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Homogeneous Assays for Simplified Screening of HLA-conferred Genetic Disease Risk

by

Minna Kiviniemi

TURUN YLIOPISTO
UNIVERSITY OF TURKU
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From

Department of Biochemistry and Food Chemistry / Biotechnology
University of Turku
Turku, Finland

Supervised by

Professor Timo Lövgren, Ph.D.
Department of Biochemistry and Food Chemistry / Biotechnology
University of Turku
Turku, Finland

Reviewed by

Docent Marja-Liisa Lokki
Transplantation Laboratory, Haartman Institute
University of Helsinki
Helsinki, Finland

and

Alice Ylikoski, Ph.D.
PerkinElmer, Life and Analytical Sciences, Wallac Oy
Turku, Finland

Opponent

Professor Ann-Christine Syvänen
Department of Medical Sciences
Uppsala University
Uppsala, Sweden

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To my family

Contents

List of original publications

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I Minna Kiviniemi, Jussi Nurmi, Hannu Turpeinen, Timo Lövgren & Jorma Ilonen (2003) A homogeneous high-throughput genotyping method based on competitive hybridization. *Clin Biochem* **36**: 633-640

II Minna Kiviniemi, Jussi Nurmi, Timo Lövgren & Jorma Ilonen (2005) Locked nucleic acid (LNA) probes in high-throughput genetic analysis: Application to an assay for type 1 diabetes-related HLA-DQB1 alleles. *Clin Biochem* **38**: 1015-1022

III Minna Kiviniemi, Robert Hermann, Jussi Nurmi, Anette G. Ziegler, Mikael Knip, Olli Simell, Riitta Veijola, Timo Lövgren, Jorma Ilonen & the TEDDY Study Group (2007) A high-throughput population screening system for the estimation of genetic risk for type 1 diabetes: An application for the TEDDY (The Environmental Determinants of Diabetes in the Young) study. *Diabetes Technol Ther* **9**: 406-472

IV Minna Kiviniemi, Jorma Ilonen & Timo Lövgren (2009) A homogeneous HLA-B*27 genotyping assay using dried reagent mixtures. *Dis Markers* **27**(2): 85-91

In addition some unpublished data are presented.

Abbreviations

AS	ankylosing spondylitis
ASPE	allele-specific primer extension
DAISY	Diabetes Autoimmunity Study in the Young
DIPP	Diabetes Prediction and Prevention
Eu	europium
IDDM	insulin-dependent diabetes mellitus
HLA	human leukocyte antigen
LNA	locked nucleic acid
MHC	major histocompatibility complex
OLA	oligonucleotide ligation assay
PANDA	Prospective Assessment in Newborns for Diabetes Autoimmunity
PCR	polymerase chain reaction
PVR	poliovirus receptor
SBCE	single base chain extension
SNP	single nucleotide polymorphism
SSO	sequence-specific oligonucleotide
SSP	sequence-specific priming
T1D	type 1 diabetes
Tb	terbium
TEDDY	The Environmental Determinants of Diabetes in the Young
T_m	melting temperature
TRIGR	Trial to Reduce IDDM in the Genetically at Risk

Abstract

The increasing incidence of type 1 diabetes has led researchers on a quest to find the reason behind this phenomenon. The rate of increase is too great to be caused simply by changes in the genetic component, and many environmental factors are under investigation for their possible contribution. These studies require, however, the participation of those individuals most likely to develop the disease, and the approach chosen by many is to screen vast populations to find persons with increased genetic risk factors. The participating individuals are then followed for signs of disease development, and their exposure to suspected environmental factors is studied.

The main purpose of this study was to find a suitable tool for easy and inexpensive screening of certain genetic risk markers for type 1 diabetes. The method should be applicable to using whole blood dried on sample collection cards as sample material, since the shipping and storage of samples in this format is preferred. However, the screening of vast sample libraries of extracted genomic DNA should also be possible, if such a need should arise, for example, when studying the effect of newly discovered genetic risk markers.

The method developed in this study is based on homogeneous assay chemistry and an asymmetrical polymerase chain reaction (PCR). The generated single-stranded PCR product is probed by lanthanide-labelled, LNA (locked nucleic acid)-spiked, short oligonucleotides with exact complementary sequences. In the case of a perfect match, the probe is hybridised to the product. However, if even a single nucleotide difference occurs, the probe is bound instead of the PCR product to a complementary quencher-oligonucleotide labelled with a dabcyl-moiety, causing the signal of the lanthanide label to be quenched.

The method was applied to the screening of the well-known type 1 diabetes risk alleles of the HLA-DQB1 gene. The method was shown to be suitable as an initial screening step including thousands of samples in the scheme used in the TEDDY (The Environmental Determinants of Diabetes in the Young) study to identify those individuals at increased genetic risk.

The method was further developed into dry-reagent form to allow an even simpler approach to screening. The reagents needed in the assay were in dry format in the reaction vessel, and performing the assay required only the

addition of the sample and, if necessary, water to rehydrate the reagents. This allows the assay to be successfully executed even by a person with minimal laboratory experience.

1 Introduction

Type 1 diabetes (T1D) is one example of a multitude of multifactorial diseases with a genetic and an environmental component. In T1D, like many other autoimmune diseases in this group, genes within the HLA (human leukocyte antigen) region are most important in the genetic component. T1D has a special position as a childhood disease with a large number of cases in small children and a very strong, although complex, HLA association. Approximately fifty percent of the genetic component is contributed by the HLA genes on chromosome 6, the DR and DQ being the most important factors (Mehers and Gillespie 2008; Ounissi-Benkalha and Polychronakos 2008). The genetic component alone is not sufficient for the development of the disease, since many of those with a strong genetic risk are not affected, but the contribution of environmental factors triggering the disease is crucial. The disease itself results from the destruction of insulin producing pancreatic beta cells by an autoimmune mechanism, and after the loss of insulin production, lifelong treatment with insulin is needed.

The incidence of T1D is increasing worldwide, with Finland having the highest incidence in the world. In 2005, the incidence rate in Finland was greater than 60 affected children per 100 000 children per year, and even further increases in the incidence rates in the future are predicted (Harjutsalo *et al.* 2008; Mehers and Gillespie 2008). The increasing incidence rate over recent decades remains unexplained; the genetic component can not be accountable due to the rapidity of the growth of the incidence rates (Mehers and Gillespie 2008; Patterson *et al.* 2009). Therefore, many environmental factors have become the focus of interest in studies attempting to find the causes behind the development of the disease.

Studies searching for the environmental factors of T1D need tools for finding those individuals with an increased genetic risk for disease development. These individuals are then included in follow-up programs with the purpose of tracing the possible factors triggering the autoimmune process. Since the contribution of the HLA genes is well-known and a considerable factor of the genetic component, many assays have been developed for deciphering the alleles of the genes in the region. An ideal method would be fast and inexpensive, have a high-throughput, and produce all the genetic information required to assess the genetic risk of an individual according to the knowledge available. With the enormity of genetic factors affecting disease susceptibility, many still unknown

restrictions to the numbers of analytes used in the risk assessment have usually been made for the assays to be conceivable.

2 Review of the literature

2.1 Human leukocyte antigen (HLA)

2.1.1 Structure and function

HLA (human leukocyte antigen) is the human version of the major histocompatibility complex (MHC) containing many genes related to the functions of the immune system. It is located on chromosome 6 and contains over 200 genes, and it is known to be the most polymorphic genetic system of the human genome. The HLA region is divided into three groups of genes: class I, class II and class III, of which the class III genes are structurally and functionally different from the two other classes, but are named thus due to their location on chromosome 6 between class I and II genes (Figure 1).

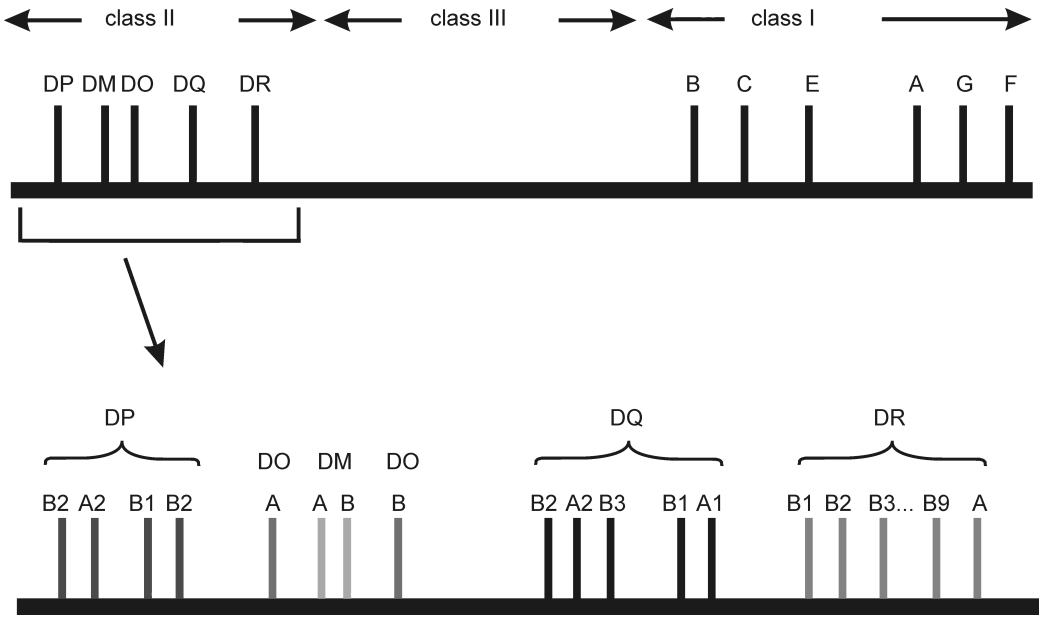

Figure 1. A simplified diagram of the HLA region in chromosome 6 with a limited number of genes shown.

Among class I genes there are three classic transplantation genes, HLA-A, -B and –C, which encode the α-polypeptide chains of the class I molecules. There are also the non-classical HLA–E, -F and -G–genes in addition to some non-expressed pseudogenes. The β-chain of the class I molecules is coded by the beta$_2$-microglobulin gene outside the HLA region in chromosome 15, and this chain combines with the α-chain to form the complete molecule (Figure 2). (Marsh *et al.* 2000)

There are five isotypes of class II molecules, HLA-DM, -DO, -DP, -DQ and -DR, each consisting of two chains, α and β, encoded by the A and B genes, respectively. The α and β chains are roughly similar in size and they form heterodimeric (αβ) protein receptors molecules. Both the α and β chains have two extracellular domains, a transmembrane region, and a cytoplasmic tail (Figure 2). Among the functional class II genes there are also pseudogenes, such as DQA2 and DQB2. HLA-DR is rather complex in structure as it consists of several functional β-chain genes as well as pseudogenes, the number of which can vary. (Marsh *et al.* 2000)

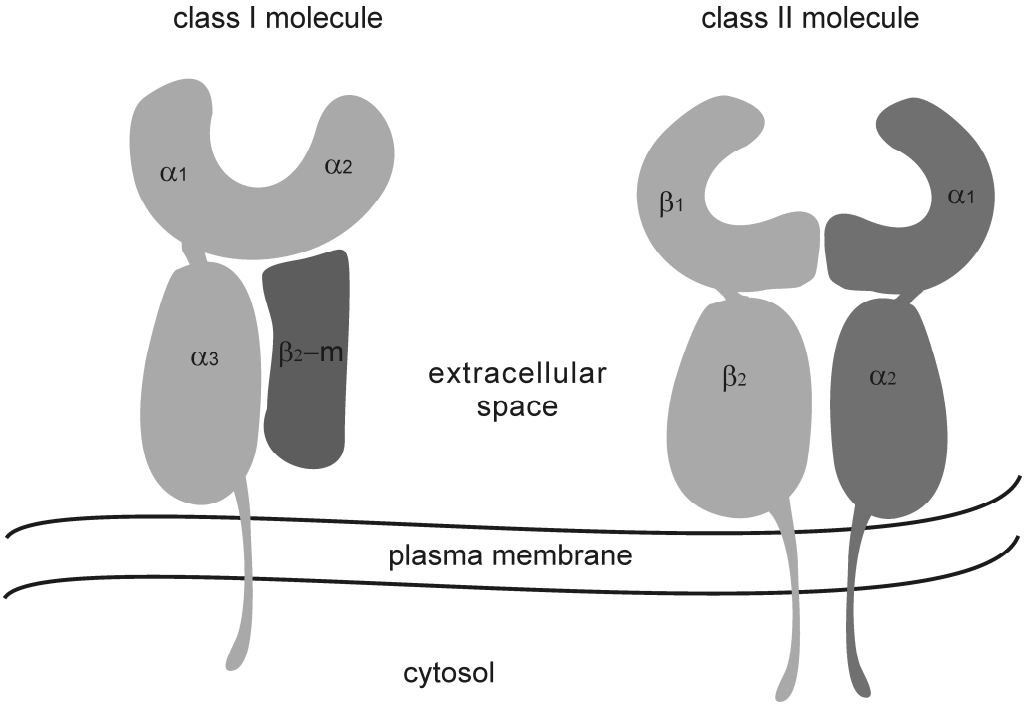

Figure 2. The structure of HLA class I and class II molecules.

Class II genes are typically expressed by a subgroup of immune cells, including B cells, activated T cells, macrophages and dendritic cells, while class I genes are expressed in most somatic cells although at variable levels depending on the tissue. Both class I and II molecules function as the presenters of short peptides to T cells, class I molecules mostly for endogenous and class II for exogenous proteins, although this division is not absolute (Klein and Sato 2000a; Turner 2004).

The polymorphisms in class I and II genes are not evenly distributed but are concentrated in regions coding for the peptide binding domains of the HLA molecule: the α1 and α2 domains of class I molecules, coded by the exons 2 and 3; and the α1 and β1 domains of class II molecules coded by exon 2 of the respective genes. The DR isoform is an exception with a monomorphic α chain (Marsh *et al.* 2000). The amino acid changes in these positions modify the peptide-binding specificity by altering the shapes of the antigen-binding groove, resulting in a repertoire of molecules with different specificities. The number of different alleles discovered for these highly polymorphic HLA loci has been increasing steadily. According to the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/, accessed June 9th 2009) (Robinson *et al.* 2009) the sequence of almost 2500 class I alleles and over 1000 class II alleles has been deciphered.

An important aspect of the HLA region is linkage disequilibrium, referring to the phenomenon of recombination occurring very rarely between two loci. Thus certain alleles of, for example, DQB1- and DQA1-genes are almost always inherited together (Bodmer 1987; Tomlinson and Bodmer 1995). This phenomenon can be utilised by deducing the allele of a gene from the typing information of another gene that is known to be in linkage disequilibrium. For example, the HLA-DQB1*0302-allele is found to be in linkage disequilibrium with DQA1*03-alleles (Klitz *et al.* 2003).

2.1.2 The nomenclature of HLA alleles

A committee for HLA nomenclature was formed in 1965, and the names were originally based on serological results. The current system was introduced in 1987 with each HLA allele name having a four, six or eight digit number, of which all alleles have the four digit number and the longer ones are used if necessary. The first two digits specify the group, often corresponding to the

serological antigen. For example, DRB1*04 signifies a group of alleles which encode the DR4 antigen. The third and fourth digits are used to list the subtypes, which are assigned according to the order in which the sequences are determined, such as DRB1*0401 and DRB1*0402, which refer to two different alleles. The fifth and sixth digits are used to separate non-coding substitutions, for example, DRB1*040101 and DRB1*040102. The seventh and eight digits separate alleles that differ only in the introns or in the 5' or 3' untranslated regions that flank the exons and introns. (Marsh *et al.* 2004, The HLA Informatics Group, Anthony Nolan Research Institute –website: http://www.anthonynolan.org.uk/research/hlainformaticsgroup/, accessed May 26th 2009.)

Alleles that are not expressed due to substitutions introducing premature stop codons or insertions or deletions altering the reading frame are called null alleles and a suffix 'N' is added to the number. Other suffixes are 'L' for low expression as compared to normal and 'S' denoting a secreted molecule not present on the cell surface.

2.2 HLA and disease

Initial interest in HLA was due to the need for avoiding rejection reactions in transplantations, in which the matching of the HLA type of the donor and the recipient is of utmost importance (Marsh *et al.* 2000). Since then the classical HLA class I and II genes have been associated with over 100 diseases, including most autoimmune diseases, and interest in HLA typing has stretched beyond the field of transplantation medicine to diseases such as T1D, rheumatoid arthritis, celiac disease, narcolepsy and asthma. For a review see Shiina *et al.* 2004; Gough and Simmonds 2007; Caillat-Zucman 2009. The relationship between the HLA and disease is often complicated, and nearly all HLA-associated diseases are multifactorial polygenic diseases with other genetic as well as environmental factors involved in disease susceptibility (Caillat-Zucman 2009). In addition to autoimmune diseases, the HLA region is also associated with the course of many infectious diseases, such as human immunodeficiency virus infections, malaria and tuberculosis (Klein and Sato 2000b; Blackwell *et al.* 2009).

2.2.1 Type 1 diabetes

In T1D the insulin production of an individual is compromised due to the destruction of the pancreatic β cells by an autoimmune process eventually leading to an inability to maintain normal blood glucose levels. Although the exact trigger of the onset of the disease is not known, both genetic and environmental factors are known to contribute to the process. For a recent review see Morran *et al.* 2008.

Although multiple genes have been suggested to play a role in disease susceptibility, the HLA region remains the major genetic risk factor. There is also strong evidence for the association of the insulin gene region in susceptibility to the disease (Bell *et al.* 1984; Bennett *et al.* 1995). Other genes involved in disease susceptibility include the CTLA-4- (cytotoxic T-lymphocyte antigen) (Nistico *et al.* 1996), PTPN22-(the gene encoding the lymphoid protein tyrosine phosphatase) (Bottini *et al.* 2004) and the IFIH1- (interferon induced with helicase C domain 1) (Smyth *et al.* 2006) genes. (For a review see Kelly *et al.* 2001; Mehers and Gillespie 2008.) Among the suspected environmental influences are dietary factors, such as exposure to cow's milk or cereals, and different viral infections (Gillespie 2006; TEDDY Study Group 2008).

2.2.2 The genetic risk for type 1 diabetes conferred by the HLA region

At least three major genes from the HLA region, namely HLA-DRB1, -DQB1 and -DQA1, contribute to the genetic risk for T1D, but other genes may also be involved (Singal and Blajchman 1973; Nerup *et al.* 1974; Cudworth and Woodrow 1975; Platz *et al.* 1981; Owerbach *et al.* 1988; Erlich *et al.* 1990; She 1996; Noble *et al.* 2002; Valdes *et al.* 2005; Nejentsev *et al.* 2007). The identification of the effects of individual genes is complicated by the strong linkage disequilibrium between the HLA genes; recombination events are rare and certain alleles of the loci are usually inherited together. The contribution of the HLA region to genetic risk is estimated to be approximately 50 percent (Risch 1987; Cox *et al.* 2001; Ounissi-Benkalha and Polychronakos 2008). The most important DQB1 alleles influencing disease risk are *02 and *0302, which increase disease susceptibility, and *0602 which has a protective effect. Table 1 shows examples of a number of haplotypes combined from the DQB1-,

DQA1- and DRB1-alleles conferring either disease susceptibility or protection. The DQA1- and DRB1-alleles further define the calculated risk. For example, in Caucasian populations of the DQB1*0302-DRB1*0401/2/4/5 and the DQB1*0302-DRB1*0403 haplotypes the latter is moderately protective, and of the DQB1*0302-DRB1*0401 and the DQB1*0302-DRB1*0404 haplotypes the latter is only moderately predisposing (Thomson *et al.* 2007; Erlich *et al.* 2008). The disease risk of an individual is estimated from the combination of both haplotypes, the DQB1*02-DQA1*05/DQB1*0302-DRB1*0401/2/4/5-genotype being associated with the most increased risk. (Nejentsev *et al.* 1999; Ilonen *et al.* 2000; Ilonen *et al.* 2002)

Table 1. HLA-DR-DQ haplotypes associated with increased or decreased disease risk in Caucasian populations (Ilonen et al. 2002; Hermann et al. 2004; Ilonen 2004; Erlich et al. 2008).

	DQB1	DQA1	DRB1
susceptible	*02	*05	*03
	*0302	*03	*0401/2/4/5
	*0304	*03	*0408
neutral	*02	*0201	*07
	*0301	*03	*0401/7/8
	*0303	*03	*09
moderately protective	*0301	*05	*11/12/1303
	*0603	*01	*13
	*0302	*03	*0403
strongly protective	*0303	*0201	*07
	*0503	*01	*14
	*0602	*01	*15

2.2.3 Ankylosing spondylitis and HLA-B*27

Ankylosing spondylitis (AS) is a chronic, inflammatory arthritis, causing pain in the lower back, and resulting eventually in the fusion of the spine and thus causing rigidity of the spine. Disease onset is typically around 20-30 years, and males are more often affected than females. For a review see Gladman 1998; Kataria and Brent 2004; Reveille 2006. The association of the HLA-B27 antigen with AS was demonstrated already in 1973 (Brewerton *et al.* 1973), and it has, in addition to narcolepsy and celiac disease, remained among the strongest of the variable HLA disease associations reported (Pile 1999; Lin *et al.* 2001; Choo 2007; Caillat-Zucman 2009). The presence of HLA-B*27 is almost necessary for disease development with over 90 percent of patients of

European ancestry being HLA-B*27 positive (Brown *et al.* 1996; Marsh *et al.* 2000; Thorsby and Lie 2005). However, the presence of HLA-B*27 is not alone sufficient for disease development. The HLA-B*27 allele is rather common in the Caucasian population, 7-14 % of the population being HLA-B*27 positive, while only 2-5 % actually develop the disease. Consequently, the test for the presence of HLA-B*27 can be used as a confirmatory assay if, based on other symptoms the patient may have, AS is suspected. (Kataria and Brent 2004; Laitinen and Hakala 2005; Caillat-Zucman 2009, dbMHC database: www.ncbi.nlm.nih.gov/gv/mhc/, accessed June 9th 2009)

The HLA-B*27 allele is also associated with other diseases, such as reactive arthritis and acute anterior uveitis; however, the association is not as strong as in the case of AS (Pile 1999; Marsh *et al.* 2000; Bowness 2002).

2.3 Methods used for typing the genetic risk for type 1 diabetes conferred by the HLA region

Initially, HLA-typing relied on serological methods, but it later became evident that these methods were not able to discriminate between all the differences in the HLA molecules. When typing down to the nucleotide level became possible, the unique complexity of the HLA genes became evident. This typing can be low-resolution, which resembles serological typing in the sense that a group of alleles, rather than individual alleles, are recognised. With high-resolution typing, discrimination is down to the individual allele level, and an intermediate resolution can be defined as distinguishing some individual alleles in addition to groups of alleles. (Marsh *et al.* 2000)

2.3.1 Studies using HLA screening to identify subjects at risk for type 1 diabetes

Several research programs have been screening or are currently screening newborns to identify those having an increased genetic risk for type 1 diabetes. These high-risk children are then followed with the hope of elucidating the factors contributing to disease pathogenesis (Carmichael *et al.* 2003). Table 2 lists some of the studies which are using various methods for typing HLA-based genetic risk, and which are investigating different aspects of T1D development, such as environmental and genetic risk factors, and also possible

approaches for the prevention of the disease (Rewers *et al.* 1996; Nejentsev *et al.* 1999; Schenker *et al.* 1999; Dorman and Bunker 2000; Schatz *et al.* 2002; Hagopian *et al.* 2006; Hood *et al.* 2006; TRIGR Study Group 2007).

Table 2. Projects using various methods for the screening of HLA-based genetic risk for T1D.

The study	Typing method	Aim of the study	Location	Reference
DAISY – Diabetes Autoimmunity Study in the Young	Dot-blot	To learn how genes and environment interact to trigger the onset of type 1 diabetes	Denver	Rewers *et al.* 1996 http://www.teddy-colorado.org/
BABYDIAB	Dot-blot	To investigate the development of autoantibodies in children of mothers or fathers with T1D	Germany	Schenker *et al.* 1999
DIPP – Diabetes Prediction and Prevention	DELFIA	To study the prediction and possible prevention of T1D	Finland	Nejentsev *et al.* 1999 http://research.utu.fi/dipp/index.php
PANDA – Prospective Assessment in Newborns for Diabetes Autoimmunity	Luminex	To understand the mechanisms leading to the disease and to develop a prevention therapy	Florida	Hood *et al.* 2006 http://www.uf-diabetes.org/research_PANDA.asp
TEDDY – The Environmental Determinants of Diabetes in the Young	DELFIA	To investigate the role of environmental factors in the development of T1D	Finland, Sweden, Germany, USA	Hagopian *et al.* 2006 http://teddy.epi.usf.edu/
TRIGR – Trial to Reduce IDDM in the Genetically at Risk	Pyro-sequencing and DELFIA	To test whether hydrolysed infant formula compared to cow's milk-based formula decreases the risk of developing T1D	Europe, USA, Canada, Australia	The TRIGR Study Group 2007 http://trigr.epi.usf.edu/

One option for the screening of genetic risk is a step-wise approach. Different factors influencing risk are analysed with several consecutive assays, and the screened population is thus narrowed down to those eligible for the study. The number of screened individuals is diminished after each step and the order of

the analytes needs to be carefully selected to provide the most economical screening program.

A second option for screening is to include all the genetic risk factors in one step, requiring a multiplex assay capable of typing all the loci in a single assay. The latter approach is usually technically more complex than step-wise typing, and is better suited for studies where it is essential to have the full information of the genetic risk factors of all the screened individuals. Even with the intention of selecting only a small percentage of the population, including those with the highest genetic risk, the number of screened samples easily expands to several thousand and the selected screening method becomes a critical aspect in the study budget. However, once the progress of the disease can be prevented or the disease cured the full genetic risk panel of those individuals receiving treatment might have a major effect on the chosen therapy, and the all-at-once approach of screening would thus be the most advantageous.

2.3.2 Dot-blot and reverse dot-blot

The first assay using the basic principle of a dot-blot assay with labelled, sequence-specific oligonucleotides (SSO) to detect PCR product immobilised onto a membrane (Figure 3) was published in the 1980s (Saiki *et al.* 1986) and has been a popular method ever since. The method was also quickly applied to the typing of HLA polymorphisms, such as DQA1- and DQB1 alleles (Morel *et al.* 1988; Dorman *et al.* 1990; Bugawan and Erlich 1991; Heimberg *et al.* 1992). The primers used were designed to include all the polymorphisms of interest within the amplicon, and the resulting PCR product was usually immobilised onto a membrane by baking or UV cross-linking. Sequence specific oligonucleotides with radioactive labels (Dorman *et al.* 1990; Khalil *et al.* 1990; Heimberg *et al.* 1992; Berzina *et al.* 2002) or nonradioactive labels with chromogenic dyes or chemiluminescent substrates for detection (Bugawan and Erlich 1991; van der Auwera *et al.* 1995; Rewers *et al.* 1996) were then used to identify the alleles of the immobilised PCR products under investigation.

In the reverse dot-blot method (Figure 3) the positions of the PCR product and the probes are reversed with the probes being those immobilised onto a solid surface and the PCR product labelled for detection and hybridised to the probes. With this reversed dot-blot method microtiter plates have been used,

instead of membranes, as the solid phase for immobilizing the probes thus enabling automation and high-throughput screening of HLA genes (Giorda *et al.* 1993; Kawai *et al.* 1994; Cinek *et al.* 2000; Geranton *et al.* 2003).

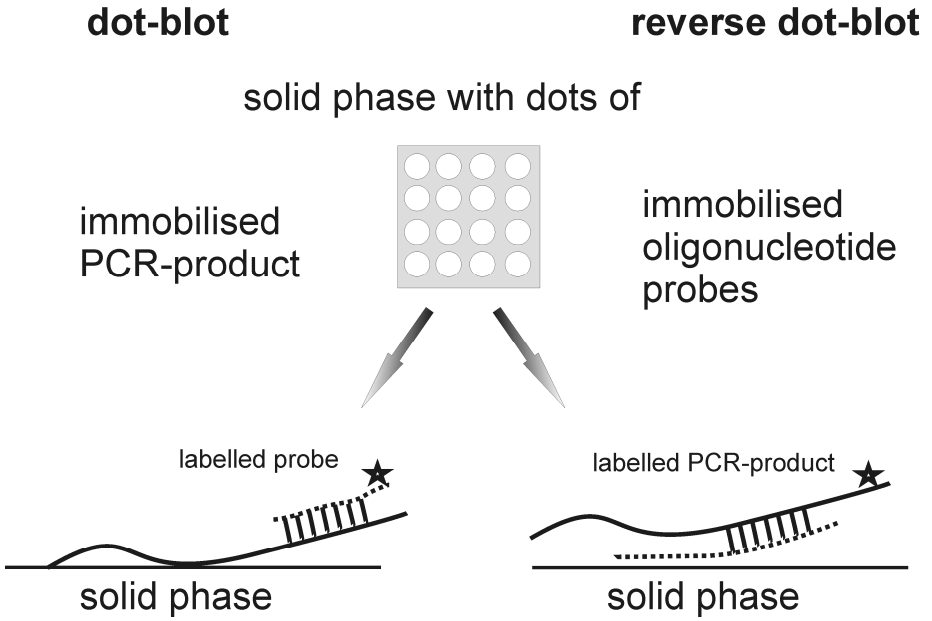

Figure 3. The principle of dot-blot and reverse dot-blot assays. In dot-blot assays PCR products are immobilised onto the solid phase and labelled oligonucleotide probes are hybridised to the PCR product, whereas in reverse dot-blot assays the positions of the PCR products and probes are reversed.

Studies that have used dot-blot for typing the HLA-DQB1 alleles include the NOBADIA (Norwegian Babies against Diabetes) study (Ronningen 1997), the German BABYDIAB study (Schenker *et al.* 1999) and the ABIS (All Babies in Southeast Sweden) study (Berzina *et al.* 2002). In the DEW-IT (Diabetes Evaluation in Washington) study glass slides were used instead of nylon membranes for immobilizing the PCR products (Wion *et al.* 2003). The DAISY (Diabetes Autoimmunity Study in the Young) study, conducted in Denver, Colorado, and initiated in 1994 (Rewers *et al.* 1996), initially also used the dot-blot method for their screening. In addition to these screening programs to find individuals with an increased genetic risk for T1D, the dot-blot method has also been used in research to further elucidate the genetic risk behind T1D (van der Auwera *et al.* 1995; Erlich *et al.* 2008) or to evaluate the best possible inclusion criteria for follow-up studies (Lampasona *et al.* 1995).

A commercial kit for typing HLA-DQB1 alleles called RELI™ SSO with a reverse dot-blot principle is available from Invitrogen (Carlsbad, CA, USA). Kits for other HLA genes, such as DRB1, are also available in the same product line. The RELI™ SSO method, to evaluate the HLA-based genetic risk for T1D, has been used in the DIABFIN (Diabetes Finalized Italian Network) (Buzzetti *et al.* 2004) and Prefin Italy (Lorini *et al.* 2005) studies as well as by the JDRF (The Juvenile Diabetes Research Foundation) / Wellcome Trust Diabetes and Inflammation Laboratory (DIL) (https://www-gene.cimr.cam.ac.uk/todd/public_data/HLA/HLA.shtml, accessed 4th March, 2009). The DAISY study has also proceeded in 2002 to type the samples with a reverse line-blot method using strips, performed in the HLA typing laboratory at Roche Molecular Systems Inc. (Alameda, CA, USA) (Blair *et al.* 2002; Emery *et al.* 2005). In this method biotinylated, amplified DNA is hybridised to nylon membrane strips containing immobilised probes in linear arrays, and detected with streptavidin-horseradish peroxsidase and chromogenic substrate.

2.3.3 DELFIA

The DELFIA® method, using sequence-specific oligonucleotides with lanthanide labels closely resembling the dot-blot method performed on a microtiter plate, is commercially available from PerkinElmer, Wallac. DELFIA® assays are available for screening certain alleles of the HLA-DQB1, -DQA1 and DRB1 genes that provide valuable information when evaluating the genetic risk for developing type 1 diabetes. This method has been used for screening for eligible participants in the DIPP (The Diabetes Prediction and Prevention) study in Finland (Nejentsev *et al.* 1999; Kukko *et al.* 2004) as well as in the TEDDY (The Environmental Determinants of Diabetes in the Young) study (The TEDDY Study Group 2007), and also in a Danish study with the objective of improving the tools used for predicting of T1D at birth (Eising *et al.* 2007).

In the DELFIA® method the region of interest of the gene under investigation is amplified by PCR using a primer pair with one of the primers labelled with biotin. As shown in Figure 4, the biotin moiety is then used to attach the PCR product onto a microtiter plate well coated with streptavidin which allows the removal of all the other components of the PCR by washes. The PCR product is then denatured using sodium hydroxide and the non-biotinylated complementary strand is removed by washes leaving a single-stranded PCR

product in the microtiter well. Lanthanide-labelled probes are added and are allowed to hybridise to their complementary sequences in the PCR products. The microtiter wells are washed once more, to remove the excess probes, and a development solution is added, after which the signals can be measured and the results interpreted. (Sjöroos *et al.* 1995; Ilonen *et al.* 1996; Sjöroos *et al.* 1998; Nejentsev *et al.* 1999; Laaksonen *et al.* 2002)

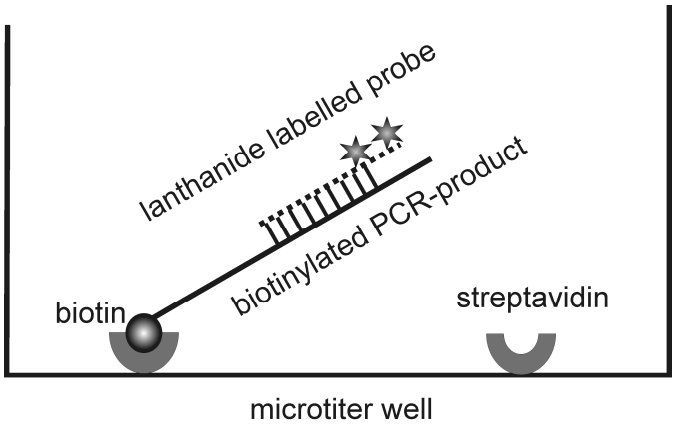

Figure 4. DELFIA hybridisation. The biotinylated PCR product is attached to the well of a streptavidin coated microtiter plate and stripped of the complementary strand. The lanthanide-labelled probes hybridise to the single-stranded PCR products, and after removal of unbound probes by washes, the signals produced by the labels are measured from the wells.

The utilisation of lanthanide labels provides an advantage. Due to their unique nature, the biological background of sample material does not interfere with the measurement allowing the use of less pure DNA material in the assays. Since the sample material need not be pre-treated excessively, valuable time and materials are saved.

2.3.4 Luminex xMAP –technology

The PANDA (Prospective Assessment in Newborns for Diabetes Autoimmunity) study is relying on the Luminex platform for typing the HLA-DQB1 and –DRB1 –alleles (Hood *et al.* 2006). Luminex xMap® technology (Figure 5) is based on microspheres that are internally labelled with two spectrally distinct fluorochromes. The ratio of the fluorochromes is specific for each type of microbead, and forms the basis of recognition of different beads.

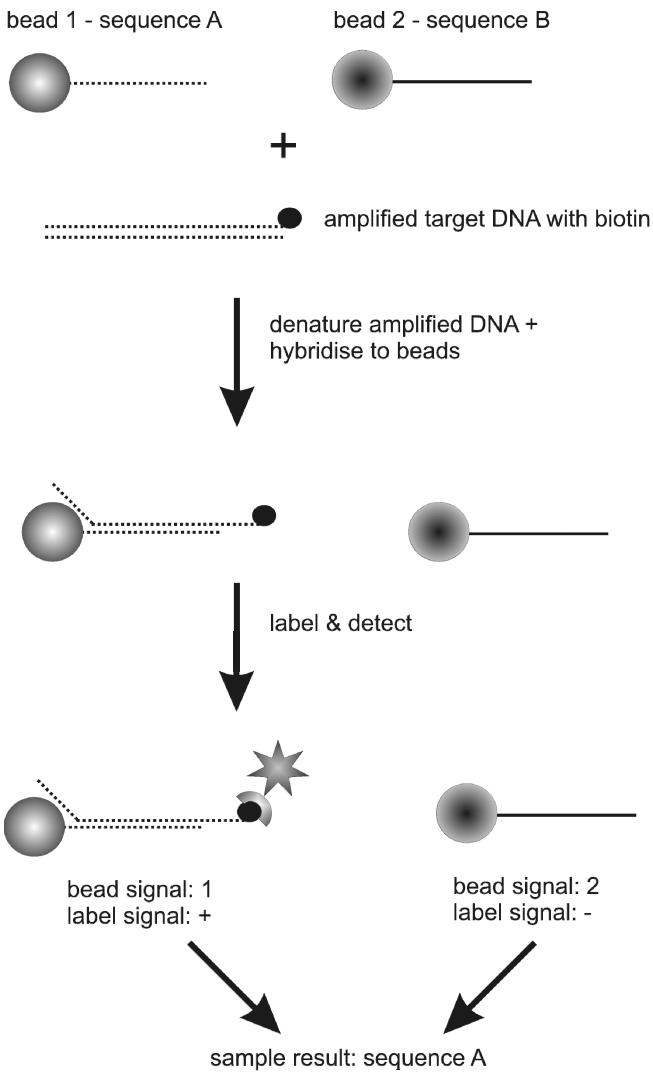

Figure 5. The Luminex assay. The labelled beads have different oligonucleotide probes attached onto their surfaces. The beads, and the particular probes attached to them, can be recognised through the varying dye ratios of the beads. The amplified and biotinylated PCR products are hybridised to the bead sequences, and the biotin is used to label the attached PCR products on the beads. The signals are then measured both from the beads, to recognise the probe sequence, and the biotin attached label, to identify if a PCR product is hybridised to the probe or not.

The beads are coated with capture probes, usually approximately 20 base pairs in length, containing a sequence complementary to a sequence in the appropriate amplified PCR product (Fulton *et al.* 1997; Dunbar 2006). The sequence of the capture probe is consequently linked to the ratio of the dyes in

the bead enabling the identification of the probe sequence through the bead. Primers are designed to amplify a region from 100 to 300 base pairs. As one of the primers is biotinylated, biotin is thus incorporated into the PCR product during amplification. This biotin is then used for labelling the PCR product hybridised to the microsphere with a streptavidin coupled label moiety. Once the PCR products are hybridised to the capture probes on the beads, the beads are analysed with a flow cytometer capable of measuring the bead fluorescence, as well as the signal from the labelled PCR product possibly hybridised to the capture sequence of the bead. (Fulton *et al.* 1997; Itoh *et al.* 2005; Dunbar 2006)

A few possible assay formats for analyzing nucleic acids with the Luminex principle have been described. In the direct hybridisation format the single-stranded PCR products, rendered such by denaturing or by asymmetric amplification, are hybridised to the capture probes. If the labelling of the target DNA is undesirable or not possible, a competitive format can be used, where biotinylated single-stranded oligonucleotide targets compete with the double-stranded PCR products for hybridisation to the capture probes. (Dunbar 2006)

The beads can also be used for analyzing the results of solution-based sequence-specific enzymatic reactions used for typing the target sequence. These reactions include the allele-specific primer extension (ASPE), the oligonucleotide ligation assay (OLA), and the single base chain extension (SBCE). In all of these approaches, a capture sequence is included in one of the oligonucleotides used in the assays to attach the product to a recognisable bead via a complementary sequence on a bead. In ASPE (Figure 6) a primer with a capture sequence is extended by the incorporation of dNTPs, one of which is biotinylated. The extension can only occur if the 3'-end of the primer, located at the site of the polymorphism, is complementary to the target. In OLA (Figure 7) a capture probe with the 3'end of the probe positioned at the site of the polymorphism and a biotinylated reporter probe are ligated by a thermostable ligase provided the probes and target are perfectly matched. Unlike in ASPE and OLA, in the SBCE method (Figure 8) the 3'-end of a primer containing a capture sequence is located one base upstream of the polymorphism. Separate reactions are required for all possible single base extensions using biotinylated nucleotides. However, after the extension step the detection can be multiplexed into a single reaction. (Dunbar 2006)

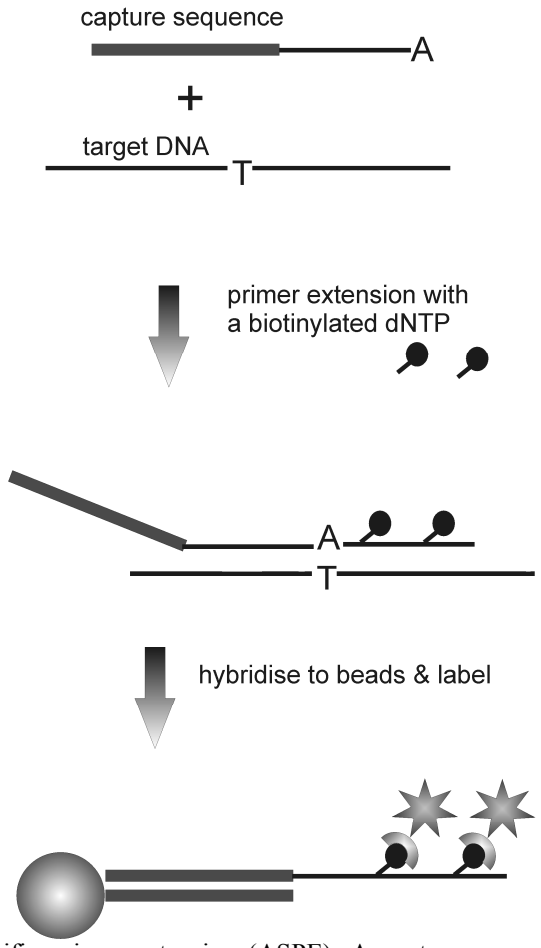

Figure 6. Allele-specific primer extension (ASPE). A capture sequence is included in the primer used for amplification of the target. The 3'-end of the primer is located at the site of the polymorphism, and amplification can occur only in the case of a perfect match of the 3'-end. One of the dNTPs is biotinylated and this biotin is incorporated into the amplification product. The capture sequence is used to attach the biotinylated PCR product to the correct bead, while the biotin moieties are used for labelling the product

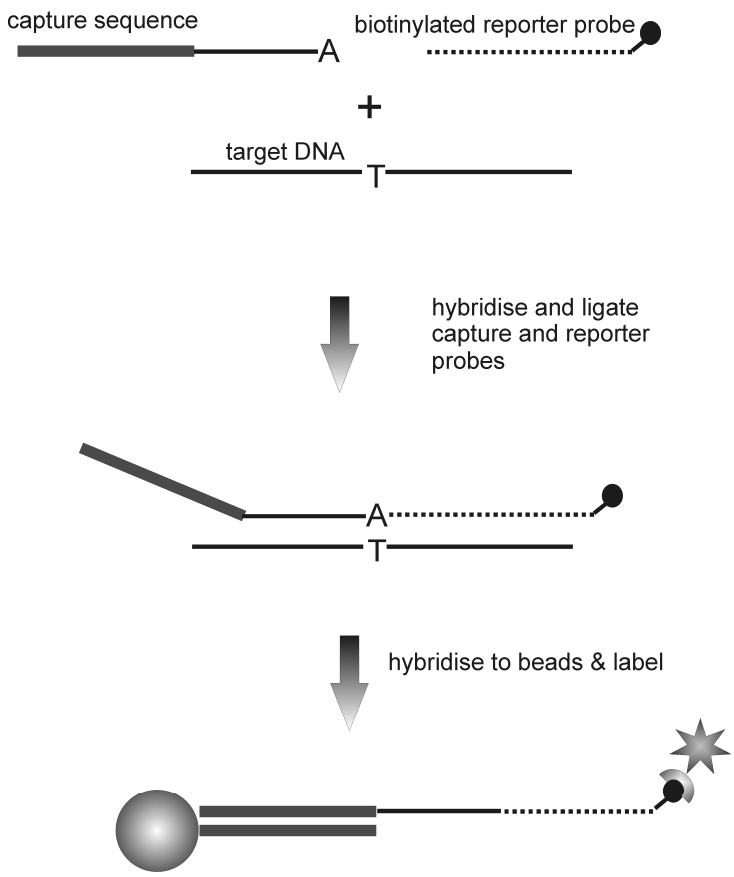

Figure 7. The oligonucleotide ligation assay (OLA). A probe with a capture sequence complementary to a sequence on a specified bead and a biotinylated reporter probe are both hybridised to the target sequence. A ligation reaction will occur only in the case of a perfect match. The capture sequence is then used to attach the formed ligation product onto a recognisable bead.

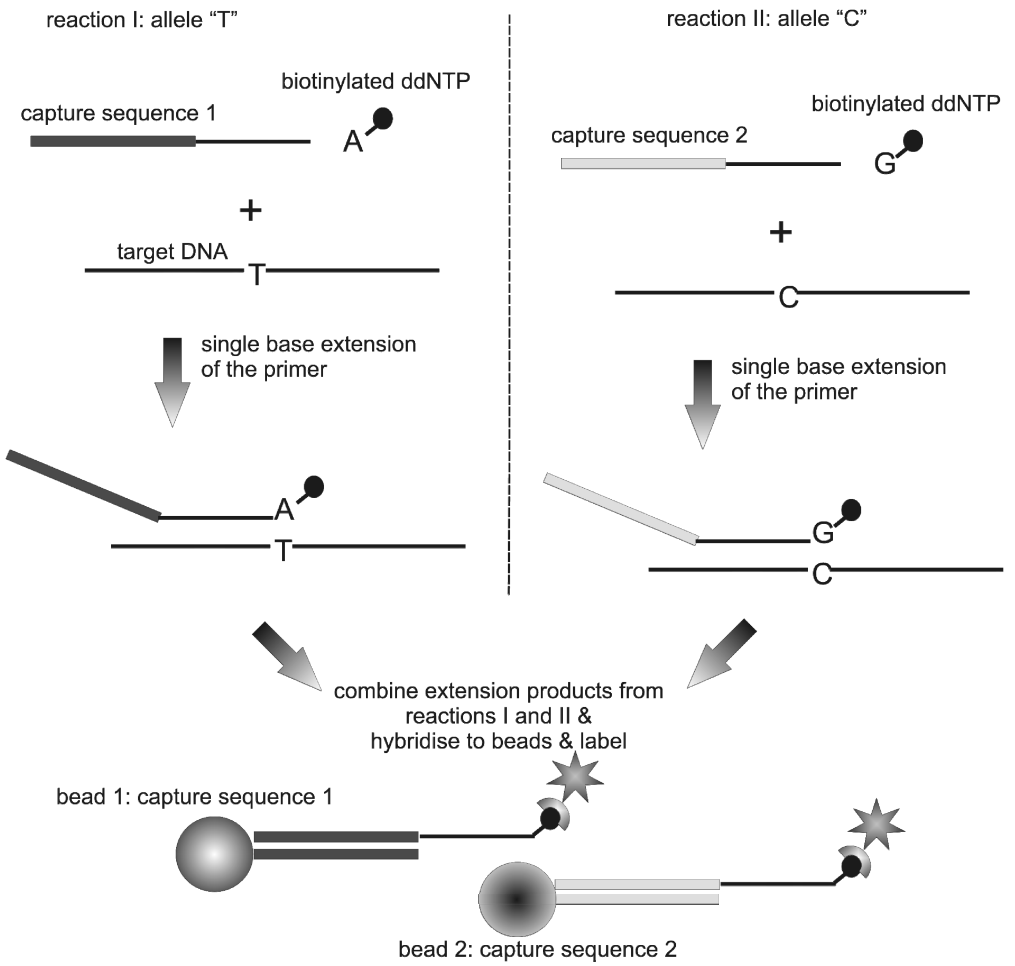

Figure 8. Single base chain extension (SBCE). A primer with a capture sequence hybridises to the target with the 3’-end next to the polymorphic site. A biotinylated ddNTP is used to extend the primer by a single base. The different ddNTPs, used for typing the different alleles, all require separate reactions, and in each reaction a different capture probe is used linking the added ddNTP to a specific sequence. The single base extension products from all reactions are then mixed with the beads. Through the capture sequence the added ddNTP can be linked to a specific bead, and the base added to the site of the polymorphism can thus be identified.

Multiple analytes are easily detected from a single reaction based on the identification of the beads and, consequently, the capture probes. The Luminex system is reported to be able to analyze up to 100 different analytes in a single reaction, thus enabling, if necessary, the typing of several HLA genes in a single run (Fulton *et al.* 1997; Dunbar 2006; Dalva and Beksac 2007). Recently, a version of the Luminex system capable of analysing up to 500 analytes in a single reaction has been released (Luminex-website

http://www.luminexcorp.com/flexmap3d/index.html, accessed 14th October 2009). In this version, three different dyes are used in the beads, instead of two, for identification purposes, and thus the number of beads can be increased. The Luminex assay principle can also be applied to other analytes beyond nucleic acid analysis, such as antigens, antibodies, and enzyme substrates (Fulton *et al.* 1997).

Kits for typing HLA alleles, including the DQB1-gene polymorphisms, are available from One Lambda Inc. (Canoga Park, CA, USA) and Tepnel Lifecodes (Stamford, CT, USA) (Dunbar 2006; Dalva and Beksac 2007). Both kits use the direct hybridisation approach, but there are slight differences between the kits, for example in the assay protocol. Tepnel Lifecodes relies on asymmetric PCR, whereas the One Lambda –kit uses regular PCR. There are also differences in the number of probes used for typing of the alleles. (Dunbar 2006; Dalva and Beksac 2007) Products for typing polymorphisms in other HLA genes, such as for DRB1, are also available from both companies.

2.3.5 Pyrosequencing

A sequence-based typing method called pyrosequencing, originally designed for sequencing short stretches of approximately 10 bases of DNA, has also been adapted for typing the HLA genes DQB1 and DRB1 (Ringquist *et al.* 2002a; Ringquist *et al.* 2002b; Ringquist *et al.* 2004; Entz *et al.* 2005). It has been used in the TRIGR (Trial to Reduce IDDM in the Genetically at Risk) –project to type samples from the USA and Canada (TRIGR Study Group 2007). The pyrosequencing method requires a single-stranded DNA-template, purified of any residual PCR components, either by solid phase capture of the PCR product or by enzymatic removal of any remaining primers and nucleotides (Ronaghi 2001). The actual sequencing reaction (Figure 9) consists of four steps: (1) A sequencing primer is hybridised to the single-stranded PCR product. (2) The sequencing is performed by adding each dNTP one-by-one into the reaction, and when the correct dNTP is added to the reaction, it becomes incorporated and pyrophosphate is released. (3) The pyrophosphate is then converted by ATP sulfurylase, with exogenous adenosine 5' phosphosulfate, into ATP, followed by the production of light with the use of luciferase. In the nucleotide additions dATPαS is used instead of dATP since it is incorporated efficiently by DNA polymerases, but it is not a substrate for luciferase. (4) Unincorporated dNTPs are degraded by apyrase before the

addition of the next dNTP. The production of light is used to determine which dNTP was successfully added and thus is the correct base in the studied sequence. Steps 2 to 4 are then repeated to further decode the target sequence. (Ronaghi *et al.* 1996; Ronaghi 2001; Fakhrai-Rad *et al.* 2002; Ringquist *et al.* 2002b; Ringquist *et al.* 2004)

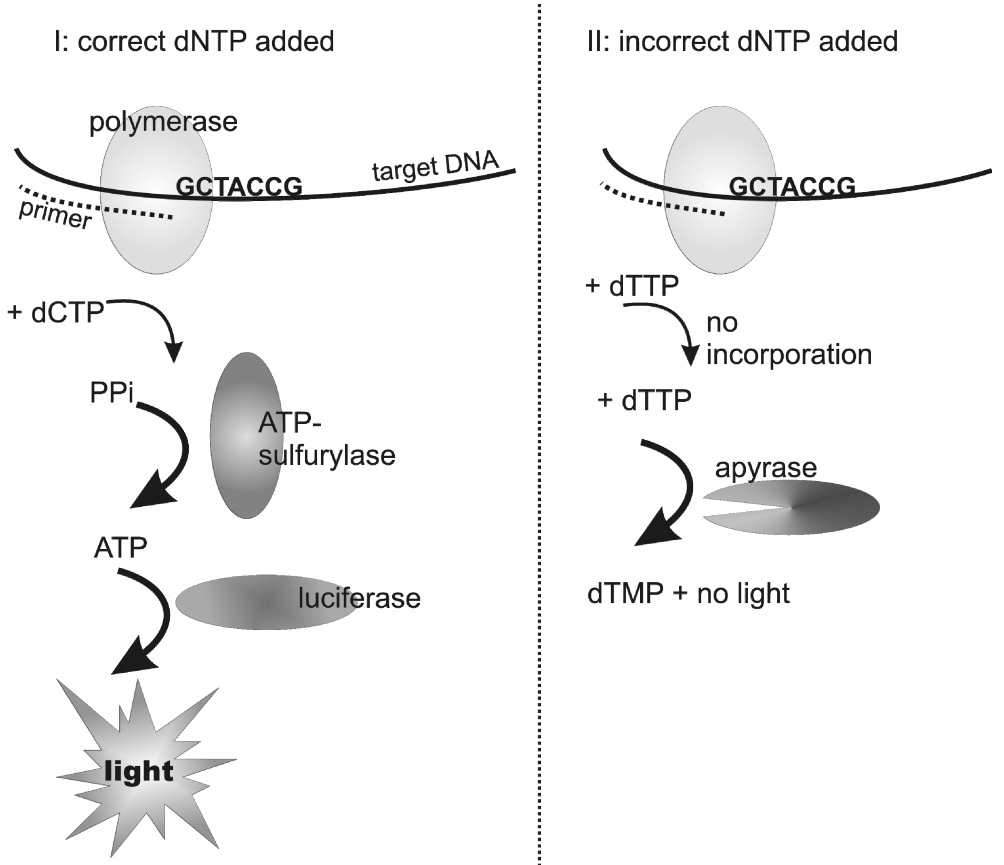

Figure 9. Pyrosequencing. I: When a correct dNTP is added, the polymerase incorporates it and a pyrophosphate is released. ATP-sulfurylase converts this pyrophosphate into ATP, which is then used by luciferase to produce light which is measured to identify a successful incorporation. II: If a wrong dNTP is added, incorporation does not occur and the added dNTP is removed from the reaction by apyrase.

Based on previous knowledge of the sequences of the region being assayed, the dispensation order of the dNTPs can be adjusted, instead of the four different dNTPs rigidly following one after another always in the same order (Ramon *et al.* 2003). This adjusted order of dNTP dispensation has also been exploited in the typing of HLA-DRB1 alleles in heterozygous DNA samples by using out-

of-phase dispensation. The order of nucleotides added is selected to cause incorporation in only one of the alleles, resulting in the sequencing of this allele to be ahead of the other. DRB1-alleles otherwise indistinguishable have been successfully typed by this method. (Ramon *et al.* 2003)

The read lengths obtained when typing the genes of the HLA region have been from 30 to 70 nucleotides (Ringquist *et al.* 2002b; Ringquist *et al.* 2004; Entz *et al.* 2005), although some methods have been reported that enable the reading of sequences up to 150 residues in length (Gharizadeh *et al.* 2002). The DQB1 and DRB1 alleles have been completely resolved by using a combination of a number of sequencing primers. The primers hybridise to different locations on the PCR product, usually in the most polymorphic exon 2 of these genes. Consequently, a more complete view of the sequence and the alleles in question can be achieved than by using the information gained from a single sequence (Ringquist *et al.* 2002b; Ringquist *et al.* 2004; Entz *et al.* 2005; Lu *et al.* 2009). To decipher the DRB1 alleles of a sample, a task complicated by the sheer number of different DRB1 alleles, a group-specific amplification as a pre-selection step combined with pyrosequencing of the identified sub-groups has also been used (Entz *et al.* 2005).

The unique, highly polymorphic nature of the genes in the HLA region requires appropriate software to resolve the alleles in question. However, pyrosequencing is a convenient method for the analysis of HLA alleles, and provides increased resolution of the region sequenced not obtainable by other methods relying on oligonucleotide probes, which dictate in advance the sequence information that can be acquired by the method in question. Thus previously unpublished polymorphisms can also be discovered by the pyrosequencing method. An added benefit of the method is the possibility to resolve some allelic combinations by out-of-phase sequencing (Ramon *et al.* 2003; Lu *et al.* 2009) that would be undecipherable by conventional sequencing.

2.3.6 Other methods for typing HLA genes

In the well-established TaqMan method (Holland *et al*. 1991), a DNA-polymerase with a 5'-nuclease activity is used in PCR to cleave a double-labelled probe hybridised to the template being amplified by the polymerase. When the probe, containing a reporter fluorophore on one end and a quencher

label on the other, is cleaved, the reporter label is released and the resulting increase in its fluorescence signal can be measured. This principle of detecting the amplified PCR product has also been applied to the testing of HLA alleles. Faas and colleagues have developed a system based on TaqMan probes to analyse HLA-DQB1 alleles where the specificity of the assay relies mostly on sequence specific primers (Faas *et al.* 1996). They noted that the TaqMan probes hybridised under PCR conditions to templates containing up to two mismatches, and thus were not able to provide the specificity required to distinguish the alleles. However, if such a method, based on a repertoire of sequence specific primers to identify the alleles, were used, fewer labelled probes would be required, since a separate probe would not be needed to detect each allele; whereas in a method where generic primers are employed, a larger panel of more specific probes would be needed to identify all required alleles. This combination of sequence-specific priming (SSP) with TaqMan detection has later also been applied to other HLA genes (Luedeck and Blasczyk 1997; Menon *et al.* 1997; Slateva *et al.* 1998; Albis-Camps and Blasczyk 1999; Gelsthorpe *et al.* 1999; Tremmel *et al.* 1999; Slateva *et al.* 2001; Reinton *et al.* 2006; Danzer *et al.* 2007), but despite these developments the utilisation of this approach in large-scale screening for the risk alleles for T1D has not been reported.

Due to the complex nature of the HLA region, an alternative approach to typing the HLA alleles, with the help of single nucleotide polymorphisms (SNP), called HLA tagging has been proposed (de Bakker *et al.* 2006; Goris *et al.* 2008; Leslie *et al.* 2008). In this method, the HLA alleles are deciphered by analysing SNPs that are located outside the HLA genes, but are strongly associated with certain HLA alleles. The use of a TaqMan-assay to screen for two SNPs identifying DR3/4-DQ8 subjects has been suggested as a primary step by Barker and colleagues, since this would identify those individuals considered at the highest risk for developing T1D (Barker *et al.* 2008). One SNP was successfully typed by the TaqMan method, but the second SNP was assayed using a restriction enzyme digestion, since nearby polymorphisms prevented the utilisation of a TaqMan assay with this SNP. Additionally, since the association of the SNP to the targeted genotype was not 100 % when compared to results obtained by a reverse dot-blot method, the HLA genotype of the individuals suspected of a high risk had to be confirmed by other methods. This SNP tagging using the TaqMan-method has also been shown to be applicable to the detection of HLA risk alleles for celiac disease (Monsuur *et al.* 2008; Koskinen *et al.* 2009).

Commercial kits based on sequence-specific primers are also available for typing the HLA genes. These methods were published already in the 1990s, and have been widely used (Olerup and Zetterquist 1992; Bunce *et al.* 1995; Welsh and Bunce 1999; Gerlach 2002). The identification of the alleles is based on the pattern of PCR products produced by an array of allele-specific primer sets, usually visualised by gel-electrophoresis. Qiagen provides Olerup SSP –kits for both low and high resolution typing of class I and class II genes (Qiagen website: http://www1.qiagen.com/Products/Assays/HLA/, accessed April 11th 2009). Invitrogen manufactures two kits relying on the same principle of sequence-specific priming: SSP UniTray® and AllSet+™Gold SSP (Invitrogen website: http://www.invitrogen.com, accessed April 11th 2009).

HLA typing can also be done by sequencing. For example, Qiagen offers the SBTexcellerator HLA kit, Invitrogen manufactures the SeCore™ kit, and Abbott Laboratories the AlleleSEQR kit (Abbot Molecular website: http://international.abbottmolecular.com/HLA_1246.aspx, accessed October 24th 2009). These methods are usually quite labour-intensive and consequently not suitable for the primary screening of large sample collections.

The different methods used in the screening for the HLA risk alleles for T1D all have their advantages as well as some disadvantages (Table 3). Some of the methods, namely the pyrosequencing and the Luminex assay, require a multi-step sample pre-treatment, while some methods allow the use of quite crude sample material, such as whole blood dried on a sample collection paper used in the DELFIA system. However, the labour required by some of the methods is compensated, for example in the Luminex assay, by the number of alleles or analytes than can be analysed simultaneously. The intended use dictates to a great extent which of the assays is the most suitable for the purpose. Considering a study aimed at the screening of thousands of samples, such as the TEDDY study, a method with minimal costs combined with fast production of results would be ideal. The costs of an assay usually mainly consist of labour and reagents. Thus an economical method would require minimal hands-on time and inexpensive reagents as well as minimal sample pre-treatment, and would not require the use of specialised equipment.

Table 3. Characteristics influencing the method of choice for screening the HLA risk alleles.

Method	Advantages	Drawbacks
dot-blot	-no complicated sample pre-treatment -simple method principle – easily applied to new analytes	-limited number of alleles detected simultaneously
Luminex	-number of alleles detected simultaneously can vary from few to hundred	-multiple steps -laborious sample pre-treatment
Pyrosequencing	-detection of unknown alleles	-laborious sample pre-treatment -specialised equipment required
DELFIA	-no complicated sample pre-treatment	-limited number of alleles detected simultaneously
TaqMan	-uncomplicated assay principle requiring only up to 2 steps	-limited number of alleles detected simultaneously -design of assay may be complicated

At present there is no cure or method of prevention for type 1 diabetes. Screening for the genetic risk for T1D is currently used to identify those individuals eligible for prevention trials (Dantonio *et al.* 2006). It has also been stated that until an effective intervention is available, this should be the only purpose of the genetic screening of the general public (Schatz *et al.* 2002). Several studies have focused on identifying environmental factors that are thought to contribute to the disease susceptibility. Possible ways of preventing the disease or delaying the disease progress can be investigated, and also deeper knowledge of the molecular mechanisms behind the disease can be gained by studying individuals with increased genetic risk (Schatz *et al.* 2002; Gillespie 2006; Mehers and Gillespie 2008). Hopefully in the future T1D can be prevented and the methods developed for screening genetic risk factors can be applied to population screening programs, and possibly even identify those preventive measures most suited for the individual with a certain genetic risk for T1D. (Knip 1998; Schatz *et al.* 2002; Kim and Polychronakos 2005)

3 Aims of the study

The aim of the present study was to improve the current methods used in the screening for the genetic risk for type 1 diabetes in order to reduce the costs and time spent in analyzing the samples. More specifically, the aim was to develop a method generally suitable for the screening of blood samples dried on sample collection paper for different polymorphisms.

The aims of the original publications were as follows:

1. To provide proof-of-principle for a method based on asymmetric PCR and competitive homogeneous hybridisation with a simple SNP assay.

2. To develop the method created further to suit a more complex assay for HLA-DQB1 screening and to develop a method allowing the use of whole blood dried on sample collection paper as sample material.

3. To show the functionality of the method developed in a large-scale screening program involving the typing of thousands of samples.

4. To provide a possible dry reagent approach to further simplify the method developed and to compare the method developed to an existing method used in a routine screening program.

4 Summary of materials and methods

A summary of the materials and methods used and some unpublished information is presented in this chapter. A more detailed description of the published materials and methods can be found in the original publications (I-IV).

4.1 Oligonucleotides

The detailed sequences and labels of the oligonucleotide primers and probes are described in the original publications (I-IV) and in Table 4. The primers and probes for study I were purchased from MWG Biotech AG (Ebersberg, Germany) and for studies II-IV from ThermoElectron GmbH (Ulm, Germany). The lanthanide probes were labelled at the Department of Biotechnology at the University of Turku (I and II) or at Abacus Diagnostica (Turku, Finland) (III and IV).

The LNA (locked nucleic acid)-spiked probes were designed according to published guidelines (Orum *et al.* 1999; Braasch and Corey 2001; Simeonov and Nikiforov 2002; Costa *et al.* 2004; Ugozzoli *et al.* 2004; You *et al.* 2006) and with the aid of web-based calculation programs (http://lnatools.com) suited for the design of LNA-spiked probes.

4.2 DNA samples and sample pre-treatment

The whole blood samples used in the studies were collected for genetic studies from Finnish type 1 diabetic families (I, II), and from population-based birth cohorts in Germany and from the three cities in Finland, Turku, Oulu and Tampere, participating in the TEDDY and DIPP studies (III). Samples originally drawn for diagnostic purposes were also used as reference material in IV for the development of a new method.

DNA was extracted from EDTA (ethylenediaminetetraacetic acid) -treated blood with a salting-out method (Olerup and Zetterquist 1992). The HLA-DQB1 genotypes of the samples were determined using a time-resolved fluorometry-based DELFIA® plate hybridisation assay (Perkin Elmer Life and

Analytical Sciences Wallac, Turku, Finland) (Sjöroos *et al.* 1995; Nejentsev *et al.* 1999). The insulin gene -2221 C/T–polymorphism (Laine *et al.* 2004; Laine *et al.* 2007) and the presence of HLA-B*27 (Välimaa *et al.* 1998) were also typed using DELFIA®-based plate hybridisation methods. For the poliovirus receptor (PVR)-A/G-SNP and the CD86-gene exon 2 A/G-SNP sequencing was used as a reference method.

EDTA-treated blood was also used in testing the possibility of using FTA® Classic Card (Whatman International Ltd., Maidstone, UK) sample collection cards as sample material. In order to use dried blood spots in the homogeneous assay format, a pre-treatment strategy to liberate the DNA from the blood spots into solution was needed. An approach where circles 3 mm in diameter were cut from the FTA cards into the wells of a PCR plate, incubated in water for ten minutes at 100 °C, and cooled to 10 °C and briefly centrifuged was found successful. 1 µl volumes of this solution per reaction were then used as the source of sample DNA. A slightly modified approach, including a washing step with 50 mM sodium hydroxide before the addition of water, was needed when the blood was spotted onto 903 ProteinSaver™ cards (Whatman International Ltd). (II, III)

4.3 The assay principle

The basic principle behind all assays developed is the same, relying on asymmetric PCR and post-PCR hybridisation of lanthanide-labelled probes with either single-stranded PCR product or complementary quencher oligonucleotides. In homogeneous assays, also known as closed tube assays, no separation steps are required: the unreacted label is not removed from the reaction; instead the PCR products can be detected without opening the reaction vessel (Higuchi *et al.* 1992). The benefits of homogeneous assays have long been accepted, compared to assays requiring separate post-PCR analysis steps (Foy and Parkes 2001). The assays developed are all homogeneous with all reagents needed to complete the analysis included in the PCR mix. After preparing the reactions and adding the samples, only thermal cycling and signal measurement are required.

The method developed requires the amplification of the PCR to be asymmetric, using an excess of one primer, in order to create single-stranded PCR product during thermal cycling (Gyllensten and Erlich 1988). This single-stranded PCR

product is then available for the probes to hybridise to after the thermal cycling is completed.

The probes used in these assays are short, and do not hybridise in the high temperature used during PCR amplification. Instead, once the amplification is finished and the temperature of the reactions is slowly lowered to room temperature, the probes hybridise to their complementary sequences. The principle behind the competitive hybridisation (Morrison *et al.* 1989; Li *et al.* 2002; Shengqi *et al.* 2002) of the probes and PCR products is described in Figure 10. Should the single-stranded PCR product contain a complementary sequence to the lanthanide-labelled probe, the probe can hybridise to this product instead of its complementary quencher oligonucleotide, and the fluorescence of the probe is not quenched. However, if the PCR product does not contain a complementary sequence, with as little as a single base difference in sequence, or if there is no PCR product due to failed amplification, the lanthanide probe binds to the complementary quencher and the fluorescence of the probe is quenched.

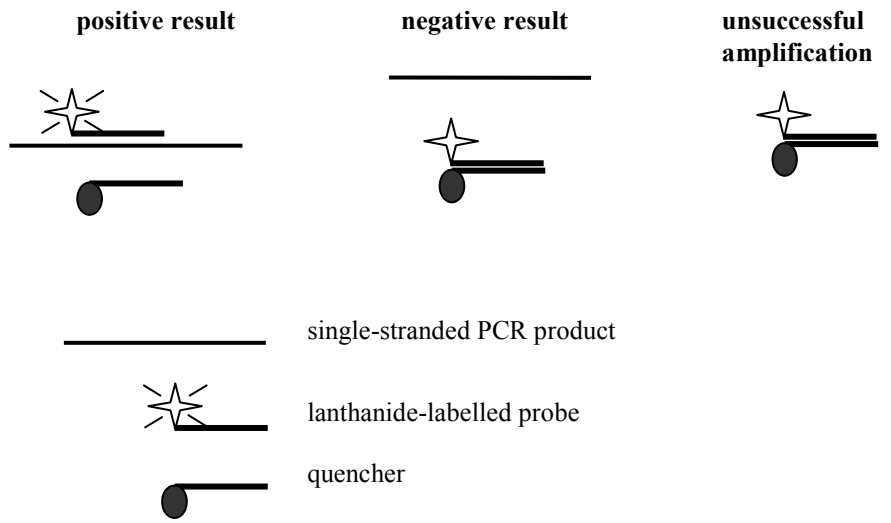

Figure 10. The possible hybridisations of the probes and quenchers. If the PCR product contains a complementary sequence the probe hybridises to it resulting in a high fluorescence signal. If the PCR product contains even a single nucleotide mismatch or there is no PCR product present due to failed amplification, the probe binds to its complementary quencher oligonucleotide and the signal from the probe is quenched.

A Victor 1420 Multilabel Counter (PerkinElmer, Wallac, Turku, Finland), with the default parameters of the instrument, was used for the measurement of

signals in all the assays developed. The signal-to-background ratios were calculated for each of the probes by dividing the mean of the measured signal from the sample by the mean of the appropriate background, this being either a no-template control reaction or a control reaction negative for the assayed parameter.

4.4 The SNP assays

The details of the reaction conditions of the SNP assays for the insulin gene -2221 C/T, the PVR-A/G and the CD86 gene exon 2 A/G polymorphisms are given in publication I. In all three assays one primer pair and two pairs of probes were used to detect the two possible alleles of each SNP according to the principle outlined above. The samples were divided into three genotype groups according to the signal-to-background ratios of both probes, since for each SNP only three genotypes were possible.

4.5 HLA-DQB1 screening

The HLA-DQB1-screening assay differed from the SNP assay in that genotyping single SNPs was not possible due to the complex nature of the typed HLA-DQB1 gene. Instead, probes that recognise short stretches of sequence, appearing in only one or a few alleles, were used. The genotypes of the samples were then deduced from the patterns produced by positive signals.

The typing scheme for the HLA-DQB1 gene was based on a heterogeneous DELFIA® assay previously developed in our laboratory (Sjöroos *et al.* 1995) and the details of the assay are described in publication II. Five probe-quencher pairs were used to recognise the DQB1 alleles *02, *0301, *0302, *0602/3 (II) and *0603/4 (unpublished). A probe hybridising to a consensus sequence found in all DQB1 alleles was also included in the assay, so that the possible failures in the amplification could be detected.

Due to the specific requirements imposed by the TEDDY study to the screening of the HLA-DQB1 conferred genetic risk, the probes used in the homogenous assay were further adjusted in publication III. The changes were made to provide a simple and fast primary screening step to be used for the

typing of thousands of samples and also, with the eligibility criteria in mind, to minimise the number of samples requiring more detailed genotyping.

The repertoire of probes currently available for typing the HLA-DQB1 alleles with the homogeneous assay developed has expanded since, and is shown in Table 4. (II, III, unpublished)

Table 4. Probes available for typing the HLA-DQB1 alleles in the homogeneous assay format. The LNA-bases are marked with underlined, bold letters. The lanthanide label, europium (Eu) or terbium (Tb) is always placed in the 5'-end of the probe and the dabcyl moiety in the 3'-end of the quencher oligonucleotide. The concentrations of the probe and the complementary quenchers in the reactions are shown. (II, III, unpublished)

target allele(s)	probe sequence 5'→3'	lanthanide label	concentration (nM)	quencher sequence 5'→3'	quencher concentration (nM)
***02**	A**A**G AG**A** **T**CG TG	Tb	0,33	CGC ACG ATC TCT	2,66
***0302**	GC**C** GCC TG**C** **C**G	Eu	6,6	GGC AGG CGG	33,2
***0301**	TGG **A**GG TGT A**C**	Eu	6,6	CGG TAC ACC TCC	33,2
***0301/3**	G**CC** GCC TG**A** **C**G	Tb	3,3	CGT CAG GCG G	16,6
***04**	A**AC** GGG **A**C**C** GAG **C**	Eu	5,0	GCT CGG TCC CGT T	39,9
***04/5**	TGC G**GG** **G**TG T**G**A C	Tb	1,66	GTC ACA CCC CGC A	5,0
***05/6**	GG**G** C**G**G C**C**T	Tb	3,3	AGG CCG CCC	6,6
***0501**	A**C**CG**G**GC**AG**TGA	Eu	13,8	TCACTGCCCGGT	13,8
***0602/3**	TA**C** CG**C** GCG	Tb	3,3	CGC GCG GTA	6,6
***0603/4**	**T**TG T**A**A C**C**A GA**C** AC	Eu	16,6	GTG TCT GGT TAC A	33,2
all (control)	CG**C** TTC **G**AC **A**G	Eu	6,6	CTG TCG AAG CG	66,4

4.6 HLA-B*27 screening with the dry reagent approach

In HLA-B*27 screening the specificity of the assay relies on both allele-specific primers and an allele-specific probe. The details of the assay are described in publication IV. In short, the B*27-specific primers are used to amplify only the B*27 alleles, if the specific alleles are present in the sample, and the terbium-labelled probe is used to recognise the PCR product. Since only the B*27 alleles are amplified with these primers, a second pair of primers amplifying a section of the β-actin gene is used to control the success of the amplification, and this PCR product is detected by a europium-labelled probe.

The feasibility of a dry reagent approach was studied by drying all the reagents needed in the PCR to a well on a PCR plate. The drying of the enzyme was tested together with the other reagents in addition to drying it separately from other reagents. The stability of the reagents at room temperature sealed into

storage packages, and protected from light was monitored over the period of eighteen weeks.

5 Summary of results and discussion

5.1 The SNP assays

The functionality of the approach developed was first demonstrated in three basic SNP assays (I). The hybridisation patterns of the europium- and terbium-labelled probes are described in the upper part of Figure 11 with respect to the results of the SNP assays, and the lower part of Figure 11 shows genotyping results produced by the insulin gene -2221 C/T and the PVR-A/G assays. The three differing genotypes of all three SNP assays, the insulin gene -2221 C/T polymorphism, the PVR-A/G-polymorphism and the CD86 gene exon 2 A/G-polymorphism, were clearly distinguishable, providing support for the functionality of the chosen approach. Moreover, of the hundreds of samples typed, only a very small number required reanalysis, varying from 0.3 to 5 percent of samples.

The chosen probe format of two complementary oligonucleotides both with a single label moiety, one with a lanthanide label and the other with a dabcyl-quencher molecule, provides an affordable approach when compared to double-labelled probes which are usually significantly more expensive than single-labelled oligonucleotides (Nurmi *et al.* 2001). Further, the design of the new probes is relatively simple since no complicated secondary structures are required for the functionality of the probes, such as for Molecular Beacons (Tyagi and Kramer 1996). The efficiency of the quenching of the signal by the quencher-oligonucleotide, in the approach where the label moiety and the quencher molecule are on opposite, complementary strands has already been demonstrated to be comparable to the characteristics of TaqMan probes and Molecular Beacons (Nurmi *et al.* 2002). The quenching efficiency was shown, also in this instance, to be quite high, with 97 % and 88 % of the signal quenched, when tested with the insulin-C-europium and insulin-T-terbium probes, respectively.

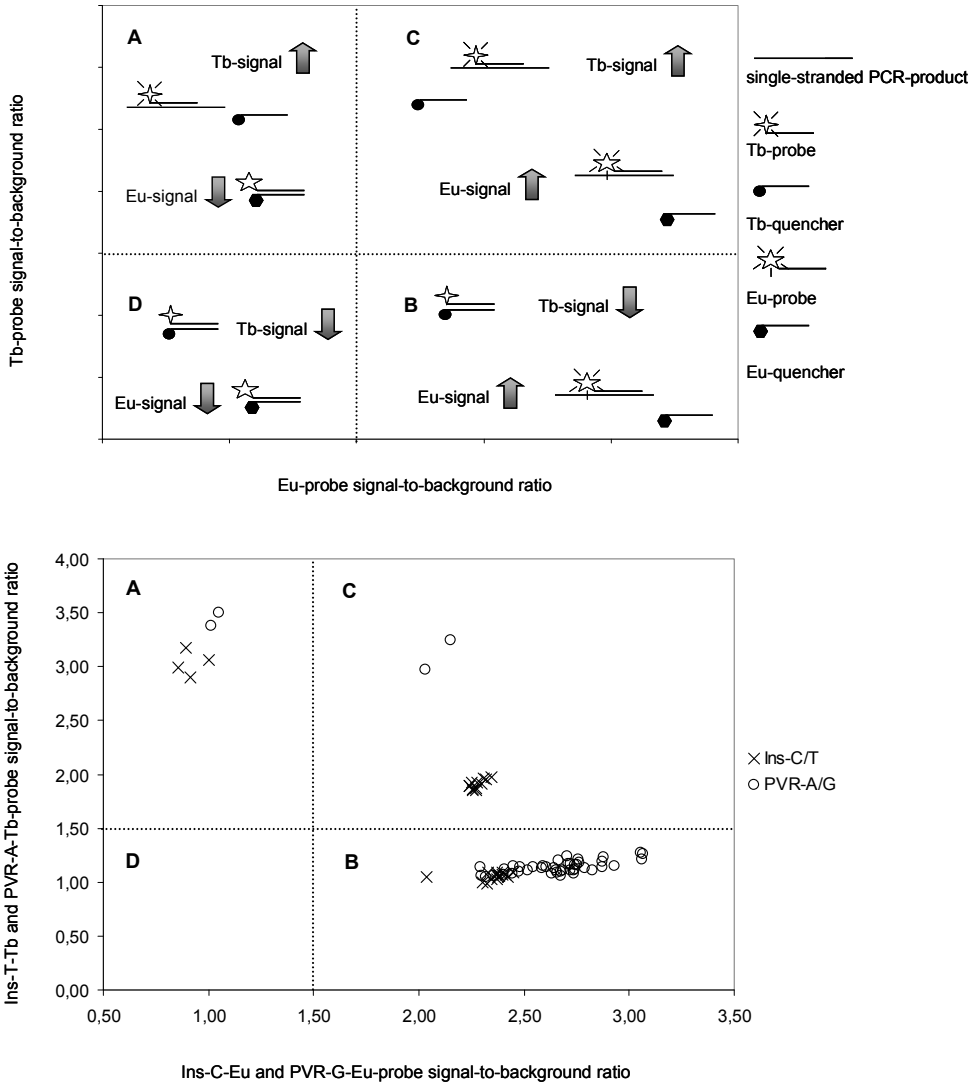

Figure 11. The hybridisation of the probes and the results of the insulin gene -2221 C/T and PVR-A/G assays. As shown in the upper part, depending on the sample genotype, in groups A and B only one of the probes produces a positive result indicating a homozygous sample, whereas in group C both probes are positive. If any of the samples failed to amplify, they would be shown in group D. In the lower part of the figure the insulin gene -2221 –results are marked with crosses and the PVR-results with open circles. The results showed the clear division of results into three distinct groups; A, B and C; based on their genotypes with no samples failing to be amplified (group D). (I)

The discrimination potential of short probes (Lipsky *et al.* 2001) was utilised by performing the hybridisation close to room temperature as opposed to the high temperatures used in TaqMan assays (Holland *et al.* 1991; Livak 1999). Even a G-T mismatch with minimal destabilising effect and cross-reactivity, that has been shown to complicate SNP typings (Ikuta *et al.* 1987; Luo *et al.* 1996; Allawi and SantaLucia 1997; Bernard *et al.* 1998; Smit *et al.* 2001), could successfully be analysed in the insulin gene assay.

An advantage in preventing the cross-reactivity is provided by the possibility of including both probes in the same reaction, enabled by the use of two lanthanide labels, europium and terbium, since only one probe can hybridise to one PCR product. Thus the probe with the completely complementary sequence has the advantage over the one with a single mismatch when competing for the same complementary strand (Livak 1999).

5.2 HLA-DQB1 assay with LNA-spiked probes

The second challenge was to apply the assay principle developed to a more complicated analyte, namely the HLA-DQB1 gene with its complex polymorphisms. The alleles are not defined by single SNPs, but rather a series of SNPs creating, in a sense, a haplotype that is called an allele. Although some alleles can be identified by probes covering a single SNP, or more typically two to three SNPs, there are alleles that can be distinguished only by a pattern produced by several probes covering multiple SNPs.

Due to the complex nature of the DQB1-gene, the approach with competing probes for a single SNP could not be used. The probes used in the initial attempts to analyse the DQB1 alleles did not have any base analogs, but contained only DNA, similar to those used in the SNP assays (II, unpublished results). However, the signal-to-background ratios of these probes did not provide acceptable results, and thus those probes with only the quencher oligonucleotide to compete with the hybridisation to the PCR product did not provide a setting strict enough to attain adequate specificity.

LNA additions were then made to the lanthanide-labelled probes to improve the functionality of the probes (II). LNAs are DNA analogs which have been shown to have strong affinity and high discriminatory power (Koshkin *et al.* 1998; Braasch and Corey 2001; Petersen and Wengel 2003; Ugozzoli *et al.*

2004). The structure of the LNA is conformationally restricted with an extra methylene bridge in the ribose ring. This structure enables a very efficient binding to complementary nucleic acids (Latorra *et al.* 2003; Vester and Wengel 2004; Yang *et al.* 2007). LNAs have been used to improve the discriminatory power of different assays. For example, LNAs have been added to probes that fail to reliably differentiate genotypes, and also to primers to enhance their specificity and strength of binding to targets (Jacobsen *et al.* 2002; Simeonov and Nikiforov 2002; Latorra *et al.* 2003; Letertre *et al.* 2003; Arjomand-Nahad *et al.* 2004; Kennedy *et al.* 2006). As shown by the comparisons of LNA-spiked probes with traditional only-DNA containing probes (II) this superior discrimination enabled a practical DQB1-assay set up.

A set-up mimicking that of an existing DELFIA® HLA-DQB1-assay designed for the analysis of T1D risk alleles was first developed with six probes identifying the DQB1*02, *0301, *0302, *0602, *0603 and *0604 alleles (II, unpublished) (Figure 12). A control probe with a consensus sequence found in all DQB1 alleles was included to ensure the successful amplification of the samples. A corresponding chart for the additional probes, *0301/3, *04, *04/5, *0501 and *05/6, designed later for a more detailed typing of the DQB1 alleles, is shown in Figure 13 (unpublished). The positive average signal-to-background ratios for each probe were calculated from all samples positive for the typed allele. (For example, the *02 probe has an average signal-to-background ratio of 3.09 for samples positive for *02 allele.) The signal-to-background ratios for those alleles not recognised by the probe were calculated from all those samples which were positive for the allele in question, but not the allele the probe hybridises to. (For example, the *02 probe has an average signal-to-background ratio of 0.86 for those *0301 positive samples which are not positive for the *02 allele.) Average signal-to-background ratios of these probes ranged from 1.7 to 4.5 with positive samples and remained below 1.2 for negative samples providing unambiguous typing results.

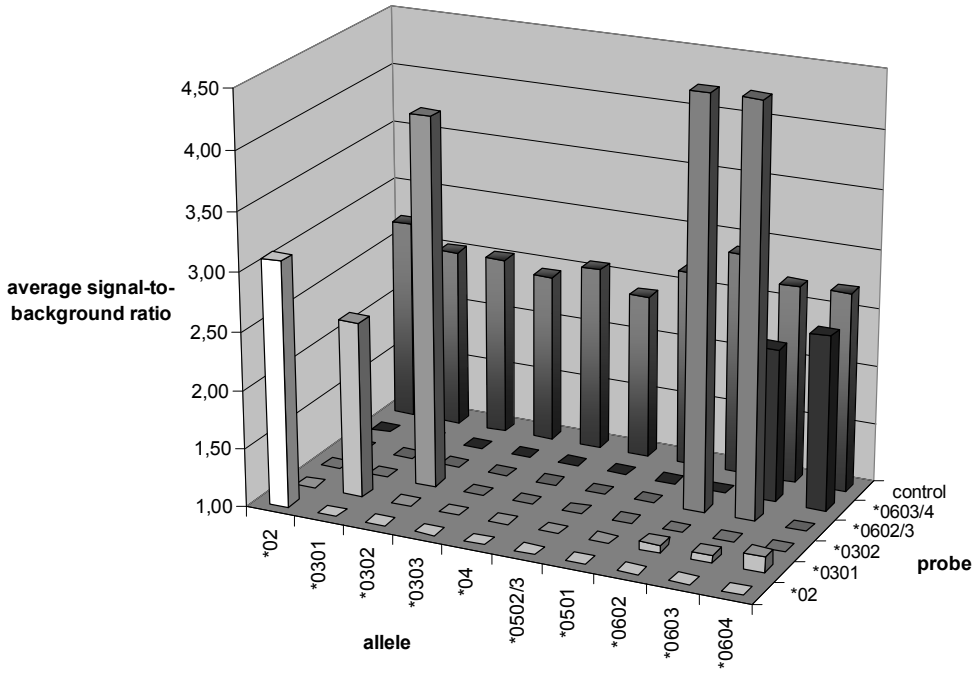

Figure 12. The average signal-to-background ratios of the HLA-DQB1 probes used for typing of the alleles important for assessing the relative genetic risk for T1D. The probes used are marked on the right and the average signal-to-background ratios of samples containing a specified allele on the left. Alleles marked with *0502/3 denote both *0502 and *0503 alleles and were not differentiated by the assay.

The bases in the probes substituted with LNA were chosen based on the differences in sequence between the allele or alleles recognised by the probe in question and all other possible sequences. LNA was usually added to the most critical bases differing between the targeted sequence and the majority of the other alleles, in order to provide the greatest possible difference between melting temperatures. However, too many LNA additions might have raised the melting temperature (T_m) of the probe too high, and destroyed the specificity achieved by the use of short probes. Therefore, the number of LNA-bases per probe was limited to between two and five, depending on the probe sequence and its GC-content (II, unpublished results).

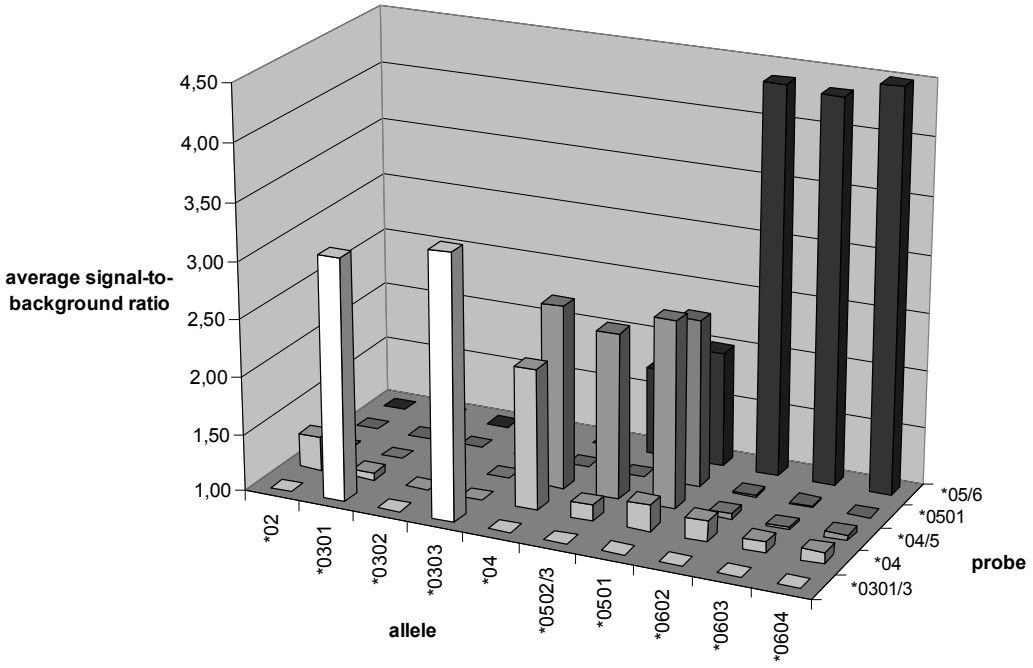

Figure 13. The average signal-to-background ratios of the HLA-DQB1 probes used for more detailed typing of DQB1 alleles. The *0301/3-probe hybridises to both *0301- and *0303-alleles; similarly the *04/5-probe recognises all *04- and *05-alleles and the *05/6-probe all *05- and *06-alleles.

The results of an assay comprising all the possible probes available in the homogeneous assay format developed for the typing of HLA-DQB1 alleles are shown in Table 5. The signal-to-background ratios from each probe and the genotype of the sample that can be deduced from these results are shown. Some of the sample genotypes can not be resolved completely due to possible homozygosity that is not revealed by the assay (only one allele as a result). Furthermore, due to the multiple alleles recognised by some probes, other possible alleles that might be present, but remain concealed, are in parenthesis. For example a *0301(0303) result might be a *0301 homozygote or the genotype of the sample could be *0301/0303. Moreover, the *0602/3/4 result could consist of a sample with a combination of *0602, *0603 and *0604 alleles or it could be the *0603 allele together with another, infrequent *06 allele.

Table 5. The results of a homogeneous assay for typing HLA-DQB1 alleles. The positive signal-to-background ratios are marked in bold. The resulting DQB1-genotype of the sample that can be deduced from the results is shown. For some of the samples a detailed genotype could not be identified and possible second alleles are set in parenthesis. The cut-off values set for each probe are also shown together with the average signal-to-background ratios for both positive and negative samples. (unpublished)

DQB1-result		probe										
allele 1	allele 2	*02	*0302	*0301	*0301/3	*04	*04/5	*05/6	*0501	*0602/3	*0603/4	control
*02	*0301	**2,54**	0,59	**2,59**	**3,00**	1,27	1,00	1,05	1,00	0,81	0,68	**3,02**
*0302	*0602	0,90	**2,20**	1,00	0,81	0,91	0,96	**2,92**	0,86	**6,04**	0,59	**2,74**
*0302	*0303	0,93	**2,04**	0,71	**2,85**	0,80	0,92	1,05	0,95	0,61	0,64	**2,56**
*0301	*0501	0,95	0,46	**2,32**	**3,29**	0,96	**2,61**	**1,98**	**2,26**	0,68	0,90	**2,57**
*04	*0602	0,92	0,45	1,07	0,72	**2,25**	**2,65**	**3,82**	1,13	**6,38**	0,65	**3,08**
*02	*0602	**2,49**	0,54	1,04	0,70	1,36	1,12	**3,67**	0,87	**6,10**	0,65	**3,10**
*04	*0602	1,06	0,53	1,12	0,74	**2,30**	**2,65**	**3,65**	1,04	**6,39**	0,77	**3,02**
*02	*0501	**2,41**	0,54	0,95	0,72	1,46	**2,76**	**2,02**	**2,45**	0,68	0,88	**2,95**
*04	*0501	0,98	0,48	0,93	0,69	**2,58**	**3,08**	**2,02**	**2,75**	0,82	0,93	**2,66**
*0302		0,94	**3,68**	0,71	1,03	1,11	1,15	1,03	0,96	0,64	0,71	**2,85**
*02	*0602	**2,46**	0,62	1,14	0,72	1,50	1,06	**3,91**	1,03	**6,46**	0,70	**3,40**
*0501	*0604	0,97	0,47	1,30	0,72	1,07	**2,41**	**5,67**	**2,38**	0,83	**2,75**	**2,63**
*0301	*0302	0,90	**2,39**	**2,28**	**2,90**	0,90	1,01	1,06	0,88	0,61	0,61	**2,45**
*02	*0501	**2,38**	0,53	0,91	0,70	1,28	**2,35**	**2,01**	**2,47**	0,62	0,96	**2,78**
*04	*0501	0,97	0,47	0,94	0,71	**2,10**	**2,57**	**1,99**	**2,32**	0,69	0,90	**2,58**
*0303	*05	0,89	0,47	0,76	**3,09**	1,06	**2,40**	**2,05**	0,86	0,63	0,86	**2,58**
*02	*05	**2,37**	0,51	0,80	0,76	1,18	**2,38**	**1,35**	1,02	0,60	0,78	**2,55**
*02	*0301	**2,26**	0,54	**2,20**	**2,45**	1,20	0,91	0,92	0,99	0,69	0,79	**2,59**
*0301	*04	0,88	0,45	**2,54**	**3,10**	**2,31**	**2,64**	0,94	1,07	0,59	0,68	**2,40**
*04	*0603	0,83	0,41	0,91	0,67	**2,07**	**2,54**	**3,25**	1,06	**5,35**	**2,05**	**2,49**
*02	*0301	**2,27**	0,54	**2,41**	**2,81**	1,24	0,97	0,93	0,88	0,70	0,62	**2,51**
*0301	*0603	0,87	0,45	**2,38**	**2,87**	1,04	1,06	**3,65**	1,00	**5,92**	**2,35**	**2,67**
*0301	(*0303)	0,96	0,51	**2,96**	**3,75**	0,90	0,82	1,15	0,98	0,61	0,68	**2,54**
*04	*0501	0,93	0,48	1,01	0,77	**2,24**	**2,65**	**2,12**	**2,59**	0,87	1,01	**2,53**
*0602/3/4		0,91	0,48	1,35	0,67	1,12	1,03	**6,10**	0,93	**6,97**	**2,87**	**2,88**
*0301	(*0303)	0,83	0,46	**2,75**	**3,51**	0,85	0,83	1,02	0,93	0,59	0,61	**2,50**
*0602		0,83	0,41	1,10	0,65	0,95	0,97	**4,89**	0,76	**6,31**	0,58	**2,51**
*0303		0,86	0,47	0,75	**3,54**	0,95	1,00	1,03	0,69	0,70	0,62	**2,46**
*0303	*05	0,89	0,44	0,70	**2,84**	0,99	**2,35**	**2,06**	0,83	0,59	0,87	**2,30**
*02		**2,51**	0,53	0,70	0,70	1,41	0,98	0,99	0,88	0,61	0,69	**2,52**
*02	*0603	**2,16**	0,49	0,98	0,65	1,22	0,97	**3,18**	0,86	**5,73**	**2,12**	**2,88**
*0602/3/4		0,88	0,44	1,15	0,63	1,08	1,08	**5,42**	1,26	**5,43**	**2,21**	**2,70**
*0301	*04	0,91	0,46	**2,49**	**2,96**	**2,33**	**2,64**	1,00	0,98	0,62	0,62	**2,43**
*0602/3/4		0,85	0,40	1,29	0,68	1,12	1,04	**5,08**	0,99	**6,46**	**2,15**	**2,62**
*02	*0301	**2,43**	0,57	**2,61**	**3,12**	1,31	1,16	1,01	1,00	0,78	0,70	**2,64**
*0301	(*0303)	0,83	0,43	**2,20**	**3,56**	0,83	0,89	1,09	0,83	0,57	0,56	**2,15**
*0301	*04	0,85	0,44	**2,68**	**3,23**	**2,20**	**2,49**	0,99	0,92	0,61	0,67	**2,43**
*02	*0301	**2,34**	0,55	**2,53**	**2,97**	1,20	0,85	0,96	0,90	0,66	0,66	**2,49**
*02	*04	**2,23**	0,49	0,66	0,65	**2,10**	**2,38**	0,96	0,96	0,57	0,62	**2,47**
*0302	*05	0,88	**2,42**	0,96	0,83	1,05	**2,43**	**1,92**	0,81	0,58	0,84	**2,31**
*02	*04	**2,17**	0,49	0,65	0,66	**2,19**	**2,47**	0,90	0,99	0,63	0,59	**2,50**
*0302	*04	0,79	**2,37**	0,70	0,93	**2,03**	**2,39**	0,87	0,68	0,55	0,60	**2,34**
*0302	*0603	0,81	**2,69**	1,04	0,81	1,00	0,90	**3,76**	0,98	**5,53**	**2,38**	**2,63**
*02	*05	**2,23**	0,49	0,68	0,68	1,37	**2,47**	**1,78**	1,01	0,71	0,90	**2,35**
*0302	*05	0,87	**2,63**	0,88	0,85	1,16	**2,60**	**1,43**	0,87	0,65	0,91	**2,34**
cut-off S/B for positive		1,50	1,50	1,50	1,50	*1,70*	1,50	*1,30*	1,50	1,50	1,50	1,50
average positive S/B		2,35	2,55	2,50	3,10	2,23	2,54	3,14	2,46	6,08	2,36	2,62
standard deviation positive		0,13	0,50	0,22	0,33	0,15	0,17	1,40	0,17	0,48	0,30	0,25
average negative S/N		0,90	0,49	0,93	0,74	1,12	0,99	1,00	0,94	0,66	0,73	
standard deviation negative		0,06	0,05	0,20	0,09	0,19	0,10	0,07	0,11	0,08	0,13	

The general levels of the signal-to-background ratios for positive samples are above 2.0 and the cut-off values were set for most probes at 1.50. The exceptions were the *04-probe, for which the cut-off was set at 1.70, and the *05/6-probe with a cut-off value of 1.30. The *05/6-probe seems to, on many occasions, generate lower signal-to-background ratios when an *05 allele is present, as is shown in Figure 13. Since the sequences of *05 and *06 alleles are identical at the probe binding site, the difference might be caused by the lower efficiency of amplification shown by the *05 alleles already in the DELFIA® based DQB1-assay (unpublished results). Further, the secondary structure of the PCR product at the probe binding site may cause the difference between the alleles, since the *05 alleles most likely have a stronger helix structure than the *06 alleles at the probe binding site (mfold web server, http://www.bioinfo.rpi.edu/applications/mfold, (Zuker 2003)) hindering the *05/6-probe from displacing the base pairing of the helix and binding to the PCR product.

The cut-off value for the *04-probe was set at 1.70, slightly higher than for the majority of the probes, to ensure correct genotyping. The cross-reactivity shown by this probe seems to be greatest for those samples positive for the DQB1*02 allele. A possible explanation for this might be the presence of a G-T-mismatch between the *04-probe and the PCR product of a DQB1*02 allele. This base pair in other non-*04 alleles is a G-C mismatch which is known to be more destabilizing than the G-T mismatch (Ikuta *et al.* 1987; Allawi and SantaLucia 1997). Since the *04/5-probe must also give a positive result for *04 positive samples, the information provided by this probe can be used to ascertain the *04 result.

When the full DQB1 genotype of a sample has to be attained, it is valuable to be able to deduce whether a sample giving a positive result for only one allele is homozygous, or if there is a second allele not typed by the assay. This, however, is not possible with the assay developed, since it has been designed to be only qualitative. No unambiguous difference in the signal-to-background ratios has been observed between homozygous and heterozygous samples even when the differences in amplification efficiency have been taken into account. If the homoxygosity of a sample has to be confirmed, sequencing is required.

Based on the application and allele information required, different combinations of the available probes can be chosen for an assay. For example, the typing of the genetic component for celiac disease would require the investigation of the DQB1*02 and *0302 alleles (Mäki *et al.* 2003; Reinton *et*

al. 2006; Ollikka *et al.* 2009), but does not benefit from the information provided by the other probes rendering them useless in this analysis. As demonstrated by the chosen approach for the primary screening step for the TEDDY study, the numbers of samples requiring further, more detailed genotyping can easily be reduced by the proper selection of the alleles typed. The most economical scheme can vary from the detection of only one or a few alleles in the primary step to the typing of as many alleles as possible at once, depending on the required genotype information.

Due to several simultaneous screening programs requiring slightly different DQB1 information, the primary screening step has been further adjusted from the one published for the TEDDY screening strategy. The availability of the array of DQB1 probes, presented in Table 4, has enabled the number of probes utilised in the primary screening step to be increased. This allows the detection of the most important DQB1 alleles influencing disease risk assessment in the current screening programs. This approach has further diminished the necessity of secondary screening steps using DELFIA® technology than was used in the original TEDDY screening strategy (unpublished).

The use of whole blood dried on sample collection paper as sample material was successfully adapted to the assay method. Since the direct use of paper discs in the reaction was not possible (unpublished), a simple method for liberating the DNA from the discs was developed that was also suited for large-scale sample processing (II, III). The collection paper is a very convenient method for the handling and shipping of blood samples: only a small volume of blood is needed, the samples can be stored at room temperature, and possible spills of potentially biologically hazardous material are avoided. The minimal space these samples take up is also a very important aspect when large numbers of samples are to be shipped or stored. An added benefit of this sample pre-treatment method is the reduced consumption of the original sample material, an important fact when the sample is scarce and multiple genetic assays are to be performed from a single sample.

5.3 HLA-B*27 screening with a dry reagent approach

In the interest of simplifying the method developed even further, a dry chemistry application aimed at the screening of the HLA-B*27 allele was tested and compared to the DELFIA® method used in routine screening

(Välimaa *et al.* 1998). The principle of the probe chemistry remained the same, but the specificity of the B*27 assay relied heavily on allele specific primers in contrast to the SNP assays presented and the HLA-DQB1 screening where the probes are solely responsible for the genotyping. Further, a second pair of primers producing a second amplicon, namely a portion of the β-actin gene, was also required to control the success of amplification: the primers used to amplify the B*27 alleles did not amplify the other HLA-B alleles. Thus, any negative B*27 result required an assurance that it was a true negative result and was not due to failure in amplification.

The dry reagents were shown to be stable at room temperature (Figure 14) at least up to eighteen weeks, and since no clear decline in the signals was shown during this period, it could be speculated that even longer storage would be possible. Storing the reagents at lower temperatures would most likely result in an even longer shelf-life (Glynou *et al.* 2007; Hagren *et al.* 2008). The reagents were successfully air-dried in sealed containers with desiccant without the use of any sophisticated instrumentation, a simple and inexpensive approach. The separation of the enzyme from the other reagents was shown to be necessary, and was accomplished by drying it into a separate well. If a method providing the separation of the enzyme from the other reagents within the reaction vessel was available, all the reagents could be placed directly into the PCR well, and the performance of the assay would consist only of the adding of the sample and optionally water.

The performance time of the homogeneous assay was compared to a corresponding heterogeneous assay based on the DELFIA® technique. The homogeneous method, using dried reagents, required hours less hands-on time when compared to the DELFIA® assay and it could be performed within three to four hours depending on the speed of the thermal cycler. The DELFIA® assay, with its multiple steps, would require at least three hours longer. In addition, the savings achieved with the homogeneous approach in reagent and material costs due to the omitted, separate hybridisation step, were significant.

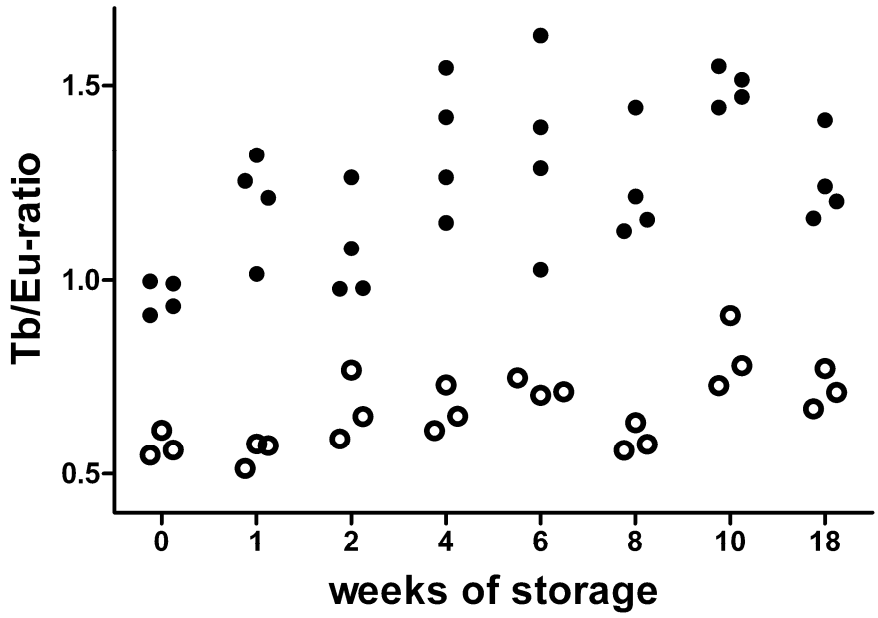

Figure 14. The stability of the dried reagents for the typing of HLA-B*27. The B*27 positive samples are marked with filled circles and the negative samples with open circles. The ratio of the terbium-labelled B*27-probe and the Eu-labelled control probe is shown on the y-axis and the time of storage on the x-axis. Four HLA-B*27 positive and three negative samples were included in each time-point tested. (IV, unpublished)

6 Conclusions

In this study, the principle of a genotyping method based on competing hybridisation (Morrison *et al.* 1989; Li *et al.* 2002; Shengqi *et al.* 2002) was described and demonstrated to be applicable to both SNP assays as well as more complicated sequence analysis. LNA-base substitutions at critical bases in the oligonucletide probes were used to enhance the specificity of the probes in the assays aimed at HLA polymorphisms. The possibility of a dry reagent approach was explored with an assay developed for the detection of the HLA-B*27 allele.

The main conclusions based on the original publications are as follows:

I The principle of the homogeneous assay based on asymmetric PCR and competitive hybridisation was shown to be applicable to three different SNP assays from three different genes investigated for possible associations to the susceptibility to type I diabetes. With the simple probe structure of a single label per oligonucleotide, the costs of the probes per assay are minimal. The expenses of the assay are mostly due to other reagents and consumables needed in the assay, all of which are commonly quite inexpensive and generally used, such as the DNA-polymerase, dNTPs and PCR plates and sealers. Combined with the use of only two instruments, a thermal cycler and a time-resolved fluorescence counter, this method is an easily adaptable and cost-effective approach to genotyping SNPs.

II The assay principle developed was successfully applied to a more complicated analyte, the HLA-DQB1-gene, where due to the unique character of the DQB1-gene sequence, the competition between two probes aimed at a single SNP used in the three previous assays could not be exploited. Instead the required specificity of the probes had to be attained with the help of LNA-base substitutions at critical bases to the lanthanide-labelled probes. The functionality and specificity of the LNA-spiked probes were shown to be superior to their DNA-only counterparts. Further, the possibility of using whole blood dried on sample collection cards instead of extracted DNA was explored, and a method of liberating the DNA from the card into solution was developed and applied.

III The HLA-DQB1-assay was further developed into a form that could be used as a primary screening step for a study comprising the genotyping of

thousands of samples for the estimation of genetic risk for type 1 diabetes. An efficient screening system enables the exclusion of ineligible individuals as early in the screening system as possible to avoid unnecessary costs by multiple screening rounds. The addition of a probe recognizing the DQB1*05 and *06 alleles in the primary screening was proven to be an effective approach with only 16.5 % of the general population samples requiring further genotyping. With little hands-on time and with only 4 hours total time needed to perform the assay, the homogeneous assay format is a suitable and cost-effective way of initiating the screening.

IV A further simplification of the method was introduced to the assay for the presence of the HLA-B*27 allele as a dry-reagent concept, where all the reagents required to perform the assay, excluding the sample, are dried into the reaction vessel. When the actual assay is performed, only water and the sample need to be added to the vessels. The reagents were shown to be stable at room temperature for at least 18 weeks, enabling easy shipping and storage of reagents. In lower temperatures the reagents could, in all probability, be stored for even longer periods. The assay developed also compared favourably with the original DELFIA®-based assay when assay performance and costs were considered.

The assay method developed has been shown be adaptable to both SNP analysis as well as more complicated sequence analysis, and it can be speculated that other types of polymorphisms, such as deletions and insertions, might also be possible targets for successful analysis. The main drawback of the assay is the current availability of only two labels, europium and terbium, which are suitable for this approach, and the limitations of multiplexing due to this fact. The use of prompt fluorophores in addition to the two time-resolved labels might offer an opportunity to increase the number of analytes detected from a single reaction. However, the unique characteristics of the lanthanide labels render them especially suitable for the analysis of crude sample material, such as whole blood dried on sample collection cards, as the impurities of this type of samples generate difficulties in the use of many other conventional fluorescent labels (Soini and Lövgren 1987; Iitiä *et al.* 1992; Nurmi *et al.* 2000a; Nurmi *et al.* 2000b). If purified DNA is to be used as a sample or if a convenient method for extracting DNA from crude samples or purifying it to an acceptable degree were available, these labels could be combined for the simultaneous detection of several analytes (Nurmi *et al.* 2002). Another possibility enabling the use of whole blood dried on sample collection cards might be the use of several different assay chemistries based on the use of

time-resolved fluorometry and lanthanide labels allowing the detection of several analytes from a single well (Laitala *et al.* 2007; Ollikka *et al.* 2009).

As our knowledge of the genetic components behind the development of T1D increases, a method for the typing of all these polymorphisms for the accurate estimation of the genetic risk becomes essential. However, as long as the other environmental factors remain elusive and a reliable prevention method or a cure is not available, the main use of these screening systems will be in studies attempting to further illuminate disease progress and possible intervention mechanisms.

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