucleotide monolayer immobilized on the gold nanoparticle and the molecular beacon design. Co-immobilization of the molecular beacon with a spacing oligonucleotide (thiolated ten-thymine oligonucleotide) in the presence of ten-adenine oligonucleotides resulted in a significant increase of the sensor response owing to improved spacing of the molecular beacons and extension of the distance from the nanoparticle surface, which renders them more available for recognition. Further increase in the response (approximately 40%) was shown to be achievable when the recognition sequence of the molecular beacon was incorporated in the stem. Improvement of the specificity of the molecular beacons was also achieved by the incorporation within their recognition sequence of a one-base mismatch. Finally, gold nanoparticles functionalized with two molecular beacons targeting the DQA1*05* and DQB1*02* alleles allowed the low-resolution typing of the DQ2 gene at the nanomolar level.

Keywords Gold nanoparticles · HLA typing · Molecular beacons · Fluorescent genosensor · Coeliac disease

Introduction

Coeliac disease (CD), a genetically and environmentally induced inflammation of the small intestine [1], is promoted by the ingestion of gluten, a protein present in wheat, barley and rye [2]. This disease has been associated with nutrient malabsorption, growth problems, gastrointestinal disorders and increased risks of osteoporosis, infertility, autoimmune diseases and lymphomas [2]. Symptomatic diagnosis of CD is quite difficult, and currently the gold standard for its diagnosis is biopsy of the small intestine. Recently, genetic testing (HLA typing) has been proposed as a tool to evaluate the predisposition to CD [3–5], because almost 100% of affected patients carry at least one of the two genotypes, *cis* DQ2 and *trans* DQ2 (approximately 95%) or DQ8 (approximately 5%). These genotypes are coded by the following combination of alleles: DQA1*0501/DQB1*0201 (*cis* DQ2) and DQA1*0505/DQB1*0202 (*trans* DQ2) or DQA1*0301/DQB1*0302 (DQ8) [2, 3, 6].

In recent years, genetic analysis has gained ground in fields such as biodiagnostics, environmental monitoring, food safety and quality control, forensics and biohazard investigations [7–9], resulting in growing interest in DNA biosensors (genosensors). However, the fact that the physical changes occurring during the recognition event

Electronic supplementary material The online version of this article (doi:10.1007/s00216-011-5493-2) contains supplementary material, which is available to authorized users.

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Published online: 16 November 2011

(hybridization) are not easily measurable resulted in the development of highly sensitive transduction approaches based on electrochemical techniques [10, 11] and optical detection [12].

Since their introduction by Tyagi and Kramer [13], fluorescent molecular beacons (MBs), have been intensively explored [13–15]. They are specifically engineered bifunctional (quencher and fluorophore) short oligonucleotide chain probes characterized by a stem–loop structure; this allows them to change their spatial conformation upon recognition of the DNA target and, as a result of this, to fluoresce [13]. In MBs, quenching was originally provided by molecular quenchers [14], but since 2001, gold surfaces [16–18] and even more widely gold nanoparticles (AuNPs) [19–25] have found application as highly efficient and universal quenchers. Moreover, the use of gold-based quenching has been shown to be characterized by minimal background fluorescence and subsequently improved signal-to-background ratio [9, 23, 26].

The high quenching efficiency of AuNPs is due to the associated surface plasmon resonance and its ability to enhance all the radiative and non-radiative properties of the nanoparticles [27]. In the case of nanoparticles, quenching has been demonstrated to be governed by the surface energy transfer [28] and to be strongly dependent on donor–acceptor distances [29].

Recently, various authors reported the use of AuNP–DNA MBs in the development of bioassays for genetic disease detection [19]. Maxwell et al. [20] and Ray et al. [22, 23] described sensing platforms based on the use of a fluorophore-modified DNA sequence immobilized on AuNPs, taking advantage of the tendency of the single-stranded DNA to organize itself into an "arch" structure when immobilized. In another approach, Mo et al. [21] reported an AuNP fluorescence sensing platform based on the displacement of a not fully complementary fluorophore modified DNA sequence by the fully complementary target. Zhang et al. [24] developed an interesting sensing platform based on a reverse MB design, where a FAM-modified linear probe immobilized onto AuNPs assumed a blocked loop structure upon hybridization with the target, resulting in quenching of the FAM fluorescence. Finally, Wang et al. [16] reported the development of a platform for the detection of single nucleotide polymorphisms based on the combination of AuNPs and ligase reaction. In this work, the discrimination was based on the different abilities of single-stranded DNA and double-stranded DNA to absorb onto AuNPs and on the resulting changes in fluorescence depending on whether the fluorescence-labelled primers were in their single strand form (no ligation occurred) or their double strand form (ligation occurred).

The first report on the use of AuNPs as quenchers in MBs and their application for the detection of a specific DNA sequence was by Dubertret et al. [19], who demonstrated the possibility of replacing the quencher in an MB with AuNPs. Following this report, other authors investigated the use of AuNP–MB conjugates for the detection of genetic disease [12], demonstrating the ability to discriminate single-point mismatches [26, 30]. Significant improvement in the state of the art was achieved by Song et al. [9], who, by taking full advantage of the quenching efficiency and scaffolding ability of AuNPs, demonstrated the possibility of having three perfectly and independently working MBs immobilized on a single AuNP, highlighting the possibility of single-particle multiplex detection of DNA markers.

In the work we report herein, the development and optimization of an AuNP-based fluorescence bioassay for the low-resolution typing of the DQ2 CD-associated genotype is presented. Key points investigated in this work were the design of the MBs, the functionalization of AuNPs and the composition of the oligonucleotide monolayer immobilized onto the AuNPs. The specificity of the MBs developed was evaluated and applied to the discrimination of single-base mismatches. Furthermore, the AuNP–MB conjugates were characterized and the MB developed was used for the low-resolution HLA typing of the DQ2 genotype.

Experimental

Materials

All chemicals were used as purchased without further purification. Potassium dihydrogen phosphate and potassium monohydrogen phosphate were purchased from Fluka (Barcelona, Spain), sodium chloride, 2 N sodium hydroxide, 6 N hydrochloric acid and concentrated nitric acid were purchased from Scharlau Chemie (Barcelona, Spain), sodium citrate, chloroauric acid, Zonyl FSN, dithiothreitol (DTT) and tris(carboxyethyl)phosphine (TCEP) and saline–sodium citrate buffer (20×) were purchased from Sigma (Barcelona, Spain) and concentrated hydrochloric acid was obtained from Merck (Darmstadt, Germany). The oligonucleotides were synthesized by biomers.net (Ulm, Germany) and were reconstituted to a 100 μM stock solution using ultrapure water and stored at −20 °C. All solutions were prepared in high-purity water obtained from a Milli-Q RG system (Millipore, Barcelona, Spain). Hybridization experiments were performed in 1× saline–sodium citrate buffer at pH 7.4. The sequences used in this work are listed in Table 1.

Design of MBs

We designed MBs for low-resolution DQ2 HLA typing. To perform HLA typing of the DQ2 genotype, the detection of

Table 1 List of the oligonucleotides used

Oligonucleotides	Sequences (5′–3′)
MBs	
DQA1*0201a	ATTO 647 N-*GCGAG* AATCTAAGTCTGTGG*CTCGC*-(10 T)-$(CH2)_3$-SH
DQA1*0201b	ATTO 647 N-*CCACA* AATCTAAGTC*TGTGG*-(10 T)-$(CH2)_3$-SH
DQB1*02 Pa	ATTO 647 N-*CTGCCT* ACTCGGCGGC*AGGCAG*-(10 T)-$(CH2)_3$-SH
DQB1*02Pb	ATTO 647 N-*CTGCCT* ACTCGG**A**GGC*AGGCAG*-(10 T)-$(CH2)_3$-SH
DQA1*05 Pa	Rh6G-*TCTGCT* AACTCTCCTC*AGCAGA*-(10 T)-$(CH2)_3$-SH
DQA1*05Pb	Rh6G-*TCTGCT* AACTCTC**A**TC*AGCAGA*-(10 T)-$(CH2)_3$-SH
"Spacing" and "spacing complementary oligonucleotide" sequences	
Poly-T oligonucleotide	TTTTTTTTTT-$(CH2)_3$-SH
Poly-A oligonucleotide	AAAAAAAAAA
Targets	
DQA1*0201	CCACAGACTTAGATT
DQB1*02	CTGCCTGCCGCCGAGTA
DQB1*0203	CTGCCTGACGCCGAGTA
DQA1*05	TCTGCTGAGGAGAGTTA
DQA1*0503	TCTTCTGAGGAGAGTTA

The recognition sequences are *underlined* and the bases in *italics* represent the complementary strands of the stems. *Bold* indicates replacement of a cytosine with an adenine

MBs molecular beacons, *Rh6G* rhodamine 6 G

the DQA1*0501, DQA1*0505, DQB1*0201 and DQB1*0202 alleles is required; a schematic representation of the combination of alleles associated with the DQ2 gene, for both its possible forms (*cis* and *trans*), is reported in Fig. 1.

In HLA typing, "low resolution" is defined as the detection of a family of alleles. We designed two MBs for low-resolution HLA typing of DQA1*05* and DQB1*02* allele families. The two probes, DQA1*05* and DQB1*02* MB probes, were designed to detect, respectively, DQA1*050101/DQA1*0505 and DQB1*0201/DQB1*0202 CD-associated alleles but not to discriminate between them. The two MBs were also able to detect some other alleles: DQA1*0508/0509/0510 in the case of the DQA1*05* probe and DQB1*0204/0205/0206 in the case of the DQB1*02* probe. These alleles, not associated with CD disease, are rare, having a prevalence below 1% [31] and because of this are not expected to generate significant false positives.

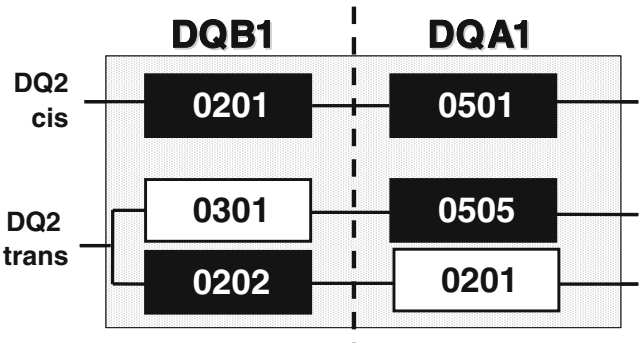

Fig. 1 The different possible allele combinations associated with the DQ2 genotypes. The alleles that have been proven to be associated with coeliac disease are highlighted in *black* and the alleles most commonly associated with them are highlighted in *white*

All MBs consisted of a 17-nucleotide-long recognition sequence and a five-nucleotide stem, with ten thymines between the functional elements of the MB and a terminating thiol group. The MBs were modified at their 5′ end with the fluorophore and at their 3′ end with a thiol. Rhodamine 6 G (adsorption 525 nm, emission 555 nm) and ATTO 647 N (adsorption 644 nm, emission 669 nm) were used as the fluorophores for the DQA1*05* and DQB1*02* MB probes, respectively (Fig. S1). Two different designs of the MB differing in the location of the recognition sequence were investigated. In the first design, the recognition sequence coincided with the loop of the MB; to achieve this, two five-base-long sequences (stem), each reverse-complementary to the other, were added at each end of the recognition sequence [9, 12–14]. In the second design, the recognition sequence was partially incorporated in the stem [32]; in this design a five-nucleotide-long appendage, complementary to the last five bases of the 3′ end of the recognition sequence, was added between the 5′ end of the loop and the fluorophore. This second design was anticipated to introduce increased stringency and consequently improved specificity [32, 33]; moreover, it was also envisaged that this design, upon hybridization, would result in increased rigidity of the duplex formed, efficiently extending the fluorophore far from the AuNPs, providing a higher fluorescence signal.

To improve the selectivity of the MB probes, the use of an amplification refractory mutation system (ARMS) [25] type approach for the design of the recognition sequences was also investigated. This approach introduces an artificial

mismatch in the recognition part of the MB with the aim of improving the selectivity for fully complementary sequence over single-nucleotide mismatch as the duplex of the MB with the mismatched sequence would be less stable than the fully complementary sequence.

Fluorescence measurements

All fluorescence measurements were performed using black quartz cuvettes with 1-cm path length and with a total maximum volume of 150 μL. The excitation and emission wavelengths used were 525 or 555 nm and 644 or 669 nm for rhodamine 6 G and ATTO 647 N, respectively. Slits of 5 nm were used for both excitation and emission. All the experiments were performed using a photomultipler tube voltage of 600 V. All measurements were performed at controlled temperature (25 °C).

The MB response was reported as the relative increase of the fluorescence intensity (R_f), which was calculated as

$$R_f = \left(I_{target} - I_{blank}\right)/\left(I_{probe} - I_{blank}\right),$$

where I_{probe} is the fluorescence intensity before hybridization, I_{target} is the fluorescence intensity after hybridization with target sequences and I_{blank} is the fluorescence intensity of the background (bare AuNPs in detection buffer).

The hybridization efficiency (H_e) was calculated as

$$H_e = \left(I_{target} - I_{probe}\right)/\left(I_{DTT} - I_{probe}\right) \times 100,$$

where I_{DTT} is fluorescence intensity after Au–S bond cleavage by 0.1 M DTT and removal of quencher AuNPs from the solution.

The discrimination ability of MBs was expressed as the discrimination factor (Df):

$$Df = \left(I_{positive} - I_{blank}\right)/\left(I_{negative} - I_{blank}\right),$$

where $I_{positive}$ is the fluorescence intensity upon addition of the complementary target and $I_{negative}$ is the fluorescence intensity upon addition of the non-complementary target.

Synthesis and functionalization of AuNPs

AuNPs with average diameters of 15 nm were prepared via hot reduction of $NaAuCl_4$ solution with sodium citrate [34, 35]. Functionalization of the AuNPs with the MBs was performed in the presence of Zonyl FSN as it has been shown to provide much greater stabilization of AuNPs even at high concentrations of Na^+ [35, 36]. Briefly, an aliquot of freshly cleaved oligonucleotides (using TCEP) was added to a suspension of the AuNPs (2.5 nM) in 10 mM phosphate buffer containing Zonyl FSN (0.05%, v/v) at pH 7.4 to obtain a final DNA-to-AuNP ratio of 500:1 [12] and the mixture was left to react at room temperature for 20 min under shaking conditions (1,400 g). The MB/AuNP mixture was then subjected to a so-called ageing process consisting of a stepwise increase of the concentration of NaCl (steps of 0.1 M) up to 0.7 M, prior to an overnight incubation at 4 °C. The MB-functionalized AuNPs were then concentrated by centrifugation (14,000 rpm, 20 min, 10 °C), washed, by a sequence of centrifugation/resuspension steps, and finally resuspended in the measurement buffer.

The immobilization of the MBs was visualized using transmission electron microscopy (TEM) imaging, and was quantitatively evaluated by monitoring the fluorescence of the AuNP–MB conjugate following cleavage of the S–Au bond by DTT. Finally, melting curve analysis was used to confirm the stability of the AuNP–MB conjugate and to evaluate the ability of the MBs to open.

Results and discussion

The envisaged mechanism of the proposed sensing platform is presented in Fig. 2. As typical with MBs, in the absence of target DNA no significant fluorescence is recorded as the MB is in the closed configuration, resulting in fluorescence quenching by the AuNPs. Upon hybridization of the immobilized MBs with the target sequences, an increase in fluorescence (fluorophores moved away from the AuNPs) is observed.

Selection of fluorophores

Owing to the strong quenching efficiency of AuNPs, a wide range of fluorophores can be used [14]; the fluorophores chosen in this work were ATTO 647 N and rhodamine 6 G, as they are pH-insensitive, relatively photostable and their

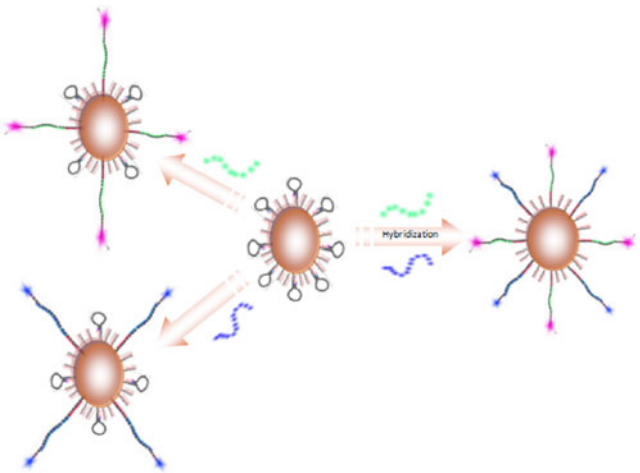

Fig. 2 The working principle of the detection platform based on gold nanoparticle–(AuNP)–molecular beacon (MB) conjugates

absorption/emission spectra are well resolved (emission maxima at 669 and 555 nm, respectively). To calculate the nanoparticle loading of the MBs, calibration curves between 1 and 1,000 nM were generated for both fluorophore-modified oligonucleotides, and a good linear response was obtained: $y=0.94x+9.67$ ($R^2=0.993$) for ATTO 647 N and $y=0.62x+4.51$ ($R^2=0.986$) for rhodamine 6 G.

Characterization of AuNPs and AuNP–MB conjugates

The size of the synthesized nanoparticles was confirmed by TEM measurements, and was found to be 15±2.6 nm ($n=100$). UV–vis measurements at 520 nm were used to calculate the concentration of the synthesized nanoparticles (approximately 4 nM); the extinction coefficient used in this measurement (2.08×10^8 L mol^{-1}cm^{-1}) was calculated assuming a spherical geometry of the nanoparticles and density equivalent to that of bulk gold (19.30 gcm^{-3}) according to the approach proposed by Liu et al. [37]. UV–vis measurements were also used to check for the presence of aggregated particles (presence of a broad peak at approximately 700 nm) as well as to confirm the presence of the DNA immobilized on the AuNPs, by measurement at 260 nm [12]. Visualization of DNA immobilized on the AuNPs was observed using TEM imaging (Fig. 3); a clear difference in the capping layer thickness, from approximately 2 nm to 6–7 nm, prior to and following functionalization with the MB was observed. This difference in capping layer thickness upon functionalization with DNA was reported previously by Stakenborg et al. [38]; in this

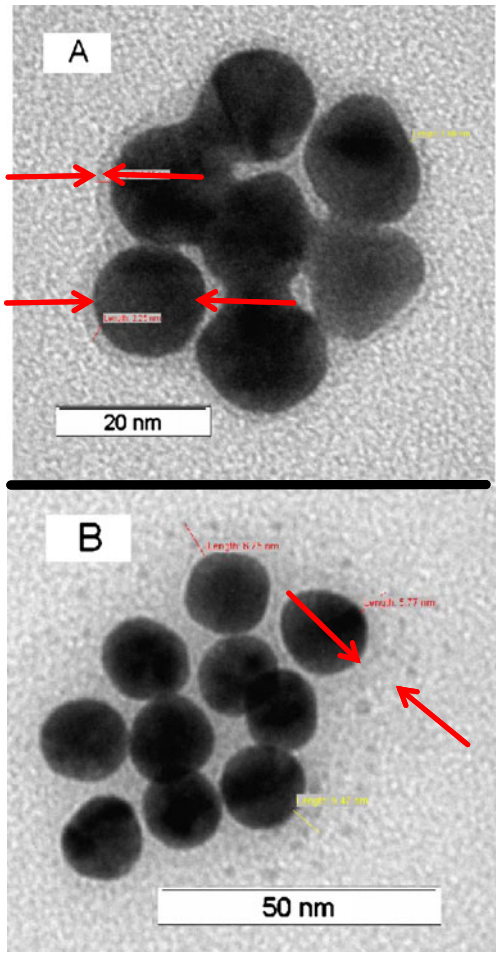

Fig. 3 Transmission electron microscopy images of the AuNPs prior to (**A**) and following (**B**) functionalization with MBs. The *red arrows* highlight the capping layer thickness

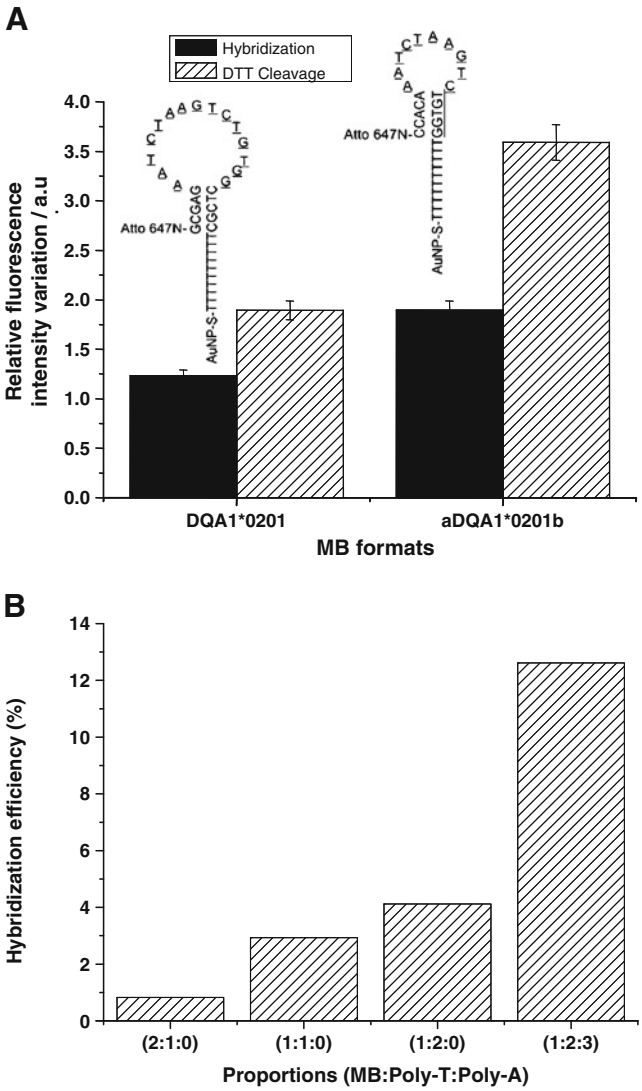

Fig. 4 **A** Evaluation of the stem position/design on MB loading (*DTT cleavage bar*) and performances (*hybridization bar*) of AuNP–MB conjugates. **B** Hybridization efficiency of AuNP–MB conjugates as a function of the composition of the oligonucleotide monolayer assembled on the AuNPs. Hybridization experiments were performed using 100 nM fully complementary target in 1× saline–sodium citrate (SSC) buffer (pH 7.4). *DTT* dithiothreitol

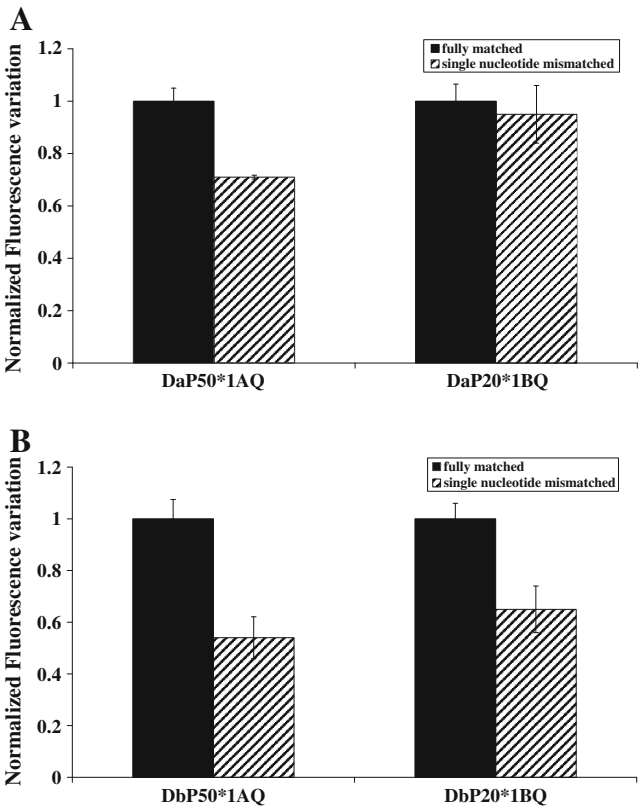

Fig. 5 Evaluation of the specificity, between sequences differing in a single base, of the AuNP–MB conjugates obtained using conventional (**A**) and amplification refractory mutation system type (**B**) MB designs. Hybridization experiments were performed using 100 nM fully complementary target in 1× SSC buffer (pH 7.4)

work this was due to the displacement of the Zonyl FSN by the MBs. A quantification of the MBs immobilized on the AuNPs was performed via DTT cleavage experiments and it was found that there were 41±7 MBs (n=3) for each AuNP.

Finally, thermal studies were performed to further confirm the presence of MB on the AuNPs and to demonstrate the level of fluorescence expected upon opening of the MBs (Fig. S2). No significant variation in fluorescence was recorded with increasing temperature until the melting temperature was reached, when a rapid increase in fluorescence, due to the opening of the MBs, was observed.

Design of MBs

The performances of the AuNP–MB conjugates were expected to be depend not only on the design of the recognition element incorporated in the MB but also on the design of the MB itself [12]. Two different designs of MBs, differing in the position of the stem, were evaluated. For the optimization of the design, an MB able to detect the DQA1*0201 allele was designed; this choice was made because the sequence of a probe specific for this allele is well known. The two MBs (DQA1*0201a and DQA1*0201b in Table 1) were immobilized, in a single-plex format, onto AuNPs and the relative increase of the fluorescence upon hybridization with 250 nM fully complementary target (DQA1*0201 in Table 1) was monitored. DTT cleavage experiments (Fig. 4a) indicated that the use of such a design can increase the loading onto the AuNPs, presumably owing to the reduced loop size, with approximately 40% higher signal being observed. The target hybridization results are consistent with this increase in MB loading, with a 40% higher signal upon target binding also being observed, although improved efficiency of MB opening can also contribute to the increase in the response.

On-particle DNA monolayer composition

The composition of the DNA monolayer immobilized onto the AuNPs has been observed to strongly influence the performance of AuNP–MB conjugates [9]. In the work reported here, the use of a poly-T spacer oligonucleotide and a complementary poly-A oligonucleotide was investigated to modulate the composition of the oligonucleotide layer, thus improving sensor performance. The use of spacer oligonucleotides with length comparable to that of the MB–thiol poly-T appendage has been demonstrated to improve the efficiency of AuNP-immobilized MBs [9]. In this work, spacer oligonucleotides (ten-base-long thiol-functionalized poly-T oligonucleotide) were used to space out the MBs. To further improve the spacing, a ten-base poly-A oligonucleotide was used for hybridization to the poly-T oligonucleotide, thus improving the steric accessibility of the target DNA to the MB.

Table 2 Comparison of the ΔG values, calculated using PerlPrimers [37], and of the discrimination factors recorded using the conventional and amplification refractory mutation system (*ARMS*)-inspired MB designs

Target	Original MB designs		ARMS-inspired MB designs	
	ΔG (kcal mol^{-1})	Discrimination factor	ΔG (kcal mol^{-1})	Discrimination factor
DQA1*05	−23.7	1.5	−19.9	2.3
DQA1*0503	−17.7		−13.9	
DQB1*02	−29.3	1.1	−24.4	1.8
DQB1*0203	−25.2		−17	

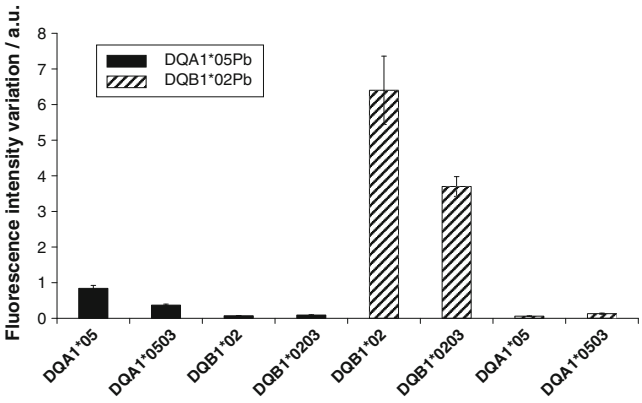

Fig. 6 Simultaneous detection of DQA1*05 and DQB1*02 target alleles and their discrimination versus single-nucleotide-mismatched alleles (DQA1*0503 and DQB1*0203). Hybridization experiments were performed using 100 nM fully complementary target in 1× SSC buffer (pH 7.4)

Furthermore, this poly-A oligonucleotide also served for hybridization to the base of the MB, effectively increasing the rigidity of this base, and forcing the MB to be extended away from the nanoparticle surface for enhanced access for target hybridization.

As expected, increasing the poly-T to MB ratio in the immobilization solution resulted in an increase in the hybridization efficiency (Fig. 4b), confirming that steric hindrance is a limiting factor for MBs immobilized on AuNPs [9]. When the poly-A sequence was added to the immobilization solution, at equivalent molar ratios of poly-T and MBs, a significant improvement in the hybridization efficiency was recorded (Fig. 4b). This was due to not only a reduction in steric hindrance, but also the fact that the MBs are forced to extend as far as possible from the nanoparticle surface, resulting in improved hybridization and a higher fluorescence signal.

DQA1*05* and DQB1*02* MB designs

Once the surface chemistry and MB design had been optimized using the DQA1*0201 allele, MB probes were designed for the detection of the DQA1*05* and DQB1*02* alleles, using the IMGT/HLA database [39] and the handbook of the International Histocompatibility Working Group [40]. Free online tools (PerlPrimer [37], DNAmelt [41] and UNAfold [42]) were used to evaluate and refine the MB designs. The specificity of the designed MBs was evaluated by comparing the fluorescence response upon hybridization with 100 nM fully complementary target with the responses recorded in the case of the one-base-mismatched sequences (DQA1*0503 and DQB1*0203). Fluorescence measurements were recorded 20 min after target addition, and as can be seen in Fig. 5a, the proposed MBs did not show very good specificity, particularly in the case of DQB1*02*. To overcome this limited specificity, a second set of MB probes (DQA105Pb and DQB102Pb in Table 1) were designed. Exploiting an ARMS-type approach [25], we introduced a mismatch into the recognition sequence of the MB, which was anticipated to have the effect of markedly reducing the stability of the duplex between the MB and the sequence with one mismatch, as effectively this duplex would now have two mismatches. Optimization of the location where the mismatch should be introduced was performed with the help of PerlPrimers [37], where different bases were substituted at various positions of the original recognition sequence and the free energy (ΔG) of the different duplexes was calculated.

For both MBs, the highest destabilization was found to occur by the replacement of a cytosine with an adenine, as highlighted in bold in the MB sequences reported in Table 1.

In Table 2 the values of the free energy calculated for the original design and for the best ARMS design are reported.

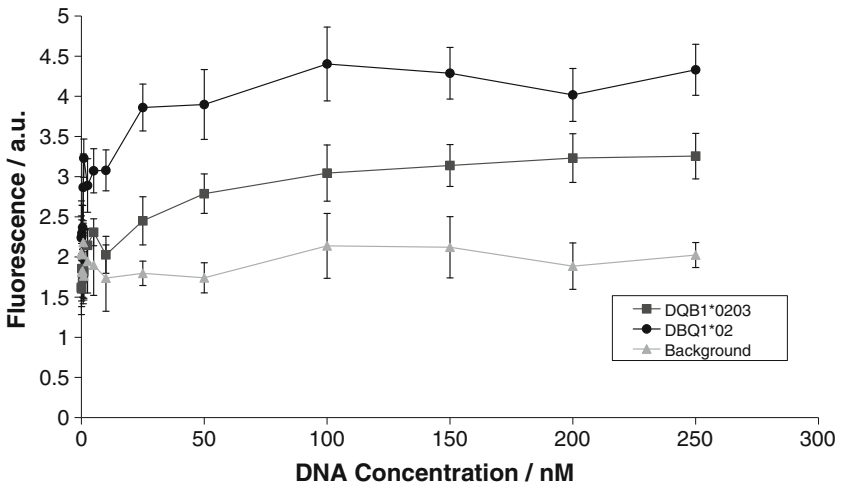

Fig. 7 Calibration curve for DQBQ02 probe specific (DQB1*02) and unspecific (DQB1*0203) targets using the multiple-parameter integrated sensing platform prepared from a starting concentration of the AuNPs of 2.5 nM and with a molar ratio of (0.5+0.5):2:3 of the MBs, poly-T spacer oligonucleotide and the complementary poly-A oligonucleotide. Measurements were performed in triplicate

As expected, the introduction of a mismatch in the recognition sequence resulted in a reduction of ΔG (stability) of the duplex, and this reduction in stability was markedly more notable, particularly for the DQB1*02* MB.

Once the new set of MB designs had been defined, the specificity of each of the AuNP–MB conjugates was again examined, and as can be seen in Fig. 5b, there is an appreciable improvement in the specificity of the two MBs.

Detection

In the experiments for the simultaneous detection of the two alleles, the AuNPs were functionalized with a solution containing a molar ratio of 0.5:0.5:2:3 DQA105Pb, DQB102Pb, poly-T spacer oligonucleotide and the complementary poly-A oligonucleotide. The simultaneous presence of the two MBs on the AuNPs was verified by DTT cleavage experiments and monitoring the fluorescence of the rhodamine 6 G and ATTO 647 N labels. These measurements indicated an average loading of 22±2 and 21±2 MBs (n=3) for AuNPs for the DQA105Pb and DQB102b MBs, respectively, loading values that are consistent with those in previous reports [9]. Furthermore, the total loading was consistent with the total loadings recorded in the case of the singleplex AuNP–MB conjugates. The AuNP–MB conjugates we had prepared were then used for the simultaneous detection of the two target alleles. This was performed following the same protocol used for the singleplex experiments, with the only difference being that in this case fluorescence measurements were performed sequentially using the wavelengths specific for each of the fluorophores (Fig. 6). Clearly the proposed detection platform can selectively detect the desired targets. Moreover no cross talk between the two fluorophores was recorded, highlighted by the fact that the opening of one of the MBs did not induce any significant increase in the fluorescence due to the fluorophore associated with the other MB. In addition, no significant variation in the intensity of the fluorescence of each MB was recorded when only one or both MBs were opened (hybridized with the fully complementary target).

We looked more closely at the analytical performances of the multiple-target nanoparticle-based assay, prepared according to the optimized oligonucleotide layer composition and MB designs. In these experiments, DQB1*02 targets were used. The sensing platform prepared using 2.5 nM starting concentration of the AuNPs showed a linear dynamic range between 0 and 10 nM (Fig. 7), with a limit of detection of approximately 0.5 nM that is consistent with the limits of detection previously reported for similar systems [12]. The linear dynamic range was narrower when compared with ranges recorded for AuNPs modified with a single MB [12]; this was expected owing to the reduced number of MBs immobilized onto the AuNP surface.

Conclusion

In the work reported, the development of a multiple-parameter fluorescence detection platform, based on MB-functionalized AuNPs, for the low-resolution HLA typing of the CD-associated DQ2 genotype was presented. The use of an MB having the recognition element partially incorporated into the stem resulted in a higher loading of the MB onto the AuNP, presumably due to the reduced size of the loop, leading to a 40% increase in fluorescence signal. Thiolated MBs with a ten-thymine appendage at the 3′ end were co-immobilized with thiolated ten-thymine spacer oligonucleotides. An increase in fluorescence was observed owing to the incorporation of the spacer oligonucleotides and further increase was observed upon addition of a ten-adenine oligonucleotide, which not only further spaced out the immobilized MBs, enhancing steric accessibility, but also gave the MB base rigidity, thus increasing the distance of the MB from the nanoparticle surface, rendering it more available for interaction with target DNA. Probes specific for the detection of the DQA1*05* and DQB1*02* alleles exploiting an ARMS-type approach consisting of introducing a mismatch facilitated the discrimination of a single-point mismatch. The AuNPs bifunctionalized with the two MBs, each labelled with a different fluorophore label, were demonstrated to selectively and simultaneously detect the DQB1*02 and DQA1*05 alleles, achieving in this way the low-resolution HLA typing of the CD-associated DQ2 genotype. The proposed platform was shown to have a limit of detection of approximately 0.5 nM, with a linear dynamic range between 0 and 10 nM.

Acknowledgements This work was conducted with partial financial support from the Commission of the European Communities, RDT programme Coeliac Disease Management Monitoring and Diagnosis Using Biosensors and an Integrated Chip System, CD-MEDICs, FP7-2007-ICT-1-216031. V.B. acknowledges the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement no. PERG-GA-2009-256542 for financial support.

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