1 Introduction

1.1 General

Every year several thousand people all over the world are in need of a tissue transplant. There are diseases, which affect vital parts of the body (heart, kidney, liver, lung), with the effect that the capacities of these organs are not sufficient for the minimal requirements. In these cases the patients will die. In certain instances, organ or tissue transplantations are the last hope to save these patients. Allogeneic stem cell transplantation has become an effective treatment for a number of haematological diseases, such as malignant diseases (e.g. acute and chronic leukaemia, myelodysplasia), aplastic anaemia, immune-deficiencies and inherited metabolic disorders. In recent years stem cells collected from blood and especially umbilical cord blood cells have been used. In 75 % of cases the source of grafted stem cells is bone marrow. These organ and tissue transplantations are accompanied by a few complications. The most known and most serious one is graft-versus-host diseases (GVHD). In some rare circumstances even host-versus-graft diseases (HVGD) has been noticed. These are initiated by an immune response, which is based on the ability of the organism to distinguish between host and graft tissue.

The recognition is based on the presentation of proteins and peptides by cell-surface molecules. These molecules are called Human Leukocyte Antigens (HLA). The HLA molecules are encoded in the human Major Histocompatibility Complex (MHC). The MHC is located on the short arm of the chromosome 6 (6p21.3), has extremely high gene density and is very highly polymorphic. The outcome of this is a huge number of gene variations; so 451 different HLA-A, 782 HLA-B and 438 HLA-DRB1 alleles are known (status of the 24.04.2006)[1], just to allude to the transplantation relevant genes.[2] In addition, with all the other genes (HLA-C, HLA-DRB2-9, HLA-DQ etc.) more then 9 billion combinations of HLA antigens, so called HLA phenotypes, are theoretically possible. On this account it is very rare for two individuals to have an identical set of HLA antigens.

HLA typing is used to identify the HLA alleles in a given individual. It helps to find the most qualified donor for a given patient. The closer the match between

[1] HLA/HTML database (www.ebi.ac.uk/imgt/hla/)

[2] Erlich, H.A.; 2001, Immunity, 14: 347 - 356

the donor and the recipient in the HLA alleles, the better the chance of survival of the transplant and consequently of the patient[3,4,5]. Therefore, family members of the patients are a favoured option to be donors for tissue and organ transplants. Identical twins have fully matching HLA phenotypes. Between siblings the likelihood to have a fully matched situation is at least 25%. The likelihood increases up to 95% with 10 siblings. Nevertheless, since it is quite uncommon to have more than two children in the modern Western Europe society[6], the likelihood to have a suitable donor within the family of the patient is quite low. In many cases a non-related donor is required.

To simplify the search for a suitable non-related donor several donor registries have been established. Today, over 8 million volunteer donors for bone marrow donations have been HLA typed and registered worldwide. This number of potential donors is increasing by hundreds of thousand every year. To register each potential donor into the registries it is necessary to carry out an HLA typing. With the current methods and technologies of HLA typing this entails high cost and effort.

Another phenomenon is the frequent occurrence of specific HLA phenotypes in different ethic groups. In 1990 one out of two donors carried a "new" (not yet registered) HLA phenotype, while nowadays one out of six carries a "new" HLA phenotype. This is partly due to the fact that donors tend to come preferentially from the same population, where the same HLA combinations are the most frequent. As a result, the costs for development and support of the registries are increasing much faster than the number of unknown and "new" HLA phenotypes in the registries.

To find a new strategy to reduce the costs, and increase the efficiency of the haematopoïetic stem cell donor registries an EU-funded project started in January 2001. This project comprised 14 partners from five countries in Europe, and is called "MADO" (Bone MArrow DOnor – Optimisation of typing policies for European Marrow Donor Registries: socio-economic evaluation of molecular and recruitment strategies; No: QLG7-CT-2001-00065)[7]. To decrease the cost a three

[3] Poli, F., et al.; 1998, Transplant International, 11: 347 - 349
[4] Sijpkens, Y., et al.; 1999, Chapther 2 in Kidney International, 56: 1920 - 1927
[5] Bartels, M.C. et al.; 2001, British Journal of Ophthalmology, 85: 1341-1346
[6] Statistisches Bundesamt Wiesbaden; 2004, „Statistisches Jahrbuch 2004"
[7] www.euromado.org

step strategy was chosen. In the first step all HLA haplotypes are divided into two groups. Group one is called “frequent HLA haplotypes”, and contains the ten most frequent HLA haplotypes of the five donor registries involved in the MADO project. These are “France Greffe de Moelle” (France), “Stichting Europdonor Foundation” (Netherlands), “Anthony Nolan Bone Marrow Trust” (United Kingdom), “Italian Bone Marrow Donor Registry” (Italy) and “National Medical Centre Budapest” (Hungeria). Group two contains all the non-frequent HLA haplotypes and is called “rare HLA haplotypes”. Since an identification of the HLA haplotype without knowledge of the HLA haplotypes of family members is technically not possible, the lists of frequent and rare HLA haplotypes was transferred into lists of frequent and rare HLA alleles. Frequent HLA alleles are those which appear in HLA haplotypes defined as frequent. In the second step all potential donors are “screened” by a suitable typing method, and sorted into one of the two groups of HLA alleles. A suitable method gives all necessary information about the HLA types of the donors to select them for one of the groups at minimal costs. In the third step all donors with a “rare HLA haplotype” are typed by a high resolution method. The main drawback of this procedure is that frequent HLA alleles do not inevitably create frequent HLA haplotypes. Therefore a rare HLA haplotype made up of frequent HLA alleles might not be identified.

1.2 Human Leukocyte Antigens (HLA)

1.2.1 Molecular structure and function of HLA molecules

The extended collection of genes on the short arm of human chromosome 6 at 6p21.3 is called Major Histocompatibility Complex (MHC) and is ~ 4 to 6 Mb in extent. This region is very gene dense and highly polymorphic. It was originally identified and named because of its role in tissue rejection after transplantation. Furthermore, many of the genes in this region show an important role in the biology of the immune system. The MHC is subdivided into three classes. The classification is based on main functional characteristics of the genes within each of the classes. The human MHC class I and class II are also called HLA class I and class II. Genes in the HLA code for HLA molecules. The main role of the HLA

molecules has little to do with transplantation. Originally these molecules are receptors, which capture peptides or protein fragments of antigens. They are present on cell surfaces where they can be recognised by appropriate T cells. With foreign antigens displaying to cells, HLA molecules evoke cytotoxic T lymphocyte (CTL). CTL is helper for a T cell response, which then regulates specific immunity.

1.2.2 HLA class II

HLA class II molecules are membrane glycoproteins formed through the non-covalent association of a 32 kDa α-chain and a 28 kDa β-chain. The α- and β-chains of all traditional HLA class II molecules have the same overall structure. The structure contains two extracellular domains, which are referred to as α1-α2, and β1-β2, respectively. The domains distal of the membrane, α1 and β1 together form a peptide-binding groove[8] (Figure 1).

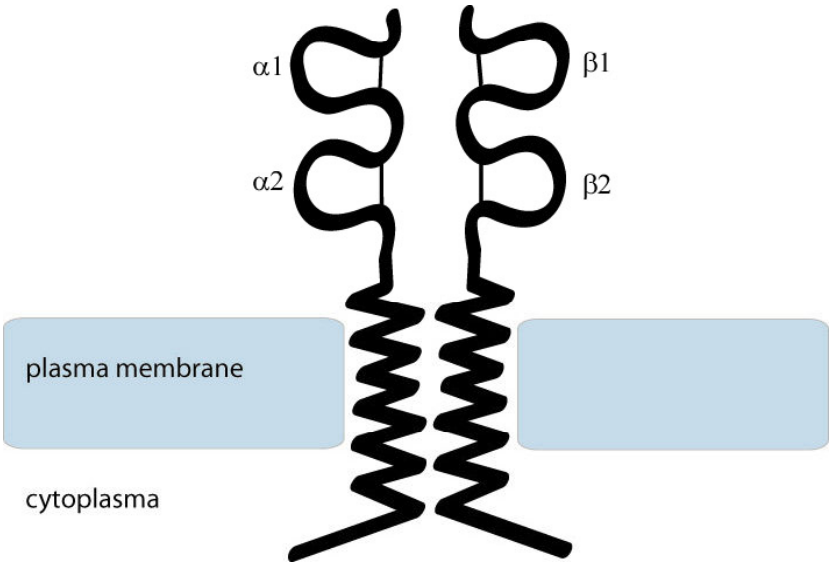

Figure 1: Schematic representation of a class II antigen

HLA class II molecules display a more limited tissue distribution than HLA class I molecules. Class II molecules are mainly expressed in the cells of the immune system such as B-cells, dendritic cells, macrophages and activated T-lymphocytes.

[8] Nelson, C.A. and Fremont, D.H.; 1999, Reviews in Immunogenetics, 1: 47 - 59

They control both the intrathymic selection and peripheral activation of CD4 T-cells[9].

MHC class II is the most centromeric class of the MHC. This class contains the genes HLA-DP, HLA-DQ and HLA-DR, as the so called classical MHC II genes, as well as HLA-DM and HLA-DO as non-classical MHC II genes. Genes of the MHC class II are found in pairs, encoding the α- and β-chains, which form the heterodimers of the MHC class II protein molecules. For HLA-DR only one non-polymorphic gene for the α-chain (HLA-DRA) exists, but in return there are 9 polymorphic β-chains (HLA-DRB1 to –DRB9). HLA-DRB1 encodes for the HLA-DR1 to HLA-DR18 molecules, and is present in all individuals. The gene HLA-DRB3 encodes molecules of the group HLA-DR52, HLA-DRB4 the molecules of the group HLA-DR53, and HLA-DRB5 the molecules of HLA-DR51. Maximum one of them occurs in an individual depending on the given haplotype. The loci for HLA-DRB2 and HLA-DRB6 to HLA-DRB9 are pseudogenes. All HLA-DR molecules are associated with the same HLA-DR α-chain.

The HLA-DQ region contains five loci. Only two polymorphic genes, HLA-DQA1 and HLA-DQB1, are expressed. HLA-DQA2, HLA-DQB2 and HLA-DQB3 are pseudogenes.

The HLA-DP region has four loci. Of them HLA-DPA1 and –DPB1 are polymorphic and expressed. The other two are pseudogenes.

For HLA-DQ and HLA-DP cis- and trans- associations between the mother and the father genes are observed, which adds to the variability of the system.

Alongside traditional HLA molecules, genes encoding for proteins that are involved in association of peptide or antigen processing are found in the MHC class II region. The latter are genes encoding transporters associated with antigen processing (TAP1 and TAP2), as well as genes of large multifunctional proteases (LMP2 and LMP7)[10, 11, 12].

1.2.3 HLA class I

In the telomeric region of the MHC are the genes of the MHC class I. This class contains genes which encode the α-chain of HLA class I molecules such as HLA-

[9] Pan, S. et al.; 1998, The Journal of Immunology, 161: 2925 - 2929
[10] Powis, S.H. and Trowsdale, J.; 1994, Behring Institute. Mitt. 94; 17-25
[11] Rhodes, D.A. and Trowsdale, J.; 1999, Reviews in Immunogenetics, 1: 21 - 31
[12] The MHC Sequencing Consortium; 1999, Nature, 401: 921 - 923

A, -B and -C, and related loci. The region of the MHC class I expands over 2 Mb. These genes are also members of the immunoglobulin gene family. The molecules of the MHC class I genes are involved in the presentation of peptides predominantly derived from intracellular proteins, to CD8+ cytotoxic T-cells.
The α-chains form heterodimers with the non-MHC coded β2-microglobulin (β2m). The heavy α-chain (45 kDa) is non-covalently associated with the β2-microglobulin (12 kDa) a polypeptide which is also found free in serum. The HLA class I α-chain consists of three extra cellular domains, a transmembrane region and the cytoplasmic C-terminus. The extra cellular domains are designated α1, α2 and α3. The domains α1 (N-terminal) and α2 together create the peptide-binding groove.

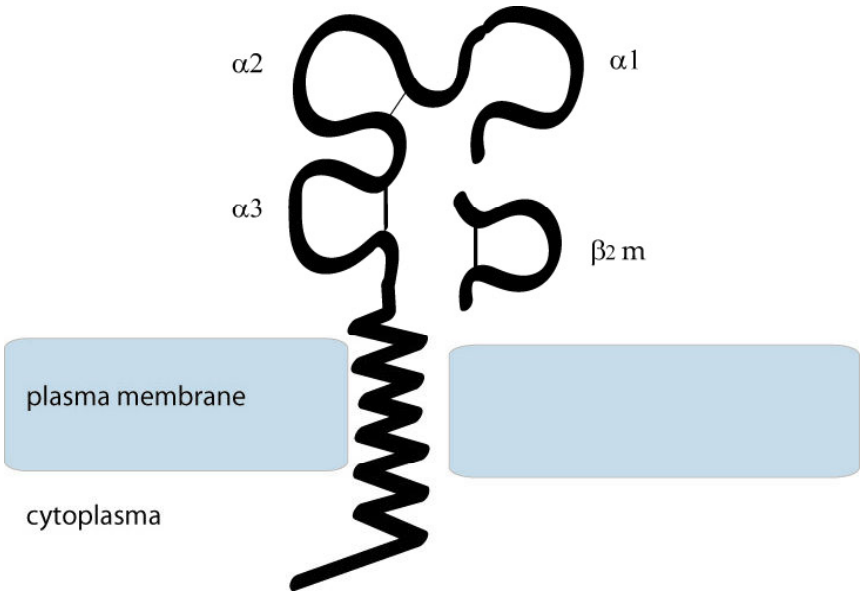

Figure 2: Schematic representation of a class I antigen

In human β2-microglobulin is a non-polymorphic protein, which is encoded on chromosome 15. It has the structure of an immunoglobulin C domain. β2m is also associated with a number of other class I-related molecules, for instance the products of the CD1 genes on chromosome 1.
The HLA-molecules of the MHC class I region are expressed ubiquitously[11].

1.2.4 MHC haplotypes

A highly relevant feature of MHC antigens is their co-dominant expression. Since both alleles contribute to the phenotype equally, it is important to examine the genotypes in disease association studies rather than the alleles on their own. Also important is the fact that the MHC is inherited *en bloc* as a haplotype[13]. An interesting group of MHC haplotypes is the ancestral or extended haplotype. These are specific MHC gene combinations in significant linkage disequilibrium in chromosomes of unrelated individuals[14,15]. They extend from HLA-B to HLA-DR and have been conserved *en bloc*. They are relatively population-specific and are believed to represent the original MHC haplotypes of our ancestors. They are easily recognized by their characteristic class III polymorphisms called complotypes[16]. In the haplotype very rare recombinational events are observed. Recombination occurs at 1 – 3 % frequency mostly at the HLA-A or HLA-DP ends. The large segment from HLA-B to HLA-DQB is almost constantly inherited as a whole. This has important inference in disease associations. Haplotypical associations are usually stronger and more meaningful than an allelic association. The co-dominant expression and transmission *en bloc* have an important consequence. Within families the HLA-identical sibling frequency should be 25 % according to the Mendelian law. However, this has been found to be higher than that in leukaemia[17,18,]. This would suggest a preferential transmission of leukaemia-associated HLA haplotypes[19]. Other examples of disease associations with MHC haplotypes are rheumatoid arthritis[20], multiple sclerosis[21], insulin-dependent diabetes mellitus[22] and systemic lupus erythematosis[23,24].

[13] Alper, C.A. et al.; 1992, Experimental and Clinical Immunogenetics, 9: 58 - 71
[14] Mariapia, A. et al.; 1992, Human Immunology, 34: 242 - 252
[15] Mariapia, A. et al.; 1995, Human Immunology, 44: 12 - 18
[16] Windsor, L. et al.; 2005, Genes and Immunity, 6: 298 - 304
[17] Carpentier, N.A. et al.; 1987, Transplantation Proceedings, 119: 2644 - 2645
[18] Dorak, M.T. et al.; 1994, Leukaemia & Lymphoma, 12: 211 - 222
[19] Dorak, M.T. et al.; 1992, Causes & Control, 3: 273 - 282
[20] Zanelli, E. et al.; 2000, Rheumatology, 39: 1060 - 1066
[21] Marrosu, M.G. et al.; 2001, Human Molecular Genetics, 10(25): 2907 - 2916
[22] Larsen, Ch. and Alper, Ch.; 2004, Current Opinion in Immunology, 16: 660 - 667
[23] Dawkins, R. et al.; 1999, Immunological Reviews, 167: 275 - 304
[24] Grant, S.F.A. et al.; 2000, Journal of Immunological Methods, 244: 41 - 47

1.2.5 Nomenclature of the HLA antigens

The nomenclature of HLA antigens is regulated by a WHO committee[25]. Rules are laid down on how to name new genes, alleles and serological specifics. The nomenclature of the HLA antigens is split by the information content of immunological methods (binding of antibodies, cellular methods) and DNA-based typing methods.

Originally HLA antigens were typed by serological methods. The "private" antigen structure, which is specific for an antigen, is detected by one specific antibody and is also called "split". The general "public" antigen structure of the HLA antigen group is detected. The public structure is shown by the name of the HLA class (e.g. HLA-A, -B, -C, etc) and a number, which stands for the supertype (e.g. HLA-A8). The subtype or private antigen structure (split) is declared by a second number (e.g. HLA-A24).

For the DNA sequence-based nomenclature the gene locus is named (e.g. HLA-A) and followed by a two digit number, which is separated from the gene name by an asterisk and indicates the serological family of the HLA antigen (e.g. HLA-A*13). A second two digit number shows coding variations of the HLA antigen (e.g. HLA-A*1303). If alleles differ by one or more silent polymorphisms a third two digit number is added (e.g. HLA-A*250101). Variations in introns or 5′ or 3′regions of the gene are denoted by the digits 7 and 8. Null alleles are marked with an "N" at the end of the name, and in cases of a low expression of alleles by an "L" (HLA-A*2411N, HLA-A*02010102L). Suffix "S" indicates an allele specifying a protein, which is expressed as a soluble "Secreted" molecule, but is not expressed on the cell surface (HLA-B*44020102S). A "C" outlines alleles where products are presented in the cytoplasm but not at the cell surface. A "Q" indicates an allele which has a mutation that has previously been shown to have a significant effect on cell surface expression, but where this has not been confirmed and its expression remains "Questionable". Finally the "A" (Aberrant) marks alleles where there is some doubt about whether a protein is expressed.

[25] Marsh, S.G.E. et al.; 2002, Tissue Antigens, 60: 407 - 464

1.3 History of HLA and HLA Typing

The discovery of the major histocompatibility antigens as so-called strong transplantation antigens, which play a major role in graft rejection after transplantation, has its origin in the animal experimental analysis of tissue transplantations with mice[26,27]. In the 50´s followed the description of the first human leukocyte antigens by Dausset[28,29], van Rood[30] and Payne et al[31]. Since then transplantation immunology became a distinct scientific discipline, since transplantations where accompanied by a very high failure rate. Tissue typing until the early 1960s was carried out by serological methods such as leukoagglutination and complement-fixation on platelets. These methods lacked reproducibility. The complexity of the genetics of leukocyte antigens was a problem.

A reversal point in the history of tissue typing was the first Workshop and Conference on Histocompatibility, which was organized by Bernhard Amos (Durham, NC) in 1964. During this workshop the participants compared the reactivity of their sera with various techniques. The results were incompatible, and could not be published.

During the second Workshop, which took place in 1965 in Leiden, the results were more consistent. Hereon Paul Terasaki and John McClelland at UCLA introduced the complement-dependent microlymphocytoxicity technique. This method has remained the standard serological test for HLA typing.

During the third Workshop organized by Ruggero Ceppelini (Torino, Italy) in 1967 the HLA system was established. Furthermore two segregated series of specificities were recognized. They were now called HLA-A and HLA-B.

After each workshop, a nomenclature committee integrated outstanding findings on the way to the definition of HLA variations. This includes the identification of additional class I locus HLA-C, by Thorsby, Sandberg and Kissmeyer-Nielson during the fourth workshop in 1970. Five years later HLA-D was added. Further

[26] Gorer, PA.; 1936, British Journal of Experimental Pathology, 17: 42 - 50
[27] Snell, G.; 1958, Journal of the National Cancer Institut, 21: 843 - 877
[28] Dausset, J.; 1954, Vox Sanguinis, 4: 190 - 198
[29] Dausset, J.; 1958, Acta haematologica, 20: 156 - 158
[30] VanRood, J.; 1958, Nature, 181: 1735 - 1736
[31] Payne, R.; 1958, Journal of Clinical Investigation, 37: 1756 - 1763

outstanding findings are the class II loci HLA-DR in 1977[32] and a few years later HLA-DQ[33] and HLA-DP[34].
The obvious success of the last workshops has assured the extension of these workshops. (Los Angeles, USA, 1970; Evian, France, 1972; Aarhus, Denmark, 1975; Oxford, England, 1977; Los Angeles, USA, 1980; Munich, Germany, 1984; New York, 1987; Yokohama, Japan, 1991; St.Malo/Paris, France, 1996; Victoria, Canada, 2002; and most recently November 2005 in Melbourne, Australia).

1.4 Current Methods for HLA Typing

Since the mid-80s serological typing methods, which were carried out exclusively[35], are complemented by analysis at the DNA level. During the 9th IHCW in 1984, several papers described the use of restriction fragment length polymorphisms (RFLP) method to study HLA at the DNA level[34]. The introduction of the PCR[36] allowed the development of a variety of simple and rapid DNA sequence based typing methods[37,38]. Nowadays several methods, which describe the HLA at DNA level, are standard methods in HLA typing laboratories. The most frequently used methods in the HLA typing laboratories are "Sequence Specific Oligonucleotide Probe Hybridization" (SSOP), "Sequence Specific Primers Amplification" (SSP), "Reverse Line Blot" and "Sequence Based Typing" (SBT). The latter method is in principle sequencing of the different HLA genes. It is the costliest method of the HLA typing methods, but at the same time it is the only technique, which allows identification of unknown HLA alleles immediately. Another high resolution technique which is used in quite a few HLA typing laboratories is "Reference Strand Conformation Analysis" (RSCA). All these DNA based methods have in common the use of polymorphisms in the DNA sequence to identify the HLA type.

[32] Bodmer, W.F. et al.; 1977, Histocompatibility Testing (Eds. 1978)
[33] Terasaki, P.I; 1980, Histocopatibility Testing (Eds. 1980)
[34] Albert, E.D. et al.; 1984, Histocompatibility Testing (Eds. 1984)
[35] Middleton D.; 1999, Revies in Immunogenetics, 1: 135 - 156
[36] Saiki, R. et al.; 1985, Sience, 230: 1350 - 1354
[37] Erlich, H.A. et al.; 1991, European Journal of Imunnogenetics, 18: 33-55
[38] Erlich, H.A. et al.; 2001, Immunity, 14: 347-356

Nevertheless serological typing methods are still standard procedures in many HLA typing laboratories. For example, many laboratories use serological typing as a pre-typing method before SBT.

1.4.1 Serological typing

In general two serological typing procedures are used for the determination of the HLA-types of donor and recipient. These are:

- Complement-dependent microlymphocytoxicity
- Mixed lymphocyte reactions

For the complement-dependent microlympho-cytoxicity test a panel of typing antisera with defined specificity (e.g. anti-HLA-B8, anti-HLA-B13) is used. If enough antibodies are bound to the HLA molecules the complement can be activated and the cell membrane is destroyed. This causes cell death, which can be visualized by trypan blue staining. Successful staining confirms that the test cells carry the antigen in question.

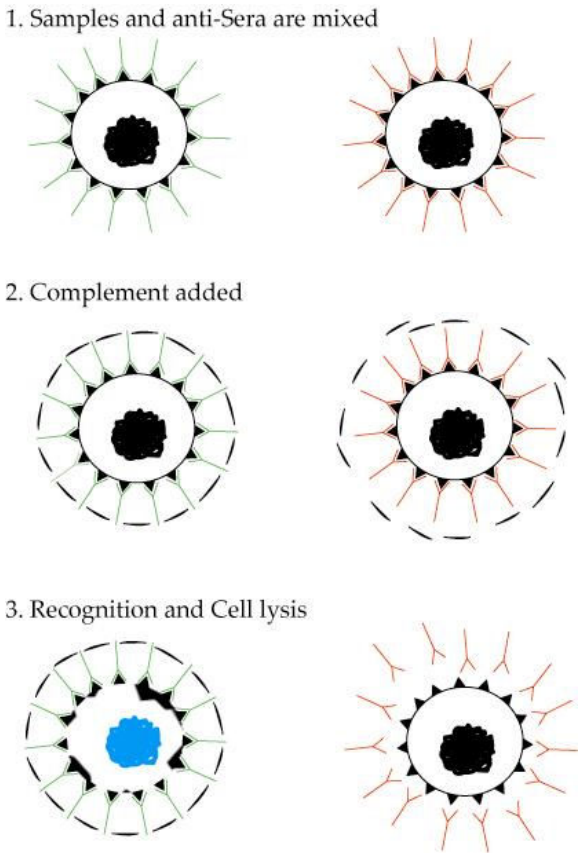

Figure 3: Principle of the complement-dependent microlymphocytoxicity test. First sample cells and specific anti-Sera are mixed.

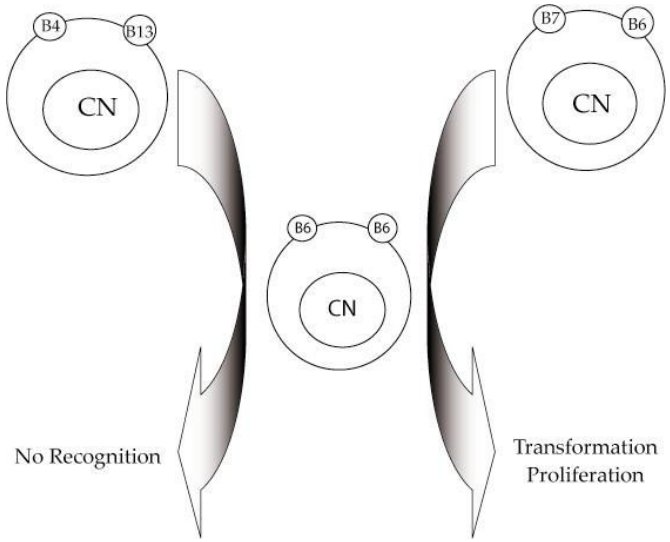

Figure 4: Principle of Mixed Lymphocyte Reaction

For the Mixed Lymphocyte Reaction (MLR) test, test cells and homozygous typing cells (for example HLA-DR6, 6) are mixed. In cases where the test cells and the typing cells share the same HLA-DR type, they do not recognize the other cells as foreign. In this case no reaction is observed. In the case of different HLA types between test cells and typing cell recognition takes place, which can be observed by a transformation and proliferation. This procedure has to be carried out with a panel of defined specific typing cells. The time necessary for this test precludes its use in most clinical organ transplantations, since organs from dead or brain-dead donors cannot be conserved for more than 24 to 48 hours. In cases where living donors are to be used, this technique is possible. The MLR test has its importance in bone-marrow transplantations and is often performed to see whether the donor bone marrow cells could respond to recipient antigens and cause graft-versus-host disease. In such a test, it is the recipient's lymphoid cells that are irradiated, and lymphocytes from the donor are proliferated.

1.4.2 Typing by hybridization to sequence-specific oligonucleotide probes

PCR based sequence-specific oligonucleotide probes hybridization (PCR-SSOP) is a very economical method for HLA typing and rather widely-used in the field of HLA typing for donor registries. As for all DNA based methods, PCR-SSOP requires a locus specific PCR, which is used as the template for hybridization of sequence-specific oligonucleotide probes. The most used method is the immobilization of a PCR product on a nylon membrane. Then a single stranded template is created by treatment with sodium hydroxide (NaOH). After washing

the membrane hybridization with labelled oligonucleotide probes is carried out. The labelling of the probes can be a radioactive label, a fluorescent dye or an enzyme, which produces a detectable signal (e.g. horse radish peroxidase). Since just one probe can be used at a time, this procedure has to be repeated for each oligonucleotide probe. This method is very time consuming, but since many different DNA samples can be immobilized on a nylon membrane, it is very powerful in terms of throughput. Therefore this method is well suited for large DNA collections. Drawbacks of the method are the high background noise. This results in a high rate of false positive signals. Another drawback is the high chemical stress for the template during the procedure, which results in the degeneration of the template and a decrease of signal quality.

An alternative to the nylon membrane method is the "Luminex" method. Luminex is a flow cytometer that is used to read a fluorescent signal on a colour coded Styrofoam bead, the microsphere. This method requires a locus-specific PCR for preparation of a template. The amplification is carried out with one biotinylated primer. The biotin molecule interacts with a streptavidin-conjugated red dye (R-Phycoerythrin) at the end of the reaction[39]. The red dye absorbs at the emission wavelength of the laser, which is used in the Luminex flow cytometer. Cy3 or Cy5 deliver very good results as well. Furthermore each of the oligonucleotide probes is covalently bound to a microsphere. The colour code, which decodes the microsphere and thus the oligonucleotide probe, is created by a mixture of orange and yellow colour dyes in different ratios (e.g. 40 % orange and 60 % yellow or 30 % orange and 70 % yellow). The decoded oligonucleotide probes are hybridized to the dye labelled PCR product. The colour code makes a high-multiplex factor possible. Commercially sets of 100 and 1000 microspheres are available, and can be used for multiplex analyses in a single reaction[40,41].

[39] Armstrong, B. et al.; 2000, Cytometry, 40: 102 - 108
[40] Colinas, R. et al.; 2000, Clinical Chemistry, 46(7): 996 - 998
[41] Yang, L. et al.; 2001, Genome Research, 11: 1888 - 1898

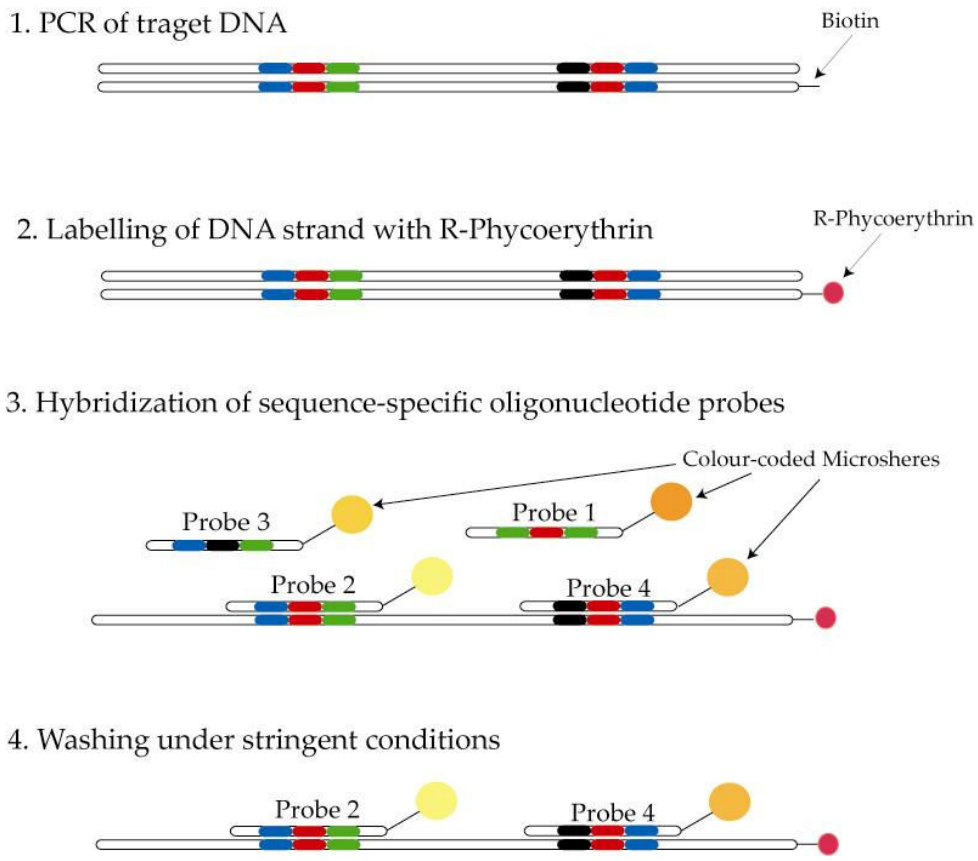

Figure 5: Principle of SSOP by Luminex. The final product is analysed by a two laser system. Laser one (green), the reporter identifies the coded beads and the second laser (red) registers the signal intensity of the fluorescence dye.

Compared to other common methods for detection of hybridization the Luminex platform is a very fast and low effort technology, however with a few drawbacks. The microspheres are quite fragile. Organic solvents like DMSO and formamide, as well as urea and temperatures over 70°C destroy the spheres or disturb decoding the microspheres.

Another approach is to use the capability of Luminex to analyse multiplex markers as described by several groups[42,43,44,45]. For this approach, the microspheres are coupled to unique 10- to 15-mer oligonucleotides, which are called "cZipCodes". Their complementary sequences (address-sequences) are tails

[42] Taylor J.D. et al.; 2001, Biotechniques, 30(3): 661 - 666, 668 - 669
[43] Ye F et al.; 2001, Human Mutation, 17(4): 305 - 316
[44] Cai, H. et al.; 2000, Genomics, 66: 135 - 143
[45] Chen, J. et al.; 2000, Genome Research, Vol. 10: 549 - 557

of specific extension primers. These primers can be extended with biotinylated or dye-labelled ddNTPs. In case of use of the biotin variant a conjugation with a streptavidin conjugated dye is required. After the extension reaction the address sequences are hybridised to the zip-sequences on the coded spheres, and analysed with the Luminex device.

1.4.3 Reverse hybridization with immobilized sequence-specific oligo-nucleotide probe arrays – Reverse Line Strip Blot

SSOP is perfectly suited for analysis of a large number of samples. An alternative technique to SSOP is the Reverse Line Strip Blot. This technique uses the same oligonucleotide probes, which are immobilised on the membrane and the labelled PCR product is hybridised to it. There are several variations of this technique. In general the amplification product is conjugated with a signal producing label, like a peroxidase[46], chemiluminescence or fluorescent dyes[47,48]. This technique is very useful, if the number of DNA samples is rather small and the number of oligonucleotide probes is high. This situation is mostly given in laboratories with low or medium throughput.

1.4.4 Typing by differential primer extension and sequence-specific amplification

Methods with primer extension and sequence-specific amplification are widely used in the field of single nucleotide polymorphism (SNP) genotyping[49,50]. In terms of HLA typing sequence-specific primer (SSP) amplification is one of the most commonly used approaches. For SSP a set of primers is used to amplify the template by PCR. Each primer has a specific sequence and only if the sequence is entirely complimentary to the sequence of the template an amplification product is obtained. The success of the amplification can be detected by separation of products by gel electrophoresis. Effectively this is an amplification refractory

[46] Bugawan, T.L. et al.; 1990, Immunogenetics, 32: 231 - 241
[47] Buyse, I. et al.; 1993, Tissue Antigens, 41: 1 - 14
[48] Cros, P. et al.; 1992, Lancet, 340: 870 - 873
[49] Syvänen, A.C.; 1999, Human Mutation, 13: 1 - 10
[50] Pastinen, T. et al.; 1997, Genome Research, 7: 606 - 614

mutation system (ARMS)[51]. The concept of ARMS was first described by Newton et al[52].

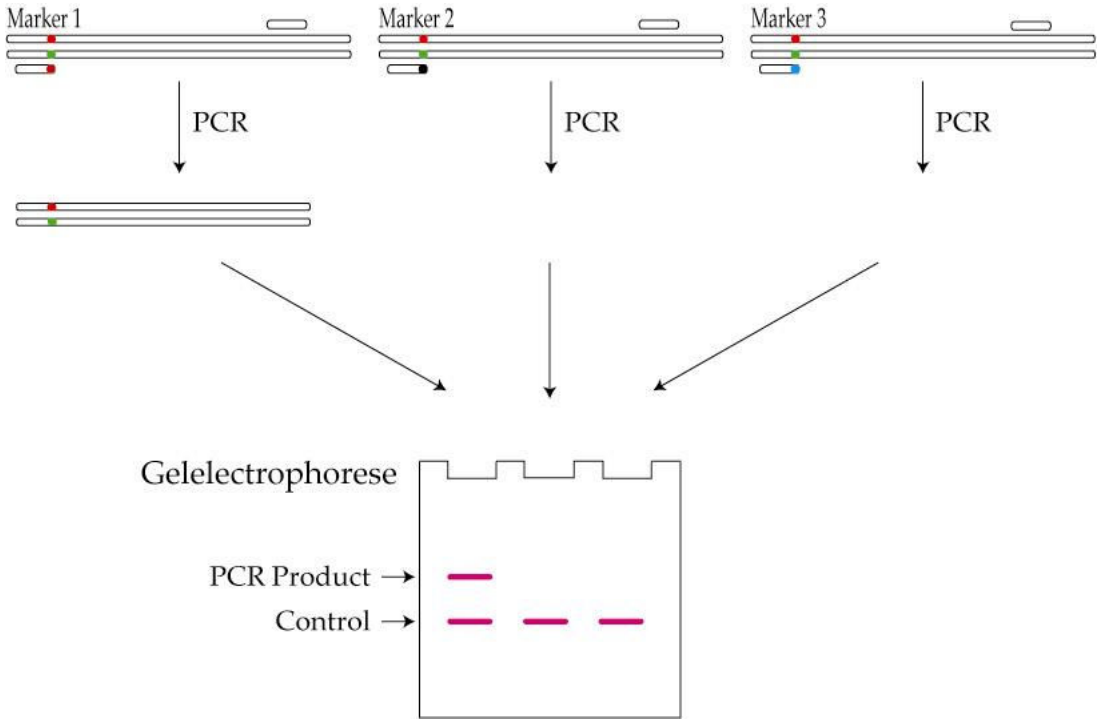

Figure 6: Principle of SSP

1.4.5 Typing based on the gel mobility of the PCR products

Reference strand mediated conformation analysis (RSCA)[53,54] is a high resolution HLA typing method which is used in several HLA typing laboratories[55]. A PCR product of a sample to be analyzed is mixed with a fluorescently labelled reference product. Denaturation and renaturation result in a mixture of two to three homoduplexes and four to six heteroduplexes (dependents on homozygous or heterozygous samples). Sequence differences between the tester and the reference result in mismatches, loops and bulges, which affect gel mobility of these products. The gel mobility is compared to a standard set of markers for

[51] Tonks, S et al.; 1999, Tissue Antigens , 53: 175-183
[52] Newton, C.R. et al.; 1989, Nucleic Acids Research, 17: 2503-2516
[53] Arguello, J.R. et al.; 1998, Nature Genetics, 18: 192-194
[54] Arguello, J.R. et al.; 1998, Tissue Antigens, 52(1): 57 - 66
[55] Corell, A. et al.; 2000, Tissue Antigens, 56: 82 - 86

each allele. The mobility analysis is usually done with capillary electrophoresis. Recently the identification of unknown HLA types by this method was reported[56].

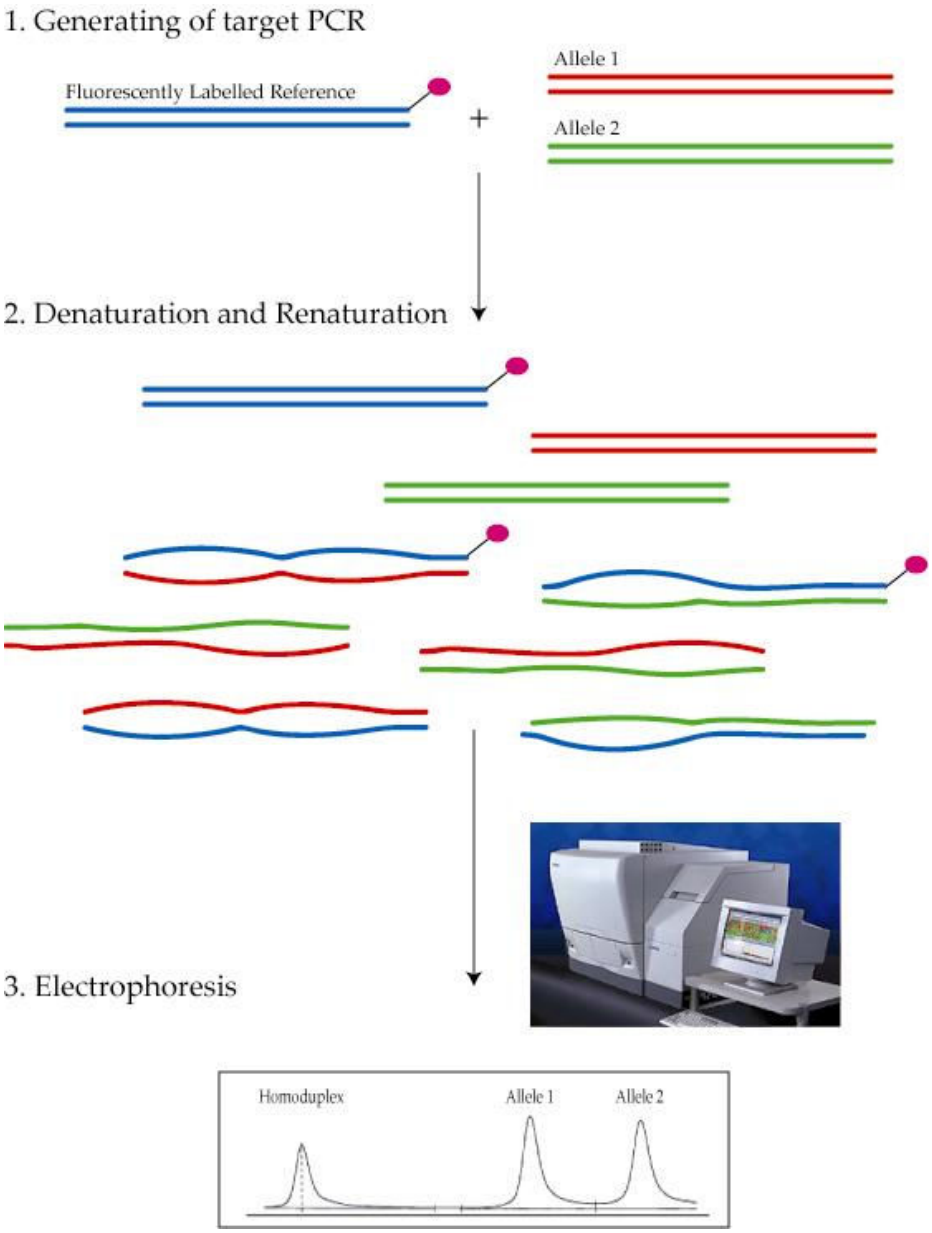

Figure 7: Schematic principle of RSCA. 1. The products of the PCR are mixed with a fluorescently labelled reference (FLR). 2. Denaturation and renaturation results in a mixture of homo- and heteroduplexes. Sequence differences between the sample products and the FLR result in loops and bulges in the renaturation products. These formations are very specific and produce very specific mobilities in polyacrylamid gels. 3. In a capillary electrophoresis only the fluorescently labelled duplexes are detected.

[56] Garcia-Ortiz, J.E. et al.; 2003, Tissue Antigens, 63:85 - 87

1.4.6 Sequence-based typing

Sequence-based typing (SBT) is the most powerful method of the DNA-based approaches. It is the only technique which gives the possibility to discover new alleles while identifying known alleles. SBT starts out like all DNA-based techniques with a locus specific PCR. This is followed by a Sanger sequencing reaction with specific primers in sense and antisense direction. Sequencing of the MHC region is associated with many complications. Since this region is highly polymorphic the reading of the sequencing traces is very difficult. Further some alleles of the HLA genes are characterized by insertions and deletions (e.g. HLA-A*6818N, HLA-A*2436N), which cause mis-alignments of the traces. In this case reading the traces is almost impossible. For this reason SBT is very time consuming. One technician needs about 25 minutes to analyse one locus for one DNA sample[57]. To this comes that the reagents for the sequencing reactions are relatively expensive. A further complication with SBT is the common use of serological typing techniques to determine HLA super-groups. The results of the serological typing are necessary to choose the set of sequencing primers. However in quite a lot of cases the serological typing provides wrong types, which leads to a failure of the SBT process.

1.5 New Methods for HLA Typing

1.5.1 HLA allele detection based on DNA array technology

The DNA array technologies are very applicable in the field of polymorphisms genotyping. Most of the approaches for high throughput analysis of polymorphisms are DNA array-based methods (e.g. Illumina[58], Affimetrix[59]). In the courses of the MADO project an HLA allele detection method based on DNA array technology was presented. It was developed by Consolandi et al[60].
First of all loci specific PCRs encompassing the three loci of interest (HLA-A, -B and -DRB1) are carried out. Subsequently a total number of 25 group-specific

[57] Eliaou, J.F.; 2003, personal communication
[58] Shen, R. et al.; 2005, Mutatin Research/ Fundamental and Molecular Mechanism of Mutagenesis, 573(1-2): 70 - 82
[59] Wang, D.G. et al.; 1998, Science, 280: 1077 - 1082
[60] Consolandi, C. et al.; 2004, Human Mutation, 24: 428 - 434

nested PCRs are required. The PCR products are templates for single base extension (SBE) reactions.
The SBE reactions are carried out with a total of 350 primers for the three loci. Each of the extension primers carries at the 5'-end a unique address sequence. The complimentary zipCode sequences are synthesized at defined positions on a chip surface. After the SBE with fluorescence dye labelled ddNTPs the extension products are hybridised to the zipCode array. The array is scanned in a fluorescence scanner and the fluorescence quantified on each feature. The results are compared with a hit table to identify the given HLA type.
The technique in general is very effective for high resolution detection of HLA alleles of a small to medium number of DNAs. The reaction steps are robust and fast, but the analysis of the huge amount of data points for a single DNA is very time-consuming.

1.5.2 Microsatellite genotyping and HLA alleles

Short tandem repeat (STR) or microsatellite (μSat) loci are tandomly repeated sequence motifs of 2-6 base pairs (bp). The length is usually less then 60 bp long[61]. 50000 to 100000 such STR are spread more or less evenly across the human genome. They have been particularly useful markers in studies of diseases and population genetics due their high degree of polymorphism and relatively high density of about one μSat locus every 20000 bases in the human genome[62]. A further characteristic that makes them highly interesting for population studies is the relative high mutation rate of 10^{-3} events per μSat per generation[61,63]. Most μSats are alternating purine/pyrimidine sequences. Alternating purine and pyrimidine sequences are able to form *in vitro* z-conformation, which means a left-rotating DNA-helix. Nearly all μSats are located in non-coding regions of the genome.
μSats in the MHC region were first described in the vicinity of the tumour necrosis factor (TNF)[64]. With these μSats linkage disequilibrium between HLA genes was first described. Because μSats have proven themselves as useful

[61] Weber, J. and Wong, C.; 1993, Human Molecular Genetics, 2: 1123 - 1128
[62] Lander, E.S. et al.; 2001, Nature, 409: 860 - 921
[63] Weissenbach, J. et al.; 1992, Nature, 359: 794 - 801
[64] Jongeneel, C. et al.; 1991, PNAS, 88: 9717-9721

genetic markers in the MHC many efforts have been made to characterize large numbers of μSats in an integrated map of the HLA region[65,66]. Recently a set of μSat markers was identified for use in disease-association studies[67,68]. 2003 Cullen used a computer-based analysis to identify μSats for future studies of MHC genetics and disease association. All in all μSats are potentially powerful markers for HLA haplotyping and association studies, but until today there are several problems. There is, for example, a lack of standardization. Different reports refer to the same μSat locus using different names and some information about μSats had to be corrected and supplemented, e.g. localization and lack of repeat pattern[69]. To solve these problems will be essential for the use of μSats as HLA typing technology.

1.5.3 HLA typing by TaqMan®

TaqMan® is a very precise method for medium- to high-throughput SNP analysis. This approach uses the 5′ to 3′ exonuclease activity of the enzyme Taq-DNA-polymerase. A set of two oligonucleotides, which are identical in the sequence except for one position, is added in to a PCR reaction. The two oligonucleotides correspond to the two alleles of the SNP. They are each labelled at the 5′-end with a different reporter fluorophore, and at the 3′-end with a common quencher dye, and the oligonucleotide is blocked at the 3′-end by a phosphate group so that it cannot act as a primer.

Each cycle of the following PCR reaction starts with a competitive hybridization of the probe oligonucleotides. This hybridization has to be extremely stringent to assure the right results. During the primer extension the Taq-polymerase degrades the hybridized probe with the 5′ to 3′ exonuclease activity. At the same time the reporter is cleaved off and freed from the quencher. This results in fluorescence. (see Figure 8)

The fluorescence signal can be measured on a real-time PCR instrument or fluorescence plate reader. This takes two minutes per 384-well plate.

[65] Foissac, A. et al.; 1997, Tissue Antigens, 49: 197 - 214
[66] Foissac, A. et al.; 2000, Tissue Antigens, 55: 477-509
[67] Matsuzaka, Y. et al.; 2001, Tissue Antigens, 57: 397-404
[68] Tamiya, G. et al.; 1999, Tissue Antigens, 54: 221-228
[69] Gourraud, PA. et al.; 2004, Tissue Antigens, 64: 543 - 555

Theoretically, a variety of fluorophores can be used. The choice of the fluorophore is dependent on the type of the detection instrument. Most commonly FAM or TET as the green fluorophore in combination with a red fluorophore either HEX or JOE is used.

Some research groups successfully use the TaqMan assay for HLA typing[70,71]. The common method for HLA typing, PCR-SSP, has been adapted for TaqMan technology. With the TaqMan® assay the elaborate post-PCR work of the PCR-SSP approach is eliminated.

Drawbacks of this approach are that only two colours can be used in one reaction. Consequently only biallelic events can be examined in a single reaction. Thus for high resolution typing a relative large number of reactions are necessary. TaqMan can be used as a fast and easy screening method, e.g. HLA-B27 typing. To detect whether a DNA sample has one of the HLA-B27 alleles or not, typing of only three polymorphisms in the HLA-B is required.

[70] Slateva K. et al.; 2001 Tissue Antigens, 58: 250-254

[71] Tremmel M. et al.; 2001, Tissue Antigens, 54: 508-516

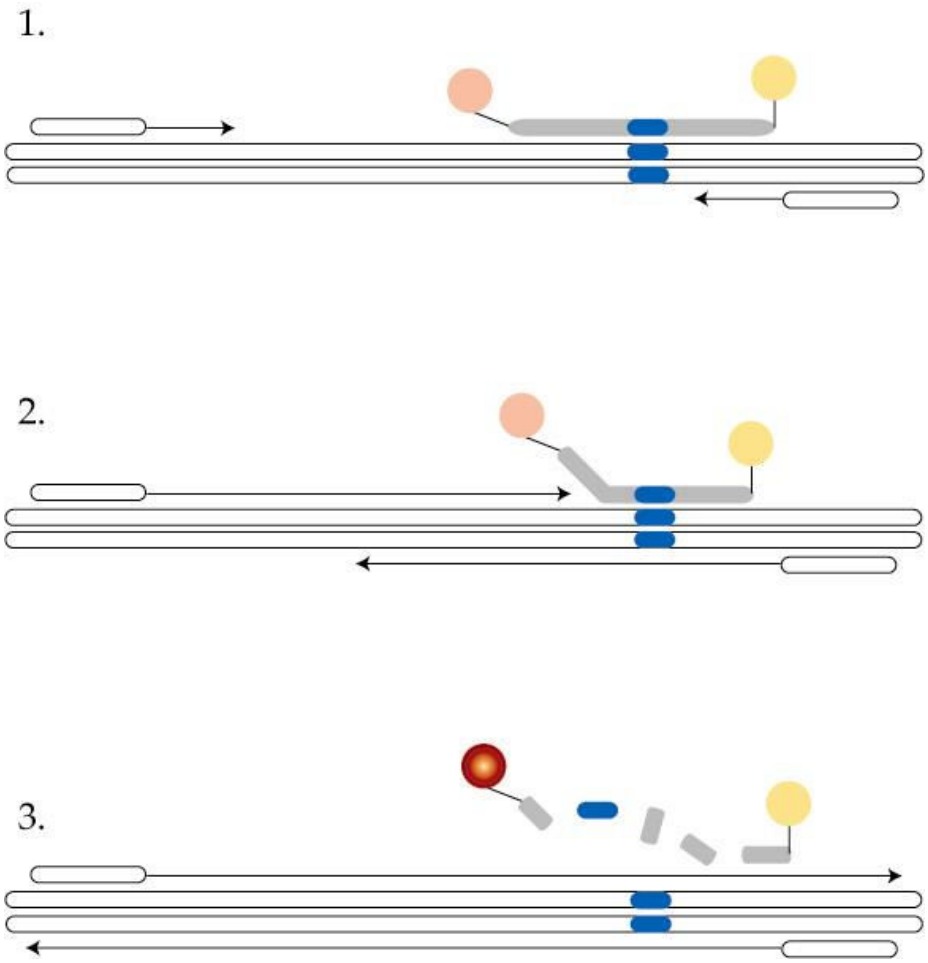

Figure 8: Principle of the TaqMan. 1. After specific hybridization of probes (specific to the SNP present in the DNA sample = dark blue) and PCR primer to the template, the Taq DNA polymerase starts extension of the PCR primers. The reporter dye on the intact probes emits very little fluorescence, because its quenched by the quencher dye, which than emits fluorescence of a longer wavelength. 2. When the extension reaches the hybridized probe it starts a probe displacement. 3. The probe displacement forms a structure which switches on the 5′ nuclease activity of the Taq DNA polymerase and therewith a cleavage of the probe. The cleavage has the effect to separate the fluorophore dye from the quencher dye which results in reduction of the quencher efficiency and at the same time an increase of the reporter signal. (According to Tremmel et al.[71])

1.5.4 Pyrosequencing™ and HLA typing

Recently Pyrosequencing™ has been applied for HLA typing[72]. Pyrosequencing™ is a real-time sequencing by synthesis method[73,74]. A single stranded template is generated by a PCR with one biotinylated primer. After amplification the PCR product is conjugated via the biotin group to a streptavidin-coated agarose gel beads. The non-biotinylated strand is removed by melting with 0.1 M NaOH. After a primer is hybridized to the single stranded template and it is incubated with a balanced enzyme-substrate mix. This mixture contains DNA polymerase, ATP sulfurylase, luciferase, apyrase and the substrates adenosine-5′-phosphosulfate (APS) and luciferin. Each of the four dNTPs is then individually added to the reaction mixture. By adding of one of the dNTPs a cycle is started. If a dNTP is complementary to the base in the template the DNA polymerase catalyzed the polymerization of the dNTP and the DNA strand. This process is accompanied by liberation of pyrophosphate (PPi) in an eqimolar quantity to the amount of incorporated nucleotide. The released PPi is quantitatively converted to ATP in the presence of adenosine-5′-phosphosulfate by the ATP sulfurylase. ATP then causes the conversion of luciferin to oxiluciferin catalyzed by the luciferase. The conversion generates visible light proportional to the amount of ATP. The quantity of light is measured and recorded. The peak height represents the amount of light which has been liberated by the luciferin-oxiluciferin conversion and is proportional to the number of dNTPs incorporated. To finish the cycle non-incorporated dNTPs and excess ATP are degraded by the apyrase. After the degradation is completed, a new cycle can be started by adding of the next dNTP and enzyme-substrate mix. The complimentary DNA strand is built up, and the sequence of the strand is determined from the peaks in the pyrogram. The principle of the Pyrosequencing™ method is shown in Figure 9.

[72] Ringquist, S et al.; 2002, Biotechniques, 33: 166 - 172
[73] Ronaghi, M et al.; 1998, Science, 281: 363 - 365
[74] Ronaghi, M et al.; 2001, Genome Research, 11: 3 - 11

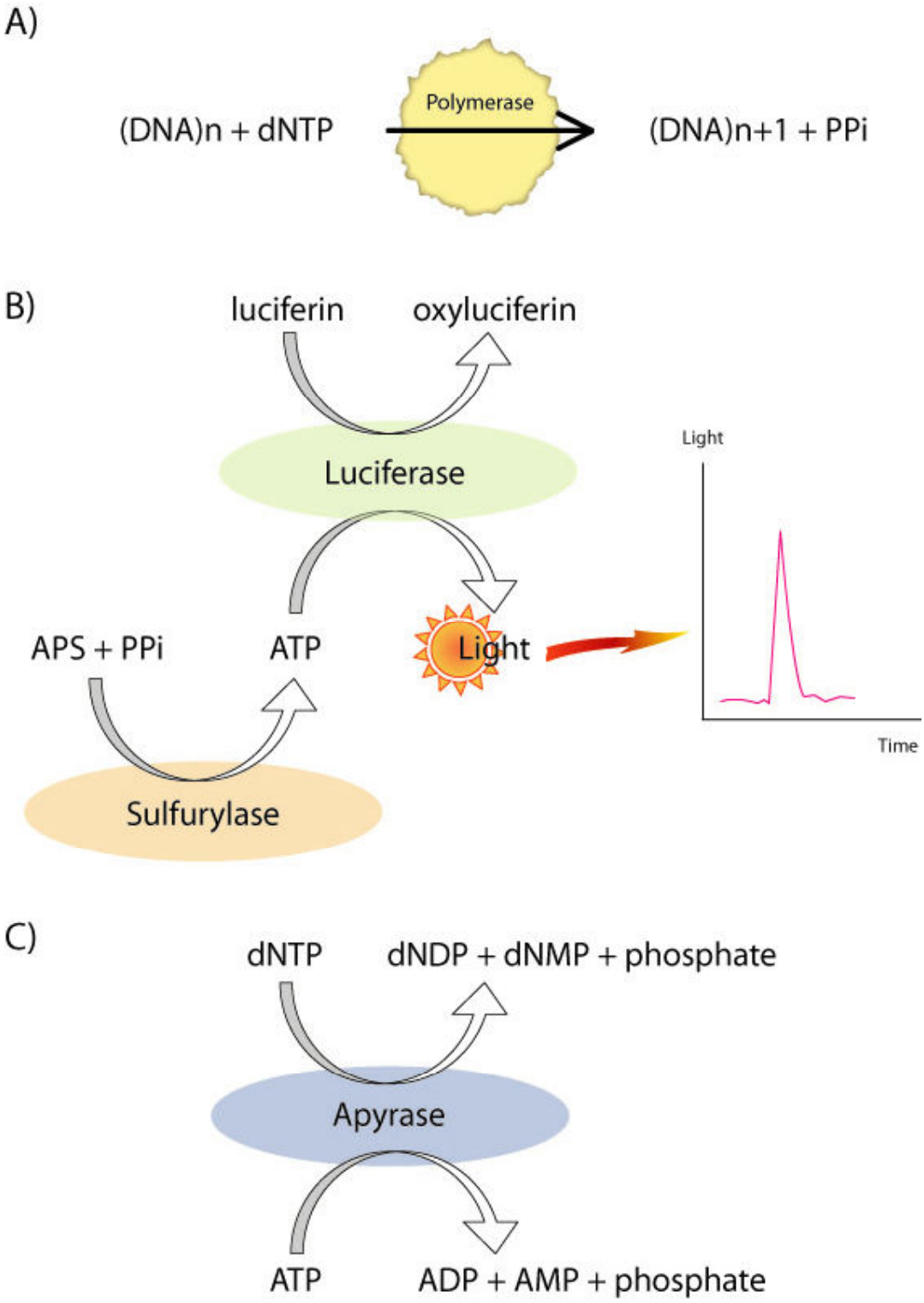

Figure 9: Principle of Pyrosequencing; Step A): The first of four dNTPs is added to the reaction. A DNA polymerase catalyzes the incorporation into the DNA strand. This event is accompanied by release of pyrophosphate (PPi). Step B): ATP sulfurylase converts quantitatively PPi to ATP in the presence of adenosine-5´-phosphosulfate (APS). The ATP drives the conversion of luciferin to oxyluciferin by the luciferase. That process generates light in amount proportional to the amount of ATP. The light is detected. Each light signal is proportional to the number of nucleotides incorporated. Step C): Apyrase degrades unincorporated dNTPs and excess ATP. When this step is complete, the next dNTP is added. (according to Biotage)

Pyrosequencing™ allows sequencing of short fragments (up to 10 – 15 bases) and longer fragments (up to 70 bases), depending on the kit. By designing the sequencing primers in a way that an allele-selection is possible, Pyrosequencing™ can easily resolve cis/trans-ambiguities of SBT and other HLA typing methods.

1.6 The GOOD Assay a potential Method for HLA Screening

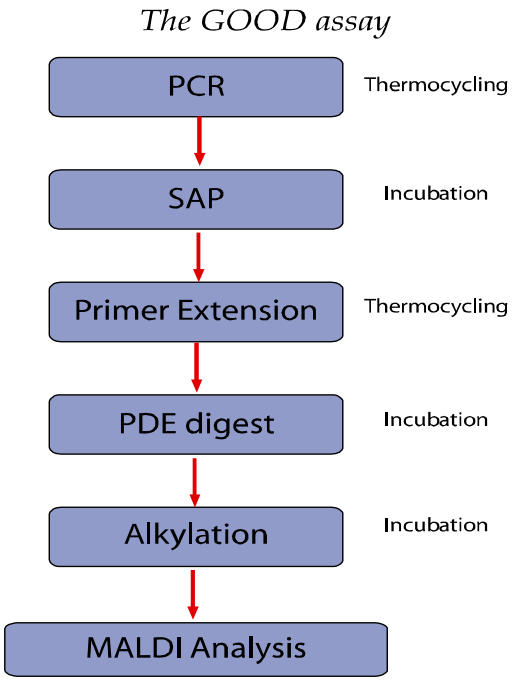

Figure 10: The flowchart shows the procedure of the GOOD assay. (According to Jörg Tost)

The GOOD assay is a method for high throughput Single Nucleotide Polymorphism (SNP) genotyping[75,76,77,78]. It is based on a mini sequencing protocol with detection by mass spectro-metry[79,80]. The greatest advantage, compared to other genotyping methods with MS detection such as PROBE[81] and INVADER[82,83] is that it is a purification free assay. Therefore it is easy to automate the liquid handling for high throughput processes. Furthermore it is a robust and stable protocol with very high sensitivity in detection. There is also not necessarily a need for high quality DNA. Even poor quality DNA results in sufficiently good genotype output. Furthermore, with this method, it is feasible to genotype multi-allele (>2 alleles) systems, with just one reaction per sample. This is an advantage over other very good high-throughput methods, e.g. TaqMan® and Amplifluor®. Since TaqMan® has, as described before, a two colour-allele-calling-system, a second reaction for

[75] Sauer, S. et al.; 2000, Nucleic Acids Research, 28 : e13
[76] Sauer, S. et al.; 2000, Nucleic Acids Research, 28 : e100
[77] Sauer, S. et al.; 2002, Nucleic Acids Research, 30: e22
[78] Tost, J. et al.; 2003, Book Chapter in "PCR Technology" 2nd Edition
[79] Pastinen, T et al.; 1997, Genome Research, 7: 606 - 614
[80] Pastinen, T et al.; 2000, Genome Research, 10: 1031 - 1042
[81] Koster, H et al.; 1996, Nature Biotechnology, 14: 1123 -1128
[82] Hosfield, D.J. et al.; 1998, Journal of Biological Chemistry, 273: 27154 - 27161
[83] Kaiser, M.W. et al.; 1999, Journal of Biological Chemistry, 30: 21387 - 21394

the same DNA sample is necessary, if three or all four bases are possible at the polymorphic position. This is found frequently within the MHC region.
The GOOD assay uses the different masses of the bases and their base-combinations for allele identification. An extension primer, with modifications at the 3'-end, is annealed immediately next to the polymorphic base. The hybridisation is followed by a single base primer extension with α-S-ddNTPs complementary to the SNP allele by a DNA polymerase.
The extension primer contains two modifications. First the last two bridges of the backbone or in some cases the last three at the 3'-end are phosphorothioates. The OH-group attached to the phosphate is replaced by a SH-group. This modification has two reasons. On one hand it inhibits the 5'-phosphodiesterase digestion of for those bases, and on the other hand it allows quantitative neutralization of the negative charges of the backbone by an alkylation reaction, which is very useful for the mass spectrometry.
The second modification of the primer is an additional amino function at the second base from the 3'-end. This amino function is a reaction site for a "charge tag" (CT). Charge tagging of the amino-modified oligonucleotides[84] is performed using different tri-alkylammoniumalkyryl-N-hydroxy succinimidyl esters, prepared according to the procedure described by Bartlet-Jones et al.[85]. The charge tags are commercially available from Bruker Saxonia, Leipzig. The main functions of the charge tag are to bring one fixed positive charge into the extension primer, and to allow a mass shift for multiplex analyses. After the phosphodiesterase digest a core sequence of the 3'-end remains. The primer fragment contains the last three or four 3'-end-bases of the primer, the charge tag and the extended base.
The last step of the assay is the preparation for the MALDI-TOF mass spectrometric analysis. For this the products have to be alkylated. This takes place for two reasons. The first aim is the charge neutralisation of the backbone of the core sequence, so that only the fixed positive charge of the charge tag is left. The other reason of the alkylation is purification by extraction. The alkylation procedure results in a biphasic system. The lower phase contains all salts and enzymes from the cycling and incubation steps. The upper phase contains the

[84] Gut, I.G. et al.;1997, Rapid Communication in Mass Spectrometry, 11: 43 - 50
[85] Bartlet-Jones et al.;1994, Rapid Communication in Mass Spectrometry, 8: 737 - 742

DNA and primer fragments. An aliquot of the upper phase is diluted in acetronitrile/water, and the resulting dilution is transferred onto a matrix-precoated MALDI target.

1.6.1 MALDI-TOF-MS technology and polymorphism analysis

In the last 15 years mass spectrometry has revolutionized the analysis of bio-molecules[86,87]. This has been appreciated by the award of the Nobelprize in Chemistry to John B. Fenn and Koichi Tanaka in 2002. In 1988 they developed soft desorption/ionisation methods for the mass spectrometric analysis of biological macromolecules. In the same year Karas and Hillenkamp developed a technology, which allows the mass spectrometric analysis of bio-molecules without fragmentation of the molecule. This technology is used for the Matrix-assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF-MS). To transfer the analyte into the gas-phase the MALDI-TOF-MS technology uses a laser with a wavelength of 337 nm and a matrix which strongly absorbs the light of the laser. The absorbed energy causes desorption of the matrix into the gas-phase. At the same time the matrix assists the analyte molecules, which were co-crystallized with the matrix, to desorb. The analyte molecules are ejected into the gas-phase.
The field of DNA analysis by mass spectrometry was recently extensively reviewed by Tost and Gut[88] and Sauer and Gut[89]. MALDI has been applied to the analysis of DNA in variations that range from the analysis of PCR products to approaches using allele-specific termination to single nucleotide primer extension reactions and sequencing[90, 91, 92, 93, 94, 95, 96 ,97, 98]. These methods are used to genotype

[86] Karas, M. and Hillenkamp, F.; 1988, Analytical Chemistry, 60: 2299 - 2301
[87] Tanaka, K. et al.; 1988, Rapid Communications n Mass Spectrometry, 2: 151 - 153
[88] Tost, J. and Gut, I.G.; 2002, Mass Spectrometry Reviews, 21: 388 - 418
[89] Sauer, S. and Gut, I.G.; 2002, Journal of Chromatography B, 782: 73 - 87
[90] Liu, Y.-H., et al.; 1995, Rapid Communications in Mass Spectrometry, 9 : 735 - 743
[91] Ch'ang, L.-Y., et al.; 1995, Rapid Communications in Mass Spectrometry, 9 : 772 - 774
[92] Little, D.P., et al.; 1997, Journal of Molecular Medicin, 75 : 745 - 750
[93] Haff, L. and Smirnov, I.P.; 1997, Genome Research, 7 : 378 - 388
[94] Fei, Z. et al.; 1998, Nucleic Acids Research, 26 : 2827 - 2828
[95] Ross, P. et al.; 1998, Nature Biotechechnology, 16: 1347 - 1351
[96] Ross, P.L. et al.; 1997, Analytical Chemistry, 69: 4197 - 4202
[97] Griffin, T.J. et al.; 1997, Nature Biotechnology, 15: 1368 - 1372
[98] Köster, H., Higgins, G.S & Little, D.P.; US Patent 6,043,031

previously identified mutations, SNPs, or insertion/deletions (indels). Spin column purification and/or magnetic bead technology, reversed-phase purification, or ion- exchange resins are frequently applied for purification prior to mass spectrometric analysis.

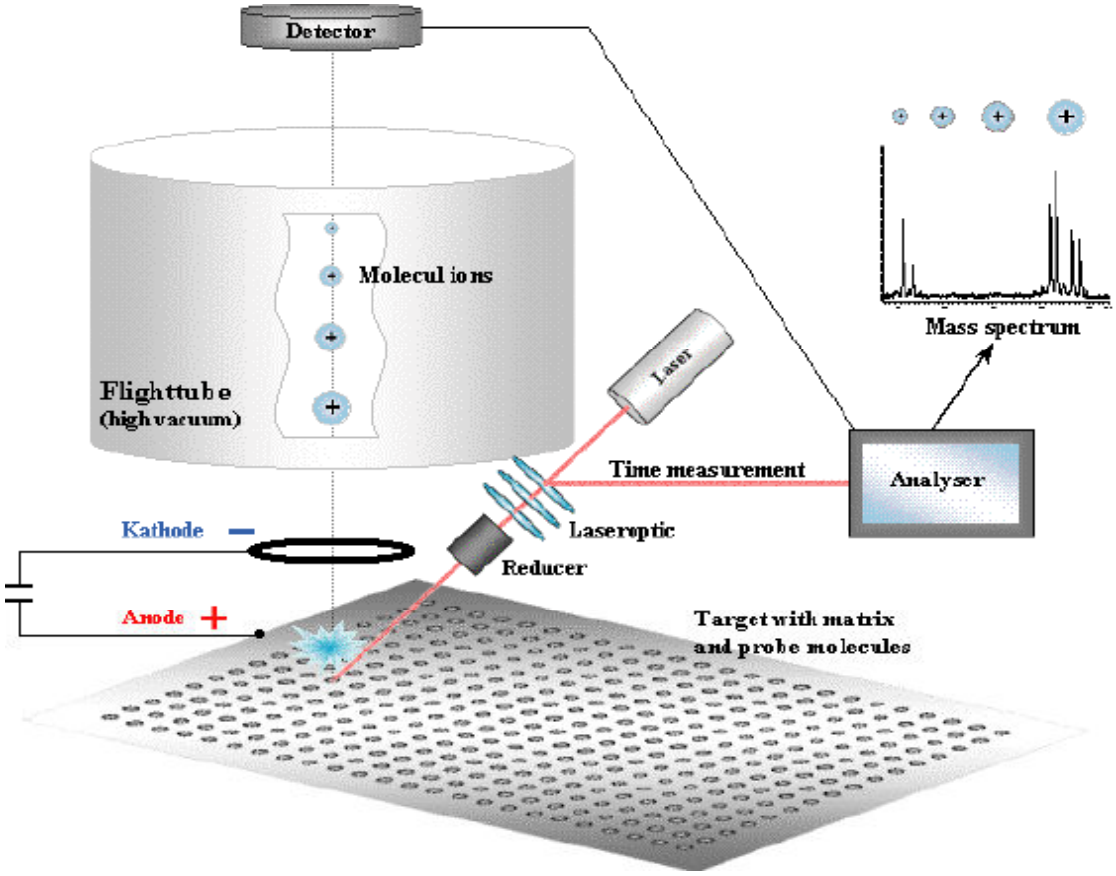

Figure 11: Principle of a MALDI-TOF mass spectrometer. The picture was kindly provided by Ole Brand.

1.7 Technologies in MADO

To find a suitable typing technology or combination of technologies, the project started with a set of six quite different methods of DNA typing. These were sequence-based typing as the "Golden Standard", since it is actually the only method to discover new alleles, and it gives the highest resolution at present, genotyping of microsatellites, DNA array technology, "Reference Strand Conformation Analysis", the Luminex based SNP genotyping and SNP

genotyping by mass spectrometry (GOOD assay). The last two methods were proposed by the CNG.

1.8 Objectives of this Thesis

In the framework of the MADO project a mass spectrometry based method should be developed. As a starting point the GOOD assay should be used. A main advantage of this assay format is the circumvention of purification steps with the help of chemical modification strategies – charge tagging and alkylation of the extension primers. The charge tagging strategy enables also accurate multiplex primer extension reaction for allele-discrimination with unambiguous assignment of the products.

The DNA sequences of the HLA genes are characterised by a very large number of polymorphisms in the exons 2 and 3 of class I genes (e.g. HLA-A and HLA-B), and in exon 2 of class II genes. The polymorphisms are SNPs, deletions and insertions. These polymorphisms should be analysed for their information content in the context of the identification of HLA alleles. The most informative polymorphisms will be selected for genotyping. The number of polymorphisms which will be selected will be based on economic aspects. The high density of polymorphisms is one of the largest difficulties for the implementation of a primer extension genotyping assay, like the GOOD assay in the HLA region. Polymorphisms are so close to each other that the extension primer will cover several polymorphisms. This can cause false-positive or false-negative results. This could be solved by using a set of primers to cover all possible sequences for a certain position in the HLA gene sequence. However, this creates other problems such as cross complementary extension of the primers.

An additional difficulty is the final analysis of the generated genotyping data to HLA alleles. All generated genotypes have to be brought together to identify the HLA type of the individual. Software, which is able to do this, needs to be developed.

1.8.1 Outlook on results

One outstanding trait of the DNA sequences of the HLA genes is the high density of polymorphisms. These were analyzed for the informativity in terms of an identification of the different alleles of the HLA genes HLA-A, HLA-B and HLA-DRB1. In this investigation the polymorphisms within the HLA gene sequences, which are polymorphisms of mostly single bases, have been combined to microhaplotypes, which expand to sequences of four to five bases. These microhaplotypes have significantly higher informativity about the present sequences than the single nucleotide polymorphisms. After investigation of the HLA sequences based on microhaplotypes a set of microhaplotype markers were selected. To genotype these markers a set of extension primers was pooled, where for each possible sequence, respectively microhaplotype, at least one primer is present. Thus pools of two to eleven extension primers were subjected to single base primer extension, which covered up to eleven microhaplotypes per marker. Microhaplotypes were measured by mass spectrometry which allows decoding the extended primer and the added base of both alleles of the tested DNA sample in a single reaction. The resulting microhaplotypes per marker and sample DNA were aligned to combinations of microhaplotypes. These alignments were compared to known HLA allele sequences. With this strategy a close estimation of the HLA types of the sample DNA could be achieved.