2 Materials and Methods

2.1 Materials

Material	Provider
Mastercycler®*Gradient* Thermocycler (96 and 384 well format)	Eppendorf AG (Hamburg, Germany)
PrimusHT Thermocycler	MWG Biotech AG (Ebersberg, Germany)
Microbiological Incubator	Binder GmbH (Tuttinglen, Germany)
Thermowell Sealing Tape	Corning Inc. (New York, USA)
Thermosealer	Applied Biosystems (Foster City, USA)
96 and 384 well plates	ABgene (Epsom, UK)
Savant Speed Vac System	Avantec (Illkirch, France)
Ultraspec spectrophotometer	Applied Biosystems (Foster City, USA)
Electrophoresis Power Supplier EPS 3501	Amersham Bioscience (Buckinghamshire, UK)
Imagemaster VDS	Amersham Bioscience (Buckinghamshire, UK)
LISCAP Image Software	Amersham Bioscience (Buckinghamshire, UK)
BRUKER Autoflex™ mass spectrometer	Bruker Daltonik (Bremen, Germany)
BRUKER Reflex III TOF-MS	Bruker Daltonik (Bremen, Germany)
Eppendorf Centrifuge 5415 D	Eppendorf AG (Hamburg, Germany)
MultiFuge 3 S-R	Heraeus GmbH (Hanau, Germany)
TAP Liquid Handler – Baseplate	The Automation Partnership (Royston, UK)
„Promega DNA“	Promega Corp. (Madison, USA)
DNA ladder	Promega Corp. (Madison, USA)

TMA31FS DNA Polymerase	Roche Molecular Diagnostics (Alameda, USA)
ThermoSequenase™	Amersham Bioscience (Buckinghamhire, UK)
Electrophoresis chamber	Bio-Rad Laboratories, Inc. (Hercules, USA)
SeaPlaqueTM low melting agarose	FMC (Philadelphia, USA)
Platinum™ Taq DNA polymerase High Fidelity	Invitrogen Life Technologies (Carlsbad, USA)
dNTPs	Amersham Bioscience (Buckinghamhire, UK)
PCR- and extension-primers	BioTez GmbH (Berlin, Germany)
Exonuclease I	New England Biolabs (Hertfordshire, UK)
Shrimp Alkaline Phosphatase (SAP)	USB Corp. (Cleveland, USA)
ThermiPol	Solis Biodyne (Tartu, Estonia)
α-S-ddNTPs	Biolog (Bremen, Germany)
TRIS-Base	Sigma-Aldrich Corporate (St. Louis, USA)
Phosphordiesterase II (PDE)	Sigma-Aldrich Corporate (St. Louis, USA)
Triethylamine	Sigma-Aldrich Corporate (St. Louis, USA)
Methyljodate	Sigma-Aldrich Corporate (St. Louis, USA)
Acetonitrile for Mass Spectrometry	Sigma-Aldrich Corporate (St. Louis, USA)
α-cyano-4-hydroxy-cinnamic acid methyl ester matrix (α-CNME)	BRUKER Saxonia (Leipzig, Germany)
Charge Tag reagents	CNG in-house synthesis by Florence Mauger and Jörg Tost, PhD, following the protocols by Bartlet-Jones et al. (1994)
Ammoniumacetat	Sigma-Aldrich Corporate (St. Louis, USA)
Ammoniumcitrat	Sigma-Aldrich Corporate (St. Louis, USA)

Ethanol	Sigma-Aldrich Corporate (St. Louis, USA)
Aluminium-nickel coated full stainless steel targets	Bruker Daltonik (Bremen, Gernamy)

Table 1: Materials and Providers

2.2 Explanatory Remarks about DNA samples

For this thesis a set of DNA samples of differing qualities were used. All basic developments for the PCR amplification and extension reactions were done with "Promega-DNA". This DNA is pooled DNA samples from different individuals and is commercially available in high quality at relatively low costs. Therefore it is very practical for material-consuming method optimizations.
For fine tuning after basic development of the assay a panel of DNA samples from the CEPH (**C**entre d'**E**tude du **P**olymorphisme **H**umain[99]) families collection was used[100]. CEPH families are of different ethic origin and their MHC is very well studied. EBV transformed cultures were prepared for these samples, so that large amounts of this DNA are available. For most of the individuals in the CEPH families the HLA types of HLA-A, HLA-B and HLA-DRB1 are known. However, in cases of unknown HLA types the family relationship can be used to verify genotyping results as well, since the results should follow Mendel's law of inheritance.

2.3 Nomenclature of PCR- and Extension-Primers

The names of the PCR primers of the loci HLA-A and HLA-B are based on the names in the technical manual of the IHWG[101,102,103]. A suffix was added to the published primer names that indicates the orientation and the length of the primer. (e.g. AAmp1_f21) AAmp1 is the name used by Wu et al. The suffix _f21 indicates that the primer is forward orientated and 21 bases long.

[99] http://www.ceph.fr
[100] http://locus.umdnj.edu/nigms/ceph/ceph.html
[101] Wu, J. et al.; http://www.ihwg.org/tmanual/TMcontents.htm, Chapter 10-B.
[102] Hurley, CK. Et al.; http://www.ihwg.org/tmanual/TMcontents.htm, Chapter 2-B-1
[103] Pozzi, S. et al.; http://www.ihwg.org/tmanual/TMcontents.htm, Chapter 8-B

The names of the markers of the extension reaction indicate the gene, location of the microhaplotype which is genotyped by this marker and the orientation of the microhaplotype. First the gene in which the microhaplotype is located is named (e.g. HLAA = HLA-A, HLAB = HLA-B and DRB1 = HLA-DRB1). The next two or three digits indicate the position of the base, which is added in the extension reaction, in the cDNA sequence of the HLA gene (HLAA_81, DRB1_125). The final two positions in the name indicate the orientation of the microhaplotype (r = reverse and f = forward), and the length of the microhaplotype. 1 denotes three bases underneath the primer and gives a four-base microhaplotype, 2 denotes four bases underneath the primer and gives a five-base microhaplotype. For example the marker HLAA_123r2 means that the corresponding microhaplotype is located in the gene HLA-A, and this microhaplotype is to the base at position 123 plus four bases in reverse direction. This marker is therefore used to analyze the five-base microhaplotypes spanning from position 123 to 127.
The nomenclature of the extension primers is based on the names of the markers they belong to. For instance, the primer DRB1_1255_1r20 is the fifth primer in the pool of the marker DRB1_125r1 and 20 bases long, which is indicated by the last two digits in the name.

2.4 Generic PCR Amplifications of Target Loci

For HLA-DRB1 amplification each sample contained 1.5 µl of each PCR primer (10 pmol/µl), 2.5 µl of a mixture of all four dNTPs (2 mM each dNTP), 4.2 µl of a 10x buffer (provided with the polymerase), 1.7 µl $MgSO_4$ (50 mM) 20 µl DNA (5 ng/µl) and 1 µl Platinum™ Taq DNA polymerase High Fidelity (5 U/µl) and 5.6 µl bi-distilled water. The reactions were denatured for 3 min at 94°C, then thermocycled 40 s at 94°C, 40 s at 61°C and 40 s at 72°C 30 times. This amplification was concluded with an incubation step for 4 min at 72°C.

Primer	Alleles which are amplified	Primer Sequences
Amp1_DRB1_f20	DRB1*0101 to DRB1*0111	5′-TTCTTGTGGSAGCTTAAGTT-3′
Amp2_DRB1_f21	DRB1*150101 to DRB1*1608	5′-TTCCTGTGGCAGCCTAAGAGG-3′
Amp3_DRB1_f22	DRB1*110101 to DRB1*1448 (except all alleles of Amp3-2_DRB1_f22, DRB1*1410, DRB1*1130 and DRB1*1122)	5′-CACGTTTCTTGGAGTACTCTAB-3′
Amp3-2_DRB1_f23	DRB1*1105, DRB1*120101 to DRB1*1209, DRB1*1317, DRB1*140301, DRB1*1411, DRB1*1415, DRB1*1428, DRB1*1431	5′-CGTTTCTTGGAGTACTCTACGGG-3′
Amp3-3_DRB1_f23	DRB1*030101 to DRB1*140302	5′-CGTTTCTTGGAGTACTCTACGTC-3′
Amp4_DRB1_f21	DRB1*0401 to DRB1*0450, DRB1*1122, DRB1*1410	5′-GTTTCTTGGAGCAGGTTAAAC-3′
DR7_DRB1_f20	All alleles of DRB1*07xx	5′-CCTGTGGCAGGGTAARTATA-3′
DR9_DRB1_f18	All alleles of DRB1*09xx	5′-CCCAACCACGTTTCTTGA-3′
DR10_DRB1_f19	All alleles of DRB1*10xx	5′-AGACCACGTTTCTTGGAGG-3′
AmpB_DRB1_r18	All alleles of DRB, used as reverse primer	5′-TCGCCGCTGCACYGTGAA-3′

Table 2: PCR primers of the HLA-DRB1 amplification

For HLA-A amplification each sample contained 7.5 µl of each PCR primer (7.5 pmol/µl), 5 µl of a mixture of all four dNTPs (2 mM each dNTP), 5 µl of a 10x buffer (provided with the polymerase), 1.5 µl $MgSO_4$ (50 mM) 20 µl DNA (5 ng/µl) and 0.9 µl Platinum™ Taq DNA polymerase high fidelity (5 U/µl) and 2.6 µl bi-distilled water. The reactions were denatured for 3 min at 94°C, then thermocycled 40 s at 94°C, 45 s at 70°C and 60 s at 72°C, 30 times. The reaction was concluded with incubation for 4 min at 72°C.

For HLA-B amplification each sample contained 2.5 µl of each PCR primer (20 pmol/µl), 2.5 µl of a mixture of all four dNTPs (2 mM each dNTP), 4.2 µl of a 10x buffer (provided with the polymerase), 1.7 µl $MgSO_4$ (50 mM) 20 µl DNA (5 ng/µl) and 1 µl Platinum™ Taq DNA polymerase High Fidelity (5 U/µl) and 8.1 µl bi-distilled water. The reaction were denatured for 3 min at 94°C, then thermocycled 30 s at 94°C, 20 s at 68.5°C and 20 s at 72°C, 30 times. The amplification was concluded with incubation for 4 min at 72°C.

Primer	**Primer Sequences**
HLA-A	
AAmp1_f21	5′-GGCCTCTGYGGGGAGAAGCAA-3′
AAmp2_r22	5′-GTCCCAATTGTCTCCCCTCCTT-3′
HLA-B	
Bx1_f22	5′-GGGAGGAGCGAGGGGACCSCAG-3′
Bx1-2_f22	5′-GGGAGGAGAGAGGGGACCGCAG-3′
Bx1-3_f22	5′-GGGAGGAGCAAGGGGACCGCAG-3′
BINT3_r23	5′-GGAGGCCATCCCCGGCGACCTAT-3′
BINT3-2_r23	5′-GGAGGCCATCCCGGGCGATCTAT-3′

Table 3: PCR primers of the HLA-A and HLA-B amplification

2.5 Gel Electrophoresis

PCR products were separated by gel electrophoresis on 1.5% agarose gel (1 g agarose dissolved in 75 ml 0.5x TBE buffer) with a migration length of about 10 cm to determine the success of the PCR. During the gel preparation 1.5 µl of 10 % ethidium bromide solution were added. 5 µl loading buffer was mixed with 5 µl of the PCR and the total volume of each mix was dispensed into wells of the agarose gel. Further 5 µl 100 bp DNA ladder (diluted 1:10 in loading buffer) was loaded into the first and last well of each row. Samples were separated by electrophoresis at 110 mA for approximately 30 minutes. Ethidium bromide intercalated into the DNA double strand and is visualized with UV light. Gels were digitally photographed.

2.6 Exonuclease I (EXO I) and Shrimp Alkaline Phosphatase (SAP) Digest

To remove residual dNTPs and PCR primers 33 µl of EXO I/SAP mixture were added to each PCR product, and the plate sealed with Thermowell Sealing Tape. The mixture contained 4 U SAP and 5 U EXO I dissolved in 50 mM Tris-buffer. After incubation at 37°C in a microbiological incubator for 1 hour the reaction was heated to 90°C for 10 min in a Mastercycler thermocycler to inactivate the enzymes.

2.7 Extension Reaction

The extension reaction is the core step for allele discrimination of this approach. The compositions for the different reactions are listed in tables 4 to 6.

Marker		**HLADR_122r2**	**DRB1_125r1**	**DRB1_196f1**	**DRB1_197r1**	**DRB1_227f1**
Water		0,02	0,52	0,62	0,125	0,72
$MgCl_2$	50 mM	0,6	0,6	0,6	0,6	0,6
α-S-ddATP	10 mM	0,02	0,02	0,02	0,025	0,04
α-S-ddCTP	10 mM	0,02	0,02	0,02	-	-
α-S-ddGTP	10 mM	0,02	0,02	0,02	0,025	-
α-S-ddTTP	10 mM	0,02	0,02	0,02	0,025	0,04
Primers	25 pmol/µl	1	0,6	0,5	1	0,4
ThermiPol	5 U/µl	0,3	0,2	0,2	0,2	0,2
Cycler-Program		Coramp56	Coramp56	Coramp56	Coramp56	Coramp56
Marker		**DRB1_261r1**	**DRB1_286f1**	**DRB1_299f1**	**DRB1_308r1**	**DRB1_341f1**
Water		0,025	0,725	0,025	0,62	0,92
$MgCl_2$	50 mM	0,6	0,6	0,6	0,6	0,6
α-S-ddATP	10 mM	0,025	0,025	0,025	0,02	-
α-S-ddCTP	10 mM	0,025	-	0,025	0,02	0,04
α-S-ddGTP	10 mM	0,025	0,025	0,025	0,02	-
α-S-ddTTP	10 mM	-	0,025	-	0,02	0,04
Primers	25 pmol/µl	1	0,4	0,9	0,5	0,2
ThermiPol	5 U/µl	0,3	0,2	0,4	0,2	0,2
Cycler-Program		Coramp56	Coramp56	Coramp58	Coramp56	Coramp56

Table 4: Composition of extension reactions of HLA-DRB (in µl)

Marker		**HLAA_98r2**	**HLAA_413r1**	**HLAA_539f1**	**HLAA_282f1**
Water		0,425	0,55	0,665	0,55
$MgCl_2$	50 mM	0,6	0,6	0,6	0,6
α-S-ddATP	10 mM	0,025	-	0,025	-
α-S-ddCTP	10 mM	-	0,04	-	0,04

α-S-ddGTP	10 mM	0,025	-	0,025	0,04
α-S-ddTTP	10 mM	0,025	0,04	0,025	-
Primer	25 pmol/µl	0,7	0,57	0,46	0,52
ThermiPoL	5 U/ µl	0,2	0,2	0,2	0,25
Cycler Program		Coramp62	Coramp58	Coramp58	Coramp56
Marker		**HLAA_268r2**	**HLAA_453r1**	**HLAA_527f1**	**HLAA_502r2**
Water		0,62	0,87	0,92	0,975
$MgCl_2$	50 mM	0,6	0,6	0,6	0,6
α-S-ddATP	10 mM	-	-	0,02	-
α-S-ddCTP	10 mM	-	-	0,02	0,025
α-S-ddGTP	10 mM	0,04	0,04	0,02	0,025
α-S-ddTTP	10 mM	0,04	0,04	0,02	0,025
Primer	25 pmol/µl	0,5	0,25	0,2	0,15
ThermiPoL	5 U/ µl	0,2	0,2	0,2	0,2
Cycler Program		Coramp54	Coramp58	Coramp58	Coramp60
Marker		**HLAA_123r2**	**HLAA_355f2**	**HLAA_241f1**	**HLAA_299r2**
Water		0,75	0,975	0,66	0,855
$MgCl_2$	50 mM	0,6	0,6	0,6	0,6
α-S-ddATP	10 mM	0,04	0,025	-	0,025
α-S-ddCTP	10 mM	-	0,025	0,04	-
α-S-ddGTP	10 mM	0,04	0,025	0,04	0,025
α-S-ddTTP	10 mM	-	-	-	0,025
Primer	25 pmol/µl	0,37	0,15	0,46	0,27
ThermiPoL	5 U/ µl	0,2	0,2	0,2	0,2
Cycler Program		Coramp62	Coramp64	Coramp64	Coramp66
Marker		**HLAA_571f2**	**HLAA_259f2**	**HLAA_81f1**	**HLAA_559r1**
Water		0,81	0,695	0,125	0,56
$MgCl_2$	50 mM	0,6	0,6	0,6	0,6
α-S-ddATP	10 mM	-	0,025	0,025	0,02
α-S-ddCTP	10 mM	-	0,025	0,025	0,02
α-S-ddGTP	10 mM	0,04	0,025	0,025	0,02
α-S-ddTTP	10 mM	0,04	-	-	0,02
Primer	25 pmol/µl	0,31	0,43	1	0,56
ThermiPoL	5 U/ µl	0,2	0,2	0,2	0,2
Cycler Program		Coramp62	Coramp66	Coramp60	Coramp58

Table 5: Composition of extension reactions of HLA-A (in µl)

Marker		**HLAB_539f1**	**HLAB_419f2**	**HLAB_559r1**	**HLAB_412f2**	**HLAB_272f1**	**HLAB_362f2**
Water		0,825	0,025	0,125	0,125	0,42	0,12
$MgCl_2$	50 mM	0,6	0,6	0,6	0,6	0,6	0,6
α-S-ddATP	10 mM	0,025	0,025	-	0,025	0,02	0,02
α-S-ddCTP	10 mM	-	-	0,025	0,025	0,02	0,02
α-S-ddGTP	10 mM	0,025	0,025	0,025	0,025	0,02	0,02
α-S-ddTTP	10 mM	0,025	0,025	0,025	-	0,02	0,02

Primers	25 pmol/µl	0,3	1	1	0,8	0,5	0,8
ThermiPol	5 U/µl	0,2	0,3	0,2	0,4	0,4	0,4
Cycler-Program		Coramp58	Coramp58	Coramp58	Coramp58	Coramp58	Coramp62
Marker		**HLAB_302f2**	**HLAB_363r2**	**HLAB_206f1**	**HLAB_369r1**	**HLAB_259f2**	**HLAB_97f2**
Water		0,32	0,525	0,32	0,825	0,32	0,825
$MgCl_2$	50 mM	0,6	0,6	0,6	0,6	0,6	0,6
α-S-ddATP	10 mM	0,04	0,025	0,02	0,025	0,04	-
α-S-ddCTP	10 mM	-	0,025	0,02	-	-	0,025
α-S-ddGTP	10 mM	0,04	0,025	0,02	0,025	0,04	0,025
α-S-ddTTP	10 mM	-	-	0,02	0,025	-	0,025
Primers	25 pmol/µl	0,8	0,4	0,6	0,3	0,8	0,3
ThermiPol	5 U/µl	0,2	0,4	0,4	0,2	0,2	0,2
Cycler-Program		Coramp58	Coramp58	Coramp56	Coramp58	Coramp58	Coramp58
Marker		**HLAB_583r1**	**HLAB_292f2**	**HLAB_222r1**	**HLAB_527f1**	**HLAB_435r1**	**HLAB_571r1**
Water		0,92	0,52	0,82	0,825	0,52	0,62
$MgCl_2$	50 mM	0,6	0,6	0,6	0,6	0,6	0,6
α-S-ddATP	10 mM	0,04	-	-	0,025	-	0,04
α-S-ddCTP	10 mM	-	-	0,04	0,025	0,04	0,04
α-S-ddGTP	10 mM	0,04	0,04	-	-	-	-
α-S-ddTTP	10 mM	-	0,04	0,04	0,025	0,04	-
Primers	25 pmol/µl	0,2	0,6	0,3	0,3	0,4	0,3
ThermiPol	5 U/µl	0,2	0,2	0,2	0,2	0,4	0,4
Cycler-Program		Coramp58	Coramp58	Coramp58	Coramp58	Coramp58	Coramp58

Table 6: Composition of extension reactions of HLA-B (in µl)

5 µl of the EXO I/SAP treated PCR products was distributed into 384-well plates, and covered with 3 µl mineral oil. Primer extension master mixes sufficient for all samples in each marker run were prepared on ice. 2 µl primer extension mix was dispensed onto the prepared PCR products. The plates were sealed with a thermosealer and thermocycled in a PrimusHT Thermocycler.

Different cycling conditions were used for extension reactions of the primer pools. These are indicated in row "Cycler Program" in tables 4 to 6. They are identical, except for the annealing temperature, which is indicated by the number in the name of the programs. All programs start with an initial denaturation step of 4 min at 94°C. This was followed by 30 cycles of 15 sec at 94°C, 20 sec at 54°C (in case of Coramp54, Coramp56 = 56°C, Coramp58 = 58°C etc.) ramping up the temperature to 72°C with 0,5°C/ sec and 20 sec at 72°C. The reactions were finished with 4 min at 72°C.

2.8 Preparation of Extension Primers

2.8.1 Charge tagging of extension primers

To make the generated specific termination products uniquely distinguishable by their masses, different charge tags were attached to the aminogroups located at the second base from the 3'-end of the individual primers of a pool. The charge tags (CT) are a series of tri-alkylammoniumalkyryl-N-hydroxy succinimidyl esters (see table 7). Besides the very advantageous effect of charging the extension primer with a fixed positive charge, these CTs allow shifting of masses of the extension primer products. This is very useful in cases were the products of the extension have identical masses (e.g. AptT(NH_2)ptGptC and CptT(NH_2)ptGptA have the same base composition. Adding a different charge tag to each one of the aminogroup will separate them by mass.). With this attribute, charge tagging enabled multiplex detection even with mass-identical sequences, and the distinction of different sequences extended with the same base.

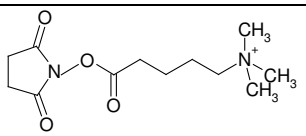
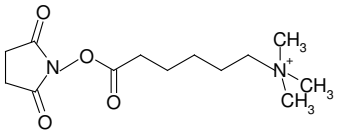
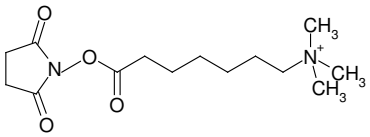
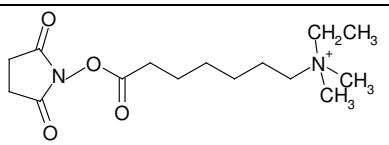
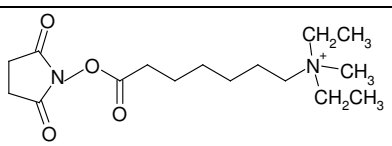
Charge Tag	**Chemical Name**	**Chemical Structure**
CT (-28)	6-trimethyl-ammoniumbutyryl-N-hydroxy-succinimidylester	
CT (-14)	6-trimethyl-ammoniumpentyryl-N-hydroxy-succinimidylester	
CT (0)	6-trimethyl-ammoniumhexyryl-N-hydroxy-succinimidylester	
CT (+14)	6-dimethyl-ethyl-ammoniumhexyryl-N-hydroxy-succinimidylester	
CT (+28)	6-diethyl-methyl-ammoniumhexyryl-N-hydroxy-succinimidylester	

Table 7: Charge Tag substrats

The charge tagging procedure is identical for all charge tags. The primers are diluted in water to a concentration c = 500 pmol/µl. 30 µl were mixed with 1.5 µl of 2 M tetraethylactate buffer (TEA/CO_2, pH ~ 8). 24 µl of a freshly prepared charge tag solution in H_2O was added and incubated on ice. The concentrations for the different CTs differ slightly. For CT (0) and CT (-14) 1 % solutions are used, for CT (-28) 0.75 % solution, for CT (+14) 1.4 % solution, and for CT (+28) 1.4 % solution.

After incubation for 30 min at 0°C the solutions were lyophilized and resuspended in 15 µl 300 mM ammonium acetate, and precipitated by adding 60 µl ethanol. To get a good yield of charge tagged extension primer the precipitate was kept at -20°C for at least 2 hours. The best yields were achieved by keeping the precipitate at -20°C for ~ 10 hours. After the precipitation was completed the tubes were centrifuged for 10 min at 13´000 rpm. The supernatants were removed and the pellets dissolved in 30 µl water. Finally the yields of products were measured and the primers diluted to a concentration of 25 pmol/µl. To determine the yield 1 µl charge tagged extension primer was diluted in 999 µl water and the optical density was measured at a wavelength of 260 nm (OD_{260}). With the OD_{260} and the mass of the primer the concentration of a primer after charge tagging was calculated.

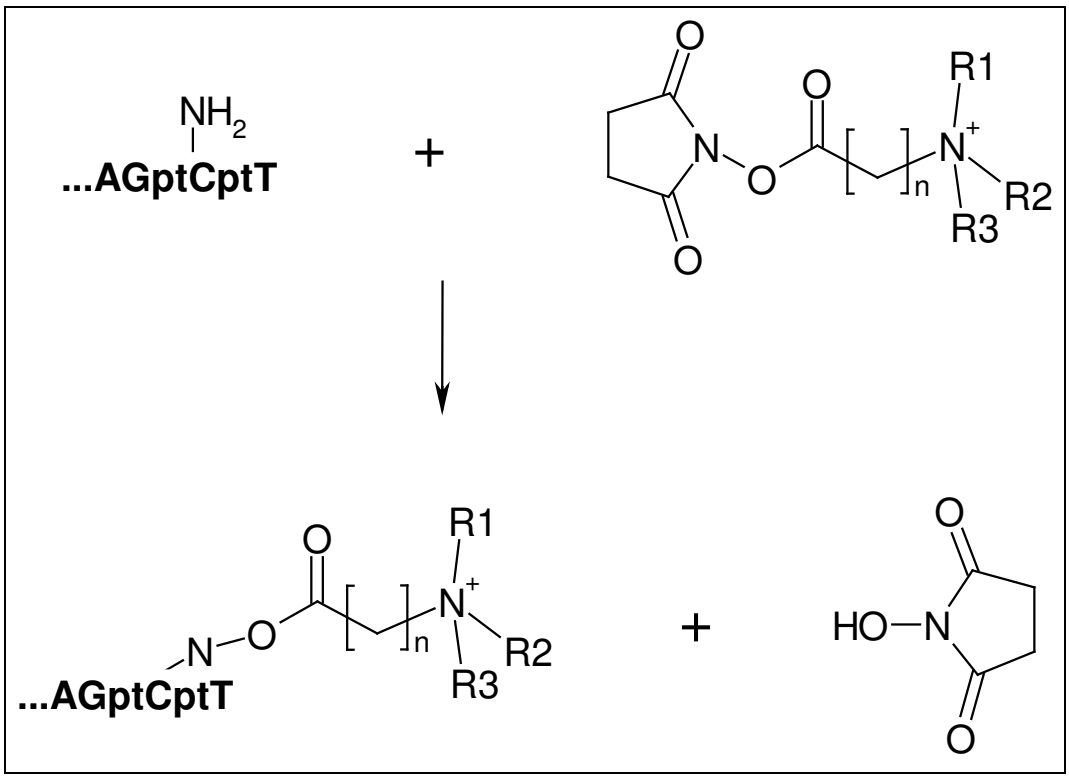


Figure 12: The charge tag reaction of an amino-modified primer (3'- sequence in bold) and a charge tag reagent (6-trialkylammoniumalkyryl-N-hydroxy succinimidylester). (Picture modified from Sascha Sauer, PhD[104])

The quality of charge tagging was routinely verified by MALDI-MS. For the quality control of charge tagged extension primers 0.5 µl HPA matrix was transferred onto a MALDI target, and 0.3 µl of solution of charge tagged primer was added to the still wet matrix. This mix dried on the MALDI target and formed a crystalline layer.
Usually, no residual starting material was observed, from which was concluded that more than 95 % of the amino-modified primers were converted into charge tagged primers.

2.8.2 Pooling of Extension Primers

For each marker a pool of extension primers is used for the extension reaction. Thus, at least one primer is in the pool that is completely complementary in sequence for each known HLA allele. Mismatches that are more than 4 bases from the 3'-end of the primer do not affect the result of the assay, as all of those

[104] Sauer, S.; 2001, PhD thesis, Free University Berlin

bases are removed by 5′ phosphodiesterase after the primer extension reaction. Primers of the pool containing mismatches in the last few bases are not extended by the DNA polymerase and duely not observable. Termination products for known alleles were generated by extending the perfectly hybridized primers with a complementary α-S-ddNTP with a DNA polymerase.

Of the primers in a pool more then one can carry the same 3′-end sequence. It is important for the MALDI-MS analysis to have almost equimolar proportions of product. Otherwise it can happen that a product, which is over-represented, suppresses another one. To avoid this situation the proportions of extension primers in the pools needs to be well balanced. Other criteria such as the creation of hairpins and self-annealing have been included in the adjustment.

The optimal primer pool compositions are listed below in tables 8 to 10. With these balances all alleles were sufficiently amplified and identified by MALDI-MS.

Marker	Name	Sequence	Parts	CT	Primer	A	C	G	T
DRB1_125r1	**DRB1_1251_1r20**	CATTGAAGAAATGACACTspC^spC	3	0	**1098,1**	**-**	**1392,3**	**-**	**-**
	DRB1_1252_1r20	CGTTGAAGAAATGACACTspT^spA	1,5	0	**1230,1**	**-**	**-**	**-**	**1548,5**
	DRB1_1253_1r20	CATTGAAGAAATGACATTspC^spA	1	0	**1113,1**	**1440,4**	**1416,3**	**1456,4**	**1431,3**
	DRB1_1254_1r20	CATTGAAGAAWTAACACTspC^spA	1	0	**1113,2**	**1440,4**	**1416,3**	**1456,4**	**1431,3**
	DRB1_1255_1r20	CRTTGAAGAAATGACACTspC^spA	1	0	**1113,3**	**1440,4**	**1416,3**	**1456,4**	**1431,3**
DRB1_196f1	**DRB1_1961_1f19**	CATCTATAACCAAGAGGspA^spA	7	-14	**1148,1**	**-**	**-**	**-**	**1466,3**
	DRB1_1962_1f19	CTTCTATCACCAAGARGspA^spG	1	0	**1178,1**	**1505,4**	**1481,3**	**1521,4**	**1496,3**
	DRB1_1963_1f19	CTTCTATAATCARGAGGspA^spG	1	0	**1178,1**	**1505,4**	**1481,3**	**1521,4**	**1496,3**
	DRB1_1964_1f19	CGTCCATAACCAAGAGGspA^spG	1	0	**1178,1**	**1505,4**	**1481,3**	**1521,4**	**1496,3**
	DRB1_1965_1f19	CATCTATAACCAAGAGGspA^spG	1	0	**1178,1**	**1505,4**	**1481,3**	**1521,4**	**1496,3**
	DRB1_1966_1f19	CTTCCATAACCRGGAGGspA^spG	1	0	**1178,1**	**1505,4**	**1481,3**	**1521,4**	**1496,3**
	DRB1_1967_1f19	CTTCGATAACCAGGAGGspA^spG	1	0	**1178,1**	**1505,4**	**1481,3**	**1521,4**	**1496,3**
	DRB1_1968_1f19	CTTCTATAACCTGGAGGspA^spG	1	0	**1178,1**	**1505,4**	**1481,3**	**1521,4**	**1496,3**
DRB1_197r1	**DRB1_1971_1r20**	CGTCGCTGTCGAAGCGCAspG^spG	2	0	**1178,1**	**1505,4**	**-**	**-**	**1496,3**
	DRB1_1972_1r20	CGTCGCTGTCGTAGCGCGspC^spG	3	0	**1154,1**	**-**	**-**	**-**	**1472,3**
	DRB1_1973_1r20	CGTCGCTGTCGAAGCGCAspA^spG	1,5	0	**1162,1**	**-**	**-**	**-**	**1480,3**
	DRB1_1974_1r20	CGTCGCTGTCGAAGYGCAspC^spG	2	-28	**1110,1**	**1437,4**	**-**	**1453,4**	**1428,3**
	DRB1_1975_1r20	CGTCGCTGTCGAASCGCAspC^spG	2	-28	**1110,1**	**1437,4**	**-**	**1453,4**	**1428,3**
DRB1_227f1	**DRB1_2271_1f20**	CGACAGCGACGTGGGGGAspC^spT	1	0	**1113,1**	**1440,4**	**-**	**-**	**-**
	DRB1_2272_1f20	CGACAGCGACGTGVGGGAspG^spT	1	0	**1153,1**	**1480,4**	**-**	**-**	**1471,3**
DRB1_261r1	**DRB1_2612_1r20**	TTCTGGCTGTTCCAGTACspC^spC	6	0	**1074,1**	**-**	**1377,3**	**-**	**-**
	DRB1_2613_1r20	TTCTGGCTGTTCCAGTAGspT^spC	2	0	**1231,2**	**-**	**1534,4**	**-**	**-**
	DRB1_2614_1r20	TTCTGGCTGTTCCAGTRCspT^spC	1,5	-14	**1177,2**	**1504,5**	**1480,4**	**1520,5**	**-**
	DRB1_2615_1r20	TTCYGGCTGTTCCAGGACspT^spC	1	-14	**1177,2**	**1504,5**	**1480,4**	**1520,5**	**-**

DRB1_286f1	**DRB1_2861_1f19**	CTGGAACAGCCAGAAGAspA^spC	2	-28	**1122,1**	**1449,4**	**-**	**-**	**-**
	DRB1_2862_1f19	CTGGAACAGCCRGAAGGspA^spC	1	0	**1138,1**	**1465,4**	**1441,3**	**-**	**1456,3**
DRB1_299f1	**DRB1_2991_In_1f20**	GAAGGACHTCCTIIAICAspG^spG	3	0	**1178,1**	**-**	**1481,3**	**-**	**-**
	DRB1_2992_In_1f20	GAAGGACATCCTIIIAIAspC^spA	1	-14	**1108,1**	**1435,1**	**-**	**1451,4**	**-**
	DRB1_2993_In_1f20	GAAGGACATCCTIIARIAspC^spA	1	-14	**1108,1**	**1435,1**	**-**	**1451,4**	**-**
	DRB1_2994_In_1f20	GAAGGACYTCCTIIAAIAspC^spA	1	-14	**1108,1**	**1435,1**	**-**	**1451,4**	**-**
	DRB1_2995_In_1f20	GAAGGACATCCTIIAICAspG^spA	3	0	**1162,1**	**1489,4**	**-**	**1505,4**	**-**
	DRB1_2996_In_1f20	GAAGGACHTCCTIIAICGspG^spA	3	0	**1178,1**	**-**	**-**	**1521,4**	**-**
	DRB1_2997_In_1f20	GAAGGACHTCCTIIAAIAspC^spG	3	0	**1138,1**	**1465,4**	**-**	**-**	**-**
DRB1_308r1	**DBR1_3081_1r20**	GTCTGCAATAGGTGTCCAspC^spG	5	0	**1138,1**	**-**	**1441,3**	**-**	**-**
	DRB1_3082_1r20	GTCTGCARTAGGCGTCCAspC^spC	1	-14	**1084,1**	**1411,4**	**1387,3**	**1427,4**	**1402,3**
	DRB1_3083_1r20	GTCTGCAGTAATTGTCCAspC^spC	1	-14	**1084,1**	**1411,4**	**1387,3**	**1427,4**	**1402,3**
	DRB1_3084_1r20	GTCTGCACACGGTGTCCAspC^spC	1	-14	**1084,1**	**1411,4**	**1387,3**	**1427,4**	**1402,3**
	DRB1_3085_1r20	GTCTGCAGTAGGTGTCCAspC^spC	1	-14	**1084,1**	**1411,4**	**1387,3**	**1427,4**	**1402,3**
	DRB1_3086_1r20	GTCTGCAATAGGTGTCCAspC^spC	1	-14	**1084,1**	**1411,4**	**1387,3**	**1427,4**	**1402,3**
DRB1_341f1	**DRB1_341_1f19**	TGCAGACACAACTACSGspG^spG	1	0	**1194,1**	**-**	**1497,3**	**-**	**1512,3**
HLADR_122r2	**HLADR_1221_2r20**	TGAAGAAATGACACTCAspTspG*spT	2	0	**1487,5**	**-**	**-**	**-**	**1805,7**
	HLADR_1222_2r20	TGCAGAAATAGCACTCGspTspG*spT	2	0	**1503,5**	**-**	**-**	**-**	**1821,7**
	HLADR_1223_2r20	TGAAGAAATGACACTCAspGspG*spT	2	0	**1512,5**	**-**	**-**	**-**	**1830,7**
	HLADR_1224_2r20	TGAAGAAATGACACTTAspTspA*spT	2	0	**1471,5**	**-**	**-**	**-**	**1789,7**
	HLADR_1225_2r20	TGAAGAAATGACACTCCspCspT*spC	2	-14	**1510,6**	**-**	**-**	**-**	**1814,8**
	HLADR_1226_2r20	TGAAGAAATRACACTCAspCspC*spC	2	-28	**1418,4**	**1717,7**	**1693,6**	**1733,7**	**-**
	HLADR_1227_2r20	TGAAGAAATGACACTCAspTspA*spC	2	-14	**1456,5**	**-**	**-**	**-**	**1760,7**
	HLADR_1228_2r20	TGAAGAAWTGACACTCAspGspA*spC	2	0	**1481,5**	**-**	**-**	**-**	**1799,7**
	HLADR_1229_2r20	TGAGGAAATGACACTCAspCspA*spC	1	-14	**1441,5**	**-**	**-**	**1770,8**	**1745,7**
	HLADR_12210_2r20	TGAAGATATGACACTCAspCspA*spC	1	-14	**1441,5**	**-**	**-**	**1770,8**	**1745,7**
	HLADR_12211_2r20	TGAAGAAATGACAYTCAspAspA*spC	2	0	**1465,5**	**-**	**-**	**-**	**1783,7**

Table 8: Sequences, proportions, charge tags and masses for MALDI-MS analysis of HLA-DRB

Marker	Name	Sequence	Parts	CT	Primer	A	C	G	T
HLAA_81f1	**HLAA_811_1f20**	TGCTCGCCCCCAGGCTCCspC^spA	4	-28	1070,1	1397,4	1373,3	-	-
	HLAA_812_1f20	TGCTCGCCCCCAGGCTCTspC^spA	1	0	1113,1	-	1416,3	1452,4	-
HLAA_98r2	**HLAA_981n_2r18**	GGCCGGGACACGGAGspGspT^spG	12	28	1658,6	-	-	-	1976,8
	HLAA_982_2r18	GGCCGGGACACGGATspGspT^spG	1	-28	1577,6	1904,9	-	1920,9	-
	HLAA_983_2r18	GGCCGGGACACGGAAspGspT^spG	1	0	1614,6	-	-	-	1932,8
HLAA_123r2	**HLAA_1231_2r20**	GCGATGAAGCGGGGCTCspCspT^spC	1	0	1510,5	-	-	1853,8	-
	HLAA_1232_2r20	GCGATGAAGCGGGGCTCspTspC^spC	2	-28	1380,4	1707,7	-	-	-
	HLAA_1233_2r20	GCGATGAAGCGGGGCTTspCspC^spC	2	0	1408,4	-	-	1751,6	-
	HLAA_1234_2r20	GMGATGAAGCGGGGCTCspCspC^spC	2,4	0	1393,4	1720,7	-	1736,7	-
HLAA_282f1	**HLAA_2821_1f20**	ACACGGAATGTGARGGGCspC^spA	1	0	1098,1	-	1401,3	1441,3	-
	HLAA_2822_1f20	ACASGGAAAGTGAAGGCCspC^spA	1	0	1098,1	-	1401,3	1441,3	-
	HLAA_2823_1f20	ACACGGCAWGTGAAGGCCspC^spA	1	0	1098,1	-	1401,3	1441,3	-
	HLAA_2824_1f20	ACACGGAACGTGAAGGCCspC^spA	1	0	1098,1	-	1401,3	1441,3	-
	HLAA_2825_1f20	ACACGGAATRTGAAGGCCspC^spA	2,5	0	1098,1	-	1401,3	1441,3	-

HLAA_453r1	**HLAA_4531_1r20**	GTCCAAGAGCGCAGGTCTspT^spC	1	0	1206,2	-	-	-	1524,4
	HLAA_4532_1r20	GTCCAAGAGCGCAGGTCCspT^spC	2	0	1191,2	-	-	1534,5	1509,4
	HLAA_4533_1r20	GTCCAGGAGCTCAGGTCCspT^spC	2	0	1191,2	-	-	1534,5	1509,4
HLAA_539f1	**HLAA_5391_1f19**	GCCCRTGAGGCGGAGCAspG^spC	5,33	0	1138,1	1465,4	-	1481,4	1456,3
	HLAA_5392_1f19	GYCCATGCGGCGGAGCAspG^spC	5,33	0	1138,1	1465,4	-	1481,4	1456,3
	HLAA_5393_1f19	GCCCGTCGGGCGGAGCAspG^spC	5,33	0	1138,1	1465,4	-	1481,4	1456,3
	HLAA_5394_1f19	GCCCATGTGGCGGAGCAspG^spC	5,33	0	1138,1	1465,4	-	1481,4	1456,3
	HLAA_5395_1f19	GTCCATGCGGCGGAGCAspG^spT	5,33	0	1153,1	-	-	1496,4	1471,3
	HLAA_5396_1f19	GCCCGTYGGGCGGAGCAspG^spT	1	0	1153,1	-	-	1496,4	1471,3
	HLAA_5397_1f19	GCCCATGAGGCGGAGCAspG^spT	1	0	1153,1	-	-	1496,4	1471,3
	HLAA_5398_1f19	GCCCWTGTGGCGGAGCAspG^spT	1	0	1153,1	-	-	1496,4	1471,3
	HLAA_5399_1f19	GCCMGTGTGGCGGAGCAspG^spT	1	0	1153,1	-	-	1496,4	1471,3
HLAA_559r1	**HLAA_5591_1r20**	GCGGAGCCACTCCACGCAspC^spT	2,5	-14	1099,1	-	1402,3	-	-
	HLAA_5592_1r20	GCGGAGCCCGTCCACGCAspC^spT	2,5	-14	1099,1	-	1402,3	-	-
	HLAA_5593_1r20	GCGGAGCCACTCCACGCAspC^spA	10	-28	1094,1	-	-	1437,4	-
	HLAA_5594_1r20	GCGGAGCCCGTCCACTCAspC^spG	1	0	1138,1	-	-	-	1456,3
	HLAA_5595_1r20	GCGGAGCCAGTCCACGCAspC^spG	1	0	1138,1	-	-	-	1456,3
	HLAA_5596_1r20	GCGGAGCCMGTCCACGCAspC^spG	1	0	1138,1	-	-	-	1456,3
	HLAA_5597_1r20	GCGGAGCCACTCCACGCAspC^spC	2,5	-28	1070,1	1397,4	-	1413,4	-
	HLAA_5598_1r20	GCGGAGCCCGTCCACGCAspC^spC	2,5	-28	1070,1	1397,4	-	1413,4	-
	HLAA_5599_1r20	GCGGAGCCACTCCACGCAspG^spG	5	0	1178,1	-	-	-	1496,3
HLAA_571f2	**HLAA_5711_2f20**	TGGAGGGCCKGTGCGTGspGspA^spG	1	0	1537,4	-	-	-	1855,6
	HLAA_5712_2f20	TGGAGGGYGAGTGCGTGspGspA^spG	1	0	1537,4	-	-	-	1855,6
	HLAA_5713_2f20	TGSAGGGCCGGTGCGTGspGspA^spG	1	0	1537,4	-	-	-	1855,6
	HLAA_5714_2f20	TGGATGSCACGTGCGTGspGspA^spG	1	0	1537,4	-	-	-	1855,6
	HLAA_5715_2f20	TGGAGGGCACSTGCGTGspGspA^spG	1	0	1537,4	-	-	-	1855,6
	HLAA_5716_2f20	TGGAGGGCACGTGMGTGspGspA^spC	2,66	-14	1483,4	-	-	1826,7	1801,6
	HLAA_5717_2f20	TGGAGGGCYGGTGCGTGspGspA^spC	2,66	-14	1483,4	-	-	1826,7	1801,6
HLAA_527f1	**HLAA_5271_1f20**	AGTGGGAGACTCCGCCCAspT^spG	1	-14	1241,3	1568,6	1544,5	-	1559,5
	HLAA_5272_1f20	CAAGTGGGAGGCGGYCCAspT^spG	1	-14	1241,3	1568,6	1544,5	-	1559,5
	HLAA_5273_1f20	CAAGTGGGAGRCGGCCCAspT^spG	1	-14	1241,3	1568,6	1544,5	-	1559,5
	HLAA_5274_1f20	CAAGTGGGAGGCGGCCCTspT^spG	2	28	1274,3	-	-	-	1592,5
	HLAA_5275_1f20	CAAGTGGGAGGCGGCCCGspT^spT	3	-14	1232,3	-	-	1575,6	-
	HLAA_5276_1f20	CAAGTGGGAGGCGGCCCGspT^spC	3	28	1259,3	-	-	1602,5	-
	HLAA_5277_1f20	CAAGTGGGAGGCGGCCMGspT^spG	4	28	1299,3	1626,6	-	-	1617,5
	HLAA_5278_1f20	CAAGTGGGAGGCRGCCCGspT^spG	4	28	1299,3	1626,6	-	-	1617,5
HLAA_413r1	**HLAA_4131_1r20**	TGCCGTCGTAGGCGTGCTspG^spT	10	28	1172,1	-	-	-	1490,3
	HLAA_4132_1r20	TGCCGTCGTAGGCGRACTspG^spG	4	14	1183,1	-	-	-	1501,3
	HLAA_4133_1r20	TGCCGTCGTAGGCATACTspG^spG	4	14	1183,1	-	-	-	1501,3
	HLAA_4134_1r20	TGCCGTMGTAGGCGTACTspG^spG	4	14	1183,1	-	-	-	1501,3
	HLAA_4135_1r20	TGCCGTCGTAAGCGTTCTspG^spC	1	0	1129,1	-	1432,3	-	1447,3
	HLAA_4136_1r20	TGCCGTCGTAGGCGTGCTspG^spC	1	0	1129,1	-	1432,3	-	1447,3
	HLAA_4137_1r20	TGCCGTCGTAGGCGACCTspG^spC	1	0	1129,1	-	1432,3	-	1447,3
	HLAA_4138_1r20	TGCCGTCGTAGGCGTACTspG^spC	1	0	1129,1	-	1432,3	-	1447,3
	HLAA_4139_1r20	TGCCGTCKTAGGCGTCCTspG^spC	1	0	1129,1	-	1432,3	-	1447,3
	HLAA_41310_1r20	TGCCGTCGTAAGCGTCCTspG^spC	1	0	1129,1	-	1432,3	-	1447,3
	HLAA_41311_1r20	TGCCGTCGTAAGCGTCCCspG^spC	10	-28	1086,1	-	-	-	1404,3

HLAA_241f1	**HLAA_2411_1f18**	TGGATARAGCRGGAGGspG^spG	1,66	-14	1180,2	-	1483,5	-	-
	HLAA_2412_1f18	TGGATAGAGCAGGAGGspG^spT	2,5	28	1197,1	-	1500,4	1540,5	-
	HLAA_2413_1f18	TGGATAGAGCAGGAGAspG^spG	1,33	-28	1150,2	-	1453,5	-	-
	HLAA_2414_1f18	TGGATAGAGCAGGAGAspG^spT	1	-14	1139,1	-	1442,4	-	-
	HLAA_2415_1f18	TGGATAGAGCAGGAGGspA^spG	1,16	14	1192,2	-	1495,5	-	-
HLAA_355f2	**HLAA_355_2f17**	GGCCAGGKWCTCACspAspC^spC	1	0	1418,4	1745,7	1721,7	1761,7	-
HLAA_299r2	**HLAA_2991_2r17**	GCGCGATCCGCAGGspTspT^spC	1	0	1565,6	-	-	1908,9	1883,9
	HLAA_2992_2r17	GCAGGGTCCCCAGGspTspT^spC	1	0	1565,6	-	-	1908,9	1883,9
	HLAA_2993_2r17	GCGCGATCCGCAGGspCspT^spC	2,6	0	1550,6	-	-	-	1868,9
	HLAA_2994_2r17	GCAGGGTCsCCAGGspTspC^spC	2,33	0	1449,5	1776,7	-	-	-
	HLAA_2995_2r17	GCAGGGTCCCCAGAspTspC^spC	2	0	1433,5	1760,8	-	-	-
HLAA_502r2	**HLAA_5021n_2r20**	GGCCGCCTCCCACTTGCspGspC^spC	2,33	-28	1405,4	-	-	-	1723,6
	HLAA_5022n_2r20	GGCCGYCTCCCACTTGTspGspC^spT	2,3	0	1463,4	-	-	-	1781,6
	HLAA_5023n_2r20	GGCCGTCTCCCACTTGCspGspC^spT	1	-14	1434,4	-	1737,6	1777,7	1752,6
	HLAA_5024n_2r20	GRCCGCCTCCCACTTGCspGspC^spT	1	-14	1434,4	-	1737,6	1777,7	1752,6
	HLAA_5025n_2r20	CGGAGTCTCCCACTTGCspGspC^spT	1	-14	1434,4	-	1737,6	1777,7	1752,6
HLAA_259f2	**HLAA_2591_2f17**	CCGGAGTATTGGGACspCspA^spG	1,6	-14	1443,5	-	-	1786,8	-
	HLAA_2592_2f17	CCKGAGTATTGGGACspGspA^spG	1	-28	1469,5	-	1772,8	1812,8	-
	HLAA_2593_2f17	CCGGAGTATTGGGACspCspG^spG	2	14	1487,5	-	-	1830,8	-
	HLAA_2594_2f17	CCGGAGTATTGGGACspCspT^spG	2	14	1564,6	-	1867,9	-	-
	HLAA_2595_2f17	GGCCGGAGTATTGACspGspA^spG	1	-28	1469,5	-	1772,8	1812,8	-
	HLAA_2596_2f17	SCGGAGTATTGGGACspGspG^spG	1	0	1513,5	1840,8	-	1856,8	-
HLAA_268r2	**HLA_2681_2r17**	TGASTGGGCCTTCACTT	1	0	1549,6	-	-	1892,9	1867,9
	HLA_2682_2r17	TGAGTGGGCCTTCACGT	2	28	1500,5	-	-	-	1818,0
	HLA_2683_2r17	TGAGTGGGCCTTCATAT	1	-28	1443,5	-	-	-	1761,8
	HLA_2684_2r17	TGASTGGSCCYTCACAT	1	-28	1428,5	-	-	1771,8	1746,8

Table 9: Sequences, proportions, charge tags and masses for MALDI-MS analysis of HLA-A

Marker	Name	Sequence	Parts	CT	Primer	A	C	G	T
HLAB_97f2	**HLAB_971_2f20**	CCCACTCCATGAGGCATspTspT^spC	1	0	**1540,3**	**-**	**1843,7**	**1883,8**	**1858,7**
	HLAB_972_2f20	CCCACTYCATGAGGTATspTspT^spC	1	0	**1540,3**	**-**	**1843,7**	**1883,8**	**1858,7**
HLAB_206f1	**HLAB_2061_1f20**	CGACGCCGCGAGTCMGAGspG^spA	2	-28	**1150,1**	**1477,4**	**1453,3**	**-**	**1468,3**
	HLAB_2062_1f20	CGACGCCACGAGTCCGAGspG^spA	2	-28	**1150,1**	**1477,4**	**1453,3**	**-**	**1468,3**
	HLAB_2063_1f20	CGACGCCGCGAGTCCRAGspA^spG	1	0	**1178,1**	**1505,4**	**-**	**1521,4**	**-**
	HLAB_2064_1f20	CGACGCCRCGAGTCCGAGspA^spG	1	0	**1178,1**	**1505,4**	**-**	**1521,4**	**-**
HLAB_222r1	**HLAB_2221_1r19**	GCCCCTCCTGCTCCACCspC^spA	1	0	**1098,3**	**-**	**1401,3**	**-**	**1416,3**
	HLAB_2222_1r19	GCCCCTCYTGCTCTATCspC^spA	1	0	**1098,3**	**-**	**1401,3**	**-**	**1416,3**
HLAB_259f2	**HLAB_2591_2f20**	GGCCGGAGTATTGGGACspGspG^spG	7	0	**1513,4**	**-**	**-**	**1856,7**	**-**
	HLAB_2592_2f20	GGCCGGAGTATTGGGACspGspA^spG	7	0	**1497,4**	**-**	**-**	**1840,7**	**-**
	HLAB_2593_2f20	GGCCGGAGTATTGGGACspCspC^spG	7	-28	**1405,4**	**-**	**-**	**1748,7**	**-**
	HLAB_2594_2f20	GGCCGGAGTATTGGGATspCspG^spG	5	0	**1488,4**	**1815,7**	**-**	**1831,7**	**-**
	HLAB_2595_2f20	GGCCGGAGTTTTGGGACspCspG^spG	1	-28	**1445,4**	**1772,7**	**-**	**1788,7**	**-**
	HLAB_2596_2f20	GGCCGGAGCATTGGGACspCspG^spG	1	-28	**1445,4**	**1772,7**	**-**	**1788,7**	**-**
	HLAB_2597_2f20	GGCCGGGATATTGGGACspCspG^spG	1	-28	**1445,4**	**1772,7**	**-**	**1788,7**	**-**
	HLAB_2598_2f20	GGCCRGAATATTGGGACspCspG^spG	1	-28	**1445,4**	**1772,7**	**-**	**1788,7**	**-**

	HLAB_2599_2f20	GGCGGGMGTATTGGGACspCspG^spG	1	-28	**1445,4**	**1772,7**	**-**	**1788,7**	**-**
	HLAB_25910_2f20	GGCCTTAGTATTGGGACspCspG^spG	1	-28	**1445,4**	**1772,7**	**-**	**1788,7**	**-**
HLAB_272f1	**HLAB_2721_In_1f20**	GGACSGGGAIACACIIAAspC^spA	2	0	**1122,1**	**-**	**-**	**-**	**1440,3**
	HLAB_2722_In_1f20	GGACGRGGAIACACIIAAspC^spA	2	0	**1122,1**	**-**	**-**	**-**	**1440,3**
	HLAB_2723_In_1f20	GGACCGGAACACACAIAAspC^spT	4	0	**1113,1**	**-**	**-**	**1456,4**	**-**
	HLAB_2724_In_1f20	GGACCGGAACACACAIACspC^spT	8	-14	**1075,1**	**-**	**-**	**-**	**1393,3**
	HLAB_2725_In_1f20	GGACCGGGAIACACAIAAspG^spT	4	0	**1153,1**	**1480,4**	**-**	**-**	**-**
	HLAB_2726_In_1f20	GGACCGGGAIATACAIATspC^spT	1	0	**1104,1**	**1431,4**	**1407,3**	**1447,4**	**1422,3**
	HLAB_2727_In_1f20	GGACCGGGASACACAIATspC^spT	1	0	**1104,1**	**1431,4**	**1407,3**	**1447,4**	**1422,3**
	HLAB_2728_In_1f20	GGACCGGGACACACAIATspC^spT	1	0	**1104,1**	**1431,4**	**1407,3**	**1447,4**	**1422,3**
	HLAB_2729_In_1f20	GGACCSGGAIACACAIATspC^spT	1	0	**1104,1**	**1431,4**	**1407,3**	**1447,4**	**1422,3**
HLAB_292f2	**HLAB_2921_2f19**	CAAGACCAACACACAGspGspC^spT	2,5	0	**1488,5**	**-**	**-**	**1831,8**	**-**
	HLAB_2922_2f19	CAAGSCCCAGGCACAGspGspC^spT	2,5	0	**1488,5**	**-**	**-**	**1831,8**	**-**
	HLAB_2923_2f19	CAAGACCAACACACGGspAspC^spT	1	-28	**1444,5**	**-**	**-**	**1787,8**	**1762,7**
	HLAB_2924_2f19	GAAGGCCTCCGCGCAGspAspC^spT	1	-28	**1444,5**	**-**	**-**	**1787,8**	**1762,7**
	HLAB_2925_2f19	CAAGGCCMAGGCACAGspAspC^spT	1	-28	**1444,5**	**-**	**-**	**1787,8**	**1762,7**
	HLAB_2926_2f19	CAAGSGCCAGGCACAGspAspC^spT	1	-28	**1444,5**	**-**	**-**	**1787,8**	**1762,7**
	HLAB_2927_2f19	GAAGACCAACACACAGspAspC^spT	1	-28	**1444,5**	**-**	**-**	**1787,8**	**1762,7**
HLAB_302f2	**HLAB_3021_2f19**	GCACAGACTGACCGAGspTspG^spG	5	0	**1528,4**	**-**	**-**	**1871,7**	**-**
	HLAB_3022_2f19	ACACAGACTTACCGAGspAspG^spG	2,5	0	**1537,4**	**1864,7**	**-**	**-**	**-**
	HLAB_3023_2f19	RCACAGACTGACCGAGspAspG^spG	2,5	0	**1537,4**	**1864,7**	**-**	**-**	**-**
	HLAB_3024_2f19	GCACAGACTGGCCGAGspTspG^spA	1,67	-28	**1481,4**	**1811,7**	**-**	**1827,7**	**-**
	HLAB_3025_2f19	ACACAGACTTACCGAGspTspG^spA	1,67	-28	**1481,4**	**1811,7**	**-**	**1827,7**	**-**
	HLAB_3026_2f19	RCACAGACTGACCGAGspTspG^spA	1,67	-28	**1481,4**	**1811,7**	**-**	**1827,7**	**-**
	HLAB_3027_2f19	ACACAGGCTGACCGAGspAspG^spA	1	-28	**1493,5**	**1820,8**	**-**	**1836,8**	**-**
	HLAB_3028_2f19	RCACAGACTGACCGAGspAspG^spA	1	-28	**1493,5**	**1820,8**	**-**	**1836,8**	**-**
	HLAB_3029_2f19	GCRCAGACTTACCGAGspAspG^spA	1	-28	**1493,5**	**1820,8**	**-**	**1836,8**	**-**
	HLAB_30210_2f19	ACACRGACTTACCGAGspAspG^spA	1	-28	**1493,5**	**1820,8**	**-**	**1836,8**	**-**
	HLAB_30211_2f19	ACACAGACTTACAGAGspAspG^spA	1	-28	**1493,5**	**1820,8**	**-**	**1836,8**	**-**
HLAB_362f2	**HLAB_3621_2f20**	CGGGTCTCACACCCTCCspAspC^spA	4	-28	**1413,4**	**-**	**-**	**1756,7**	**-**
	HLAB_3622_2f20	CGGGTCTCACAYCATCCspAspG^spA	1	-14	**1467,4**	**1794,7**	**1770,6**	**1810,7**	**1785,6**
	HLAB_3623_2f20	CGGKTCTCACACCCTCCspAspG^spA	1	-14	**1467,4**	**1794,7**	**1770,6**	**1810,7**	**1785,6**
	HLAB_3624_2f20	CGGGTCTCACACTTGGCspAspG^spA	1	-14	**1467,4**	**1794,7**	**1770,6**	**1810,7**	**1785,6**
	HLAB_3625_2f20	CGGGTCTCACATCATCCspAspG^spG	3	-14	**1483,4**	**-**	**-**	**-**	**1801,6**
	HLAB_3626_2f20	CGGGTCTCACACCCTCCspAspG^spT	3	0	**1472,4**	**-**	**-**	**1815,7**	**-**
HLAB_363r2	**HLAB_3631_2r20**	CCCASGTCGCAGCCGTAspCspA^spT	1	-28	**1442,4**	**1769,7**	**1745,6**	**1785,7**	**-**
	HLAB_3632_2r20	CCCABGTCGCAGCCATAspCspA^spT	1	-28	**1442,4**	**1769,7**	**1745,6**	**1785,7**	**-**
	HLAB_3633_2r20	CCCASGTCGCAGCCAAAspCspA^spT	1	-28	**1442,4**	**1769,7**	**1745,6**	**1785,7**	**-**
	HLAB_3634_2r20	CCCACGTCGCAGCCAGAspCspA^spT	1	-28	**1442,4**	**1769,7**	**1745,6**	**1785,7**	**-**
	HLAB_3635_2r20	CCCACGTCGCAGCCGCAspCspA^spT	1	-28	**1442,4**	**1769,7**	**1745,6**	**1785,7**	**-**
	HLAB_3636_2r20	CCCACGTCGCAGCCTTAspCspA^spT	1	-28	**1442,4**	**1769,7**	**1745,6**	**1785,7**	**-**
	HLAB_3637_2r20	CCCACGTCGCAGCCGTAspCspG^spT	9	0	**1472,4**	**1799,7**	**1775,6**	**1815,7**	**-**
HLAB_369r1	**HLAB_3691_1r20**	TCCGGCCCCAKGTCGCAGspC^spC	1	0	**1114,1**	**1441,4**	**-**	**1457,4**	**1432,3**
	HLAB_3692_1r20	TCGGGCCCCASGTCGCAGspC^spC	1	0	**1114,1**	**1441,4**	**-**	**1457,4**	**1432,3**
HLAB_412f2	**HLAB_4121_2f20**	GGCGCCTCCTCCGCGGGspTspA^spC	4	-28	**1444,4**	**-**	**1747,6**	**-**	**-**
	HLAB_4122_2f20	GGCGCCTCCTCCSCGGGspCspA^spT	1	0	**1472,4**	**1799,7**	**-**	**1815,7**	**-**
	HLAB_4123_2f20	GGCGCYTCCTCCGCGGGspCspA^spT	1	0	**1472,4**	**1799,7**	**-**	**1815,7**	**-**

	HLAB_4124_2f20	GGCGTCTCCTCCGCGGTspTspA^spT	6	0	**1462,4**	-	**1765,6**	-	-
	HLAB_4125_2f20	GGCGCCTCCTCCGCGGspTspA^spT	4	-14	**1473,4**	-	**1776,6**	-	-
HLAB_419f2	**HLAB_4191n_2f20**	TCCTCCGCGGGCATAACCspAspG^spA	3	0	**1481,4**	**1808,7**	-	-	-
	HLAB_4192n_2f20	TCCTCCGCGGGTACCACCspAspG^spC	3	-28	**1429,4**	**1756,4**	-	-	-
	HLAB_4193n_2f20	TCCTCCGCGGGCATAACCspAspG^spG	1	0	**1497,4**	**1824,7**	-	-	-
	HLAB_4194n_2f20	TCCTCCGCGGGTACCACCspAspG^spG	1	0	**1497,4**	**1824,7**	-	-	-
	HLAB_4195n_2f20	TCCTCCGCGGGTATGACCspAspG^spG	1	0	**1497,4**	**1824,7**	-	-	-
	HLAB_4196n_2f20	TCCTCCGCGGGCATAAMCspAspG^spT	1	-14	**1472,4**	**1799,7**	**1775,6**	-	**1790,6**
	HLAB_4197n_2f20	TCCTCCGCGGKTATAACCspAspG^spT	1	-14	**1472,4**	**1799,7**	**1775,6**	-	**1790,6**
	HLAB_4198n_2f20	TCCTCCGCGGGYATGACCspAspG^spT	1	-14	**1472,4**	**1799,7**	**1775,6**	-	**1790,6**
HLAB_435r1	**HLAB_4351n_1r20**	TCMTTCAGGGCGATGTAAspT^spC	2	-14	**1201,3**	-	**1504,4**	-	**1519,4**
	HLAB_4352n_1r20	TCGTTCAGGGCGATGTAAspT^spT	1	0	**1230,3**	-	**1533,5**	-	-
HLAB_527f1	**HLAB_5271_1f20**	CAAGTGGGAGGCGGCCCTspT^spG	1	0	**1246,3**	-	-	-	**1564,5**
	HLAB_5272_1f20	CAAGTKGGAGGCGGCCCGspT^spG	1	0	**1271,3**	**1598,6**	**1574,3**	-	**1589,5**
HLAB_539f1	**HLAB_5391_1f20**	GGCCCGTGYGGCGGAGCAspG^spC	2	0	**1138,1**	-	-	**1481,3**	**1456,3**
	HLAB_5392_1f20	GGCCCGTGTCGCGGAGCAspG^spG	1	0	**1178,1**	**1505,4**	-	-	-
	HLAB_5393_1f20	GGCCCGTGWGGCGGAGCAspG^spG	1	0	**1178,1**	**1505,4**	-	-	-
	HLAB_5394_1f20	GGCCCGTGAGGCGGAGCAspG^spT	2	0	**1153,1**	-	-	**1496,4**	-
HLAB_559r1	**HLAB_5591_1r20**	GCGGAGCGACTCCACGCAspC^spT	1	0	**1113,1**	-	**1416,3**	-	-
	HLAB_5592_1r20	GCGGAGCCACTCCACGCAspC^spT	1	0	**1113,1**	-	**1416,3**	-	-
	HLAB_5593_1r20	GCGGAGCCAATCCACGCAspC^spT	1	0	**1113,1**	-	**1416,3**	-	-
	HLAB_5594_1r20	GCGGAGCCACTCCACGCAspC^spG	3	0	**1138,1**	-	**1441,3**	-	**1456,3**
	HLAB_5595_1r20	GCGGAGCGACTCCRCGCAspC^spA	1	-14	**1108,1**	-	-	**1451,4**	**1426,3**
	HLAB_5596_1r20	GCGGAGCSACTCCACGCAspC^spA	1	-14	**1108,1**	-	-	**1451,4**	**1426,3**
	HLAB_5597_1r20	GCGGAGCCCGTCCACGCAspC^spA	1	-14	**1108,1**	-	-	**1451,4**	**1426,3**
HLAB_571r1	**HLAB_5711_1r20**	CTCCAGGTAYCTGCGGAGspC^spG	1	0	**1154,1**	**1481,4**	-	-	-
	HLAB_5712_1r20	CTCCAGGTRTCTGCGGAGspC^spC	1	0	**1114,1**	**1441,4**	**1417,3**	-	-
HLAB_583r1	**HLAB_583n_1r19**	ACCTGGAGAACGGGAAGspG^spT	1	0	**1169,1**	**1496,2**	-	**1512,4**	-

Table 10: Sequences, proportions, charge tags and masses for MALDI-MS analysis of HLA-B

2.9 PDE Digest

To reduce the extension products to their core sequences 1.5 µl 5′-phosphodiesterase ($3.4*10^{-3}$ U/ µl) dissolved in 0.1 M ammonium-citrate buffer (pH ~ 6.0) and 0.5 µl 0.5 M acetic acid were added into each well and incubated for 120 minutes at 37°C in a microbiological incubator.

2.10 Alkylation

To neutralize all charges of the phosphorothioate backbones, all extension reaction products were alkylated by adding 21 µl of a mixture of 377 parts of

water-free acetonitrile, 15 parts of 2 M triethylamine/CO_2 (pH ~7.5), 75 of parts 2 mM Tris-HCl and 174 parts of methyljodate and incubated in a PrimusHT Thermocyclers for 15 minutes at 40°C. The reaction was stopped by adding 10 µl deionised water. 5 µl of the resulting upper phase were diluted in 10 µl of 40 % acetonitrile. This sample solution is stable at -20°C until MALDI-MS analysis for several weeks.

2.11 Target Preparation and MALDI-TOF-MS Analysis

0.5 µl of a 20 % saturated solution of α-cyano-4-hydroxy-cinnamic acid methyl ester matrix (α-CNME) dissolved in water-free acetonitrile was spotted onto each of the 384 positions of the MALDI plates. 0.3 µl of the sample solution was transferred onto the dried matrix forming a very thin crystalline matrix layer.
Spectra were recorded automatically using a Bruker Autoflex™ mass spectrometer in positive ion linear time-of-flight mode. Two times 15 shots were accumulated. The mass spectrometer is equipped with a Scout MTP™ ion source with delayed ion extraction. Typical acceleration potentials were –20 kV, and the ion extraction was delayed by 200 ns. For the analysis of the spectra a CNG in-house created software package ("Helixir") was used. The software is discussed in detail in the section 2.14.4 "Helixir" – reading raw data.

2.12 BasePlate Liquid Handler

All liquid transfer steps were carried out with a BasePlate liquid handler. This device is an automated system for high-throughput assay assembly and plate replications and allows the precise transfer of 1 µl volumes with 96 tips at once. Further the BasePlate system is able to aspirate and dispense into 96- and 384-well plates with highly accurate serial dispensing.

2.13 Sample organisation for high-throughput genotyping

All DNA samples were arranged in 96-well plates. Besides the DNA samples these plates carry 2 control-DNA samples and 2 water-controls. The controls are at least one row and one column away from the border of the plate. Each DNA plate exists in two layouts, one original and one rearranged. On the rearranged plate the DNA samples have new positions. The DNA sample of well "A 1" of the

original plate in the rearranged plate is moved into a well in the centre of the plate (see tables 11 and 12).

	1	2	3	4	5	6	7	8	9	10	11	12
A	DNA 1	DNA 2	DNA 3	DNA 4	DNA 5	DNA 6	DNA 7	DNA 8	DNA 9	DNA 10	DNA 11	DNA 12
B	DNA 37	DNA 38	DNA 39	DNA 40	DNA 41	DNA 42	DNA 43	DNA 44	DNA 45	DNA 46	DNA 47	DNA 13
C	DNA 36	DNA 65	Water	C1	DNA 66	DNA 67	DNA 68	DNA 69	DNA 70	DNA 71	DNA 48	DNA 14
D	DNA 35	DNA 64	DNA 81	DNA 82	DNA 83	DNA 84	DNA 85	DNA 86	DNA 87	DNA 72	DNA 49	DNA 15
E	DNA 34	DNA 63	DNA 80	DNA 93	DNA 92	DNA 91	DNA 90	DNA 89	DNA 88	DNA 73	DNA 50	DNA 16
F	DNA 33	DNA 62	DNA 79	DNA 78	DNA 77	DNA 76	DNA 75	DNA 74	C2	Water	DNA 51	DNA 17
G	DNA 32	DNA 61	DNA 60	DNA 59	DNA 58	DNA 57	DNA 56	DNA 55	DNA 54	DNA 53	DNA 52	DNA 18
H	DNA 31	DNA 30	DNA 29	DNA 28	DNA 27	DNA 26	DNA 25	DNA 24	DNA 23	DNA 21	DNA 20	DNA 19

Table 11: DNA plate layout "Original", DNA samples named C1 and C2 are controls

	1	2	3	4	5	6	7	8	9	10	11	12
A	DNA 93	DNA 92	DNA 91	DNA 90	DNA 89	DNA 88	DNA 87	DNA 86	DNA 85	DNA 84	DNA 83	DNA 82
B	DNA 58	DNA 57	DNA 56	DNA 55	DNA 54	DNA 53	DNA 52	DNA 51	DNA 50	DNA 49	DNA 47	DNA 81
C	DNA 59	DNA 29	Water	C1	DNA 28	DNA 27	DNA 26	DNA 25	DNA 24	DNA 23	DNA 46	DNA 80
D	DNA 60	DNA 30	DNA 13	DNA 12	DNA 11	DNA 10	DNA 9	DNA 8	DNA 7	DNA 22	DNA 45	DNA 79
E	DNA 61	DNA 31	DNA 14	DNA 1	DNA 2	DNA 3	DNA 4	DNA 5	DNA 6	DNA 21	DNA 44	DNA 78
F	DNA 62	DNA 32	DNA 15	DNA 16	DNA 17	DNA 18	DNA 19	DNA 20	C2	Water	DNA 43	DNA 77
G	DNA 63	DNA 33	DNA 34	DNA 35	DNA 36	DNA 37	DNA 38	DNA 39	DNA 40	DNA 41	DNA 42	DNA 76
H	DNA 64	DNA 65	DNA 66	DNA 67	DNA 68	DNA 69	DNA 70	DNA 71	DNA 72	DNA 73	DNA 74	DNA 75

Table 12: DNA plate layout "Rearranged", DNA samples named C1 and C2 are controls

An aliquot was taken from these stock plates and transferred into a PCR compatible 96-well microtitre plate. After PCR the products were transferred to 384-well microtitre plates. Four 96-well plates were combined to one 384-well plate.

Failure of the assay is in most of the cases a result of evaporation during the cycling and incubation. Experience shows that samples which fail are predominantly located at the periphery of a 384-well plate. To assure that at least one unambiguous result per sample can be recorded, the system of arranging layouts was established.

2.14 Software developed and used for HLA typing

2.14.1 "Genalys" – Sequence analysis

Genalys is a software tool for the analysis of SNPs from sequencing data and available from the CNG internet page (http://software.cng.fr/). It allows the alignment of multiple sequences (up to 1000) and to analyze their differences. Originally the software was developed to read sequencing traces and identify SNPs[105,106].

Further, the software allows aligning FASTA sequences and manipulating them. The differences of these sequences can be annotated and extracted into an Excelsheet. The resulting Excel sheets of the different loci were used for processing with further software tools. In the context of this thesis Genalys was used to align FASTA sequences from the IMGT-HLA Sequence Database and annotate the polymorphisms. The IMGT-HLA Sequence Database is a public database and is accessible via internet (www.ebi.ac.uk/imgt/hla)[107]. This database is updated every few months by the HLA bioinformatics group lead by Steven Marsh (Anthony Nolan Trust, London, UK) .

2.14.2 "Trans_numerique" – transforming microhaplotypes into number codes

To perform effective marker selection for HLA typing a matrix of an alignment of all allele sequences of a given locus was created. As the aim of this thesis is to separate groups of HLA alleles from each other by testing a number of

[105] Takahashi, M et al.; 2003, Journal of Bioinformatics and Computational Biology, Vol. 1(2): 253 - 265

[106] Takahashi, M et al.; 2003 Processing IEEE CS Bioinformatics, 87-96

[107] Robinson, J et al.; 2003, Nucleic Acid Research, Vol. 31 (1): 311 - 314

polymorphic bases, the most informative positions had to be identified. The distinction is based on variations in the sequences. The matrix created by this "trans_numerique"-algorithm combines variations, which are less than five bases away, so they can be handled as one item. This makes the matrix less complex for following procedures. To create the matrix, the sequences of all HLA alleles of exon 2 and 3 for class I genes and exon 2 for the class II gene were extracted from the IMGT-HLA Sequence Database.

The extracted sequences were aligned in a text delimited table as shown Table 13, and aligned in order from base 74 to 619 for class I genes and from 101 to 370 for class II genes. The base number is relative to the cDNA sequences.

Position in cDNA	**188**	**189**	**190**	**191**	**192**	**193**	**194**	**195**	**196**	**197**	**198**	**199**	**200**	**201**	**202**
DRB1-0108	A	A	G	A	G	G	A	G	T	A	C	G	T	G	C
DRB1-0109	A	A	G	A	G	G	A	G	T	C	C	G	T	G	C
DRB1-0110	A	A	G	A	G	G	A	G	T	C	C	G	T	G	C
DRB1-0111	A	A	G	A	G	G	A	G	T	C	C	G	T	G	C
DRB1-0112	A	A	G	A	G	G	A	G	T	C	C	G	T	G	C
DRB1-030101	A	G	G	A	G	G	A	G	A	A	C	G	T	G	C
DRB1-030102	A	G	G	A	G	G	A	G	A	A	C	G	T	G	C
DRB1-030201	A	G	G	A	G	G	A	G	A	A	C	G	T	G	C
DRB1-030202	A	G	G	A	G	G	A	G	A	A	C	G	T	G	C
DRB1-0303	A	G	G	A	G	G	A	G	A	A	C	G	T	G	C

Table 13: Example of an extracted table of sequence alignment

These tables were used for a transformation of microhaplotype sequences into numbers coded via a system to the base of 5 by the following algorithm (see also Table 14):

1. Let be T = 1, C = 2, G = 3, A = 4 and dels =0 (further called base value = V_b)
    i. Unknown bases are labelled with an asterisk
2. Set positions in the microhaplotype sequence equal to numbers of a sequence of 5 (1, 5, 25, 125, 625) (further called position value = V_P)
3. Multiply $V_b * V_P$
4. Sum all products ($V_b * V_P$) of the given microhaplotype

Position of Base in Microhaplotype Sequence	1	2	3	4	5	
Sequence	T	C	G	A	T	
Base Value (V_b)	1	2	3	4	1	
Position Value (V_P)	1	5	25	125	625	
$V_b * V_P$	1	10	75	500	625	**$\Sigma = 1211$**

Table 14: Example for a five base microhaplotype. In general the focus was on four base microhaplotypes. Only if the fifth base was polymorphic as wel the transformation was carried out for a five base microhaplotype.

Encoding was carried out for every position to the last polymorphic position but maximally to the fifth base from individual position. Encoding was done in sense and antisense direction using the sense sequence. Finally all non-polymorphic individual positions were removed from the matrix. The two matrices of the sense and antisense calculation were combined to one table. A final matrix is shown in table 15.

PositioncDNA	**122**	**196**	**227**	**286**	**299**	**341**	**125r**	**197r**	**261r**	**308r**
DRB1-010101	794	2366	84	487	1848	2341	3081	212	1117	17
DRB1-010102	794	2371	84	487	1848	2341	3081	212	1117	17
DRB1-010103	794	2366	84	487	1848	2341	3081	212	1117	17
DRB1-010201	794	2366	84	487	1848	2342	3081	212	1117	17
DRB1-010202	794	2366	84	487	1848	2342	3081	212	1117	12
DRB1-010203	794	2366	84	487	1848	2342	3081	212	1117	17
DRB1-010204	794	2366	84	487	1848	2342	3081	212	1116	17
DRB1-0103	794	2366	84	489	2444	2341	3081	212	1117	17
DRB1-0104	794	2366	84	487	1848	2341	3081	212	1117	17
DRB1-0105	794	2366	84	487	1848	2341	3081	212	1117	17
DRB1-0106	794	2366	84	487	1842	2341	3081	212	1117	17
DRB1-0107	794	2366	84	487	1848	2341	3081	212	1117	17
DRB1-0108	794	2366	84	487	1848	2341	3081	214	1117	17
DRB1-0109	794	2366	84	487	1842	2341	3081	212	1117	17
DRB1-0110	794	2366	84	487	1849	2341	3081	212	1117	17
DRB1-0111	794	2366	84	487	1848	2341	3081	212	1117	17
DRB1-0112	794	2366	84	487	1848	2341	3081	212	1117	17
DRB1-030101	917	2369	81	487	1849	2341	2457	214	1117	18

DRB1-030102	917	2369	81	487	1849	2341	2457	214	1117	18
DRB1-030201	917	2369	84	487	1849	2341	2457	214	1117	18
DRB1-030202	917	2369	84	487	1849	2341	2457	214	1117	18

Table 15: Example of an output file (a "r" with the number in the head-row indicates a reverse orientated microhaplotype)

The software for this encoding procedure was written by Carine Audubert at the CNG during a practical training.

2.14.3 "select" – Marker selection software

This software was created to perform a selection of markers which have to be analysed for best distinction of a selected group of alleles from the rest of the alleles. In this case frequent alleles and rare alleles are to be separated from each other. Also the discrimination of specific alleles is possible (e.g. find all markers which discriminate HLA-A*01 alleles from all other alleles of HLA-A).
The matrix created by the "trans_numerique"-algorithm is used as the input file for this selection software. Furthermore a txt.-file with all alleles, which have to be separated from the other alleles, has to be created. The alleles that have to be written into this file depend on the query. In this particular case the alleles of the frequent groups were written into these files. Together these files are used to select the optimal microhaplotype positions by the following algorithm.
Let g_n be a selected group of microhaplotypes of n sites. S(g_n) is the score for the selection. In this case S(g_n) simply is the number of non-selected alleles indistinguishable from the selected alleles for the given g_n. G_n is a selected collection of possible sets of g_n. Let min(S(G_n)) be the minimum score S(g_n) for all g_n in G-n. Eliminate all alleles, which are totally explained by other alleles. That has the effect to clear totally associated polymorphisms. Next all alleles, which can not be distinguished using all microhaplotypes, are combined. Now let G_1 be the collection of all possible sets g_1 (i.e. try every microhaplotype in turn). The next step is to calculate S(g_1) for all sets g_1 in G_1. Now let j be equal 2 and construct G_j by:

- taking each set g_(j-1) in G_(j-1)
- adding in turn each site not already in g_(j-1) to form h_j
- calculating of the score of h_j, S(h_j)

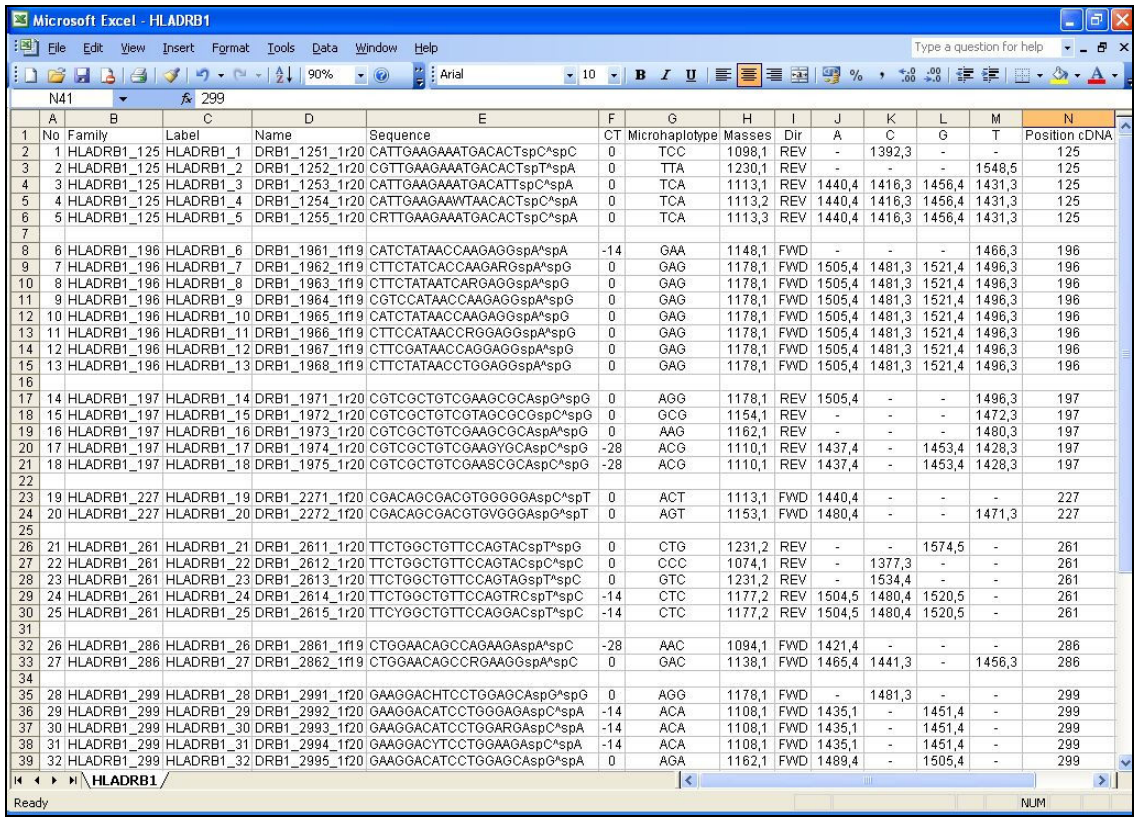
- adding h_j to G_j if S(h_j) < min(S(G_j-1))

Finally output all g_j in G_j where S(g_j) = min(S(G_j)), increase j by 1 (j -> j+1) and continue with the construction of G_j until min(S(G_j)) = 0 or reached the number of sites desired. An output of this algorithm is shown in Appendix S, figure S-3.

This software was coded by Simon Heath at the CNG.

2.14.4 "Helixir" – reading raw data

Helixir is a software tool that allows reading and analyzing of MALDI spectra. Therefore a set of input files has to be created. First all markers, which are in use, have to be listed in a file called primers.txt (see figure 13).



Microsoft Excel - HLADRB1

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N41 299

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	No	Family	Label	Name	Sequence	CT	Microhaplotype	Masses	Dir	A	C	G	T	Position cDNA
2	1	HLADRB1_125	HLADRB1_1	DRB1_1251_1r20	CATTGAAGAAATGACACTspC^spC	0	TCC	1098,1	REV	-	1392,3	-	-	125
3	2	HLADRB1_125	HLADRB1_2	DRB1_1252_1r20	CGTTGAAGAAATGACACTspT^spA	0	TTA	1230,1	REV	-	-	-	1548,5	125
4	3	HLADRB1_125	HLADRB1_3	DRB1_1253_1r20	CATTGAAGAAATGACATTspC^spA	0	TCA	1113,1	REV	1440,4	1416,3	1456,4	1431,3	125
5	4	HLADRB1_125	HLADRB1_4	DRB1_1254_1r20	CATTGAAGAAWTAACACTspC^spA	0	TCA	1113,2	REV	1440,4	1416,3	1456,4	1431,3	125
6	5	HLADRB1_125	HLADRB1_5	DRB1_1255_1r20	CRTTGAAGAAATGACACTspC^spA	0	TCA	1113,3	REV	1440,4	1416,3	1456,4	1431,3	125
7														
8	6	HLADRB1_196	HLADRB1_6	DRB1_1961_1f19	CATCTATAACCAAGAGGspA^spA	-14	GAA	1148,1	FWD	-	-	-	1466,3	196
9	7	HLADRB1_196	HLADRB1_7	DRB1_1962_1f19	CTTCTATCACCAAGARGspA^spG	0	GAG	1178,1	FWD	1505,4	1481,3	1521,4	1496,3	196
10	8	HLADRB1_196	HLADRB1_8	DRB1_1963_1f19	CTTCTATAATCARGAGGspA^spG	0	GAG	1178,1	FWD	1505,4	1481,3	1521,4	1496,3	196
11	9	HLADRB1_196	HLADRB1_9	DRB1_1964_1f19	CGTCCATAACCAAGAGGspA^spG	0	GAG	1178,1	FWD	1505,4	1481,3	1521,4	1496,3	196
12	10	HLADRB1_196	HLADRB1_10	DRB1_1965_1f19	CATCTATAACCAAGAGGspA^spG	0	GAG	1178,1	FWD	1505,4	1481,3	1521,4	1496,3	196
13	11	HLADRB1_196	HLADRB1_11	DRB1_1966_1f19	CTTCCATAACCRGGAGGspA^spG	0	GAG	1178,1	FWD	1505,4	1481,3	1521,4	1496,3	196
14	12	HLADRB1_196	HLADRB1_12	DRB1_1967_1f19	CTTCGATAACCAGGAGGspA^spG	0	GAG	1178,1	FWD	1505,4	1481,3	1521,4	1496,3	196
15	13	HLADRB1_196	HLADRB1_13	DRB1_1968_1f19	CTTCTATAACCTGGAGGspA^spG	0	GAG	1178,1	FWD	1505,4	1481,3	1521,4	1496,3	196
16														
17	14	HLADRB1_197	HLADRB1_14	DRB1_1971_1r20	CGTCGCTGTCGAAGCGCAspG^spG	0	AGG	1178,1	REV	1505,4	-	-	1496,3	197
18	15	HLADRB1_197	HLADRB1_15	DRB1_1972_1r20	CGTCGCTGTCGTAGCGCGspC^spG	0	GCG	1154,1	REV	-	-	-	1472,3	197
19	16	HLADRB1_197	HLADRB1_16	DRB1_1973_1r20	CGTCGCTGTCGAAGCGCAspA^spG	0	AAG	1162,1	REV	-	-	-	1480,3	197
20	17	HLADRB1_197	HLADRB1_17	DRB1_1974_1r20	CGTCGCTGTCGAAGYGCAspC^spG	-28	ACG	1110,1	REV	1437,4	-	1453,4	1428,3	197
21	18	HLADRB1_197	HLADRB1_18	DRB1_1975_1r20	CGTCGCTGTCGAASCGCAspC^spG	-28	ACG	1110,1	REV	1437,4	-	1453,4	1428,3	197
22														
23	19	HLADRB1_227	HLADRB1_19	DRB1_2271_1f20	CGACAGCGACGTGGGGGAspC^spT	0	ACT	1113,1	FWD	1440,4	-	-	-	227
24	20	HLADRB1_227	HLADRB1_20	DRB1_2272_1f20	CGACAGCGACGTGVGGGAspG^spT	0	AGT	1153,1	FWD	1480,4	-	-	1471,3	227
25														
26	21	HLADRB1_261	HLADRB1_21	DRB1_2611_1r20	TTCTGGCTGTTCCAGTACspT^spG	0	CTG	1231,2	REV	-	-	1574,5	-	261
27	22	HLADRB1_261	HLADRB1_22	DRB1_2612_1r20	TTCTGGCTGTTCCAGTACspC^spC	0	CCC	1074,1	REV	-	1377,3	-	-	261
28	23	HLADRB1_261	HLADRB1_23	DRB1_2613_1r20	TTCTGGCTGTTCCAGTAGspT^spC	0	GTC	1231,2	REV	-	1534,4	-	-	261
29	24	HLADRB1_261	HLADRB1_24	DRB1_2614_1r20	TTCTGGCTGTTCCAGTRCspT^spC	-14	CTC	1177,2	REV	1504,5	1480,4	1520,5	-	261
30	25	HLADRB1_261	HLADRB1_25	DRB1_2615_1r20	TTCYGGCTGTTCCAGGACspT^spC	-14	CTC	1177,2	REV	1504,5	1480,4	1520,5	-	261
31														
32	26	HLADRB1_286	HLADRB1_26	DRB1_2861_1f19	CTGGAACAGCCAGAAGAspA^spC	-28	AAC	1094,1	FWD	1421,4	-	-	-	286
33	27	HLADRB1_286	HLADRB1_27	DRB1_2862_1f19	CTGGAACAGCCRGAAGGspA^spC	0	GAC	1138,1	FWD	1465,4	1441,3	-	1456,3	286
34														
35	28	HLADRB1_299	HLADRB1_28	DRB1_2991_1f20	GAAGGACHTCCTGGAGCAspG^spG	0	AGG	1178,1	FWD	-	1481,3	-	-	299
36	29	HLADRB1_299	HLADRB1_29	DRB1_2992_1f20	GAAGGACATCCTGGGAGAspC^spA	-14	ACA	1108,1	FWD	1435,1	-	1451,4	-	299
37	30	HLADRB1_299	HLADRB1_30	DRB1_2993_1f20	GAAGGACATCCTGGARGAspC^spA	-14	ACA	1108,1	FWD	1435,1	-	1451,4	-	299
38	31	HLADRB1_299	HLADRB1_31	DRB1_2994_1f20	GAAGGACYTCCTGGAAGAspC^spA	-14	ACA	1108,1	FWD	1435,1	-	1451,4	-	299
39	32	HLADRB1_299	HLADRB1_32	DRB1_2995_1f20	GAAGGACATCCTGGAGCAspG^spA	0	AGA	1162,1	FWD	1489,4	-	1505,4	-	299

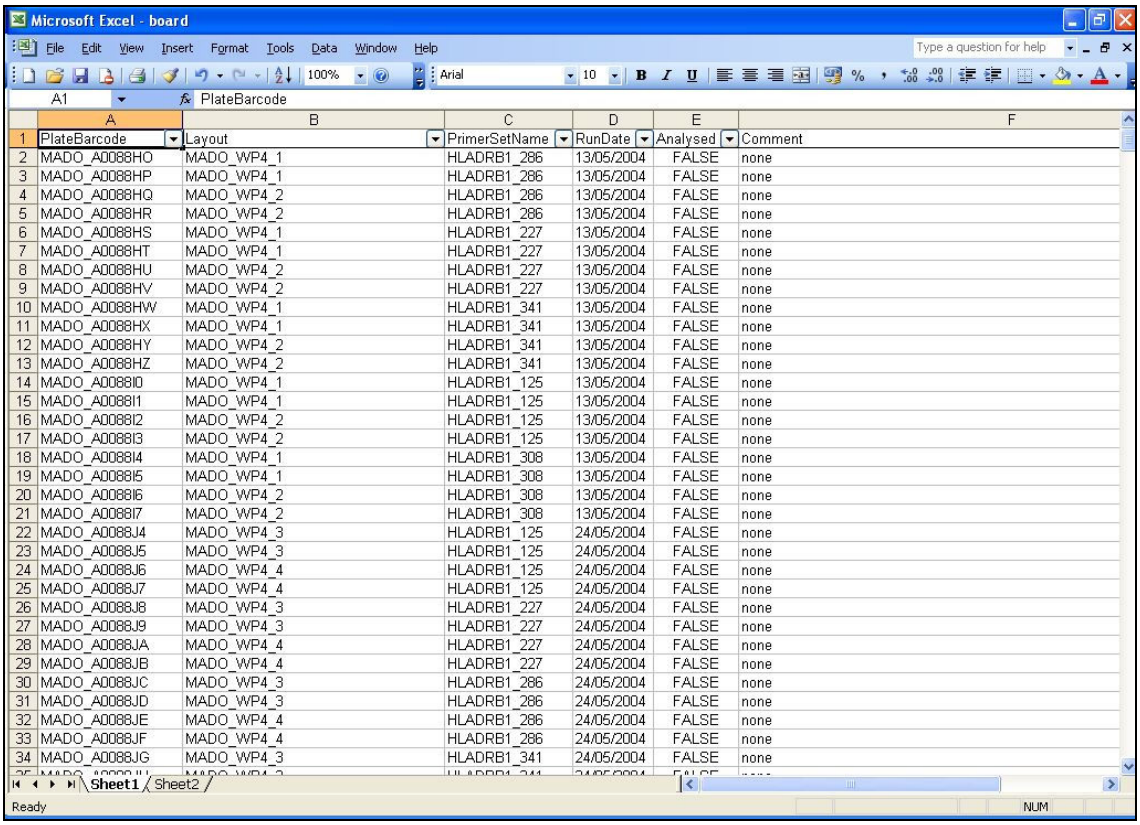
HLADRB1

Ready NUM

Figure 13: Screenshot of the file "primers.txt"

This file contains all information about the markers for the extension reaction, like marker name, primers in the pool for this marker, orientation (forward or reverse) and the masses for the expected microhaplotypes.

Further a file containing all information about the location of the DNA samples on a plate is input. This file is called layout.txt, and shows the position on the plate and the barcode of the respective DNA sample.
Finally a file called board.xls is required (see figure 14). This file contains the information about the spectra, the barcode of the target, the marker that was measured on this target and which DNA samples (layout) were on this target.



Microsoft Excel - board

File Edit View Insert Format Tools Data Window Help

A1 PlateBarcode

	A	B	C	D	E	F
1	PlateBarcode	Layout	PrimerSetName	RunDate	Analysed	Comment
2	MADO_A0088HO	MADO_WP4_1	HLADRB1_286	13/05/2004	FALSE	none
3	MADO_A0088HP	MADO_WP4_1	HLADRB1_286	13/05/2004	FALSE	none
4	MADO_A0088HQ	MADO_WP4_2	HLADRB1_286	13/05/2004	FALSE	none
5	MADO_A0088HR	MADO_WP4_2	HLADRB1_286	13/05/2004	FALSE	none
6	MADO_A0088HS	MADO_WP4_1	HLADRB1_227	13/05/2004	FALSE	none
7	MADO_A0088HT	MADO_WP4_1	HLADRB1_227	13/05/2004	FALSE	none
8	MADO_A0088HU	MADO_WP4_2	HLADRB1_227	13/05/2004	FALSE	none
9	MADO_A0088HV	MADO_WP4_2	HLADRB1_227	13/05/2004	FALSE	none
10	MADO_A0088HW	MADO_WP4_1	HLADRB1_341	13/05/2004	FALSE	none
11	MADO_A0088HX	MADO_WP4_1	HLADRB1_341	13/05/2004	FALSE	none
12	MADO_A0088HY	MADO_WP4_2	HLADRB1_341	13/05/2004	FALSE	none
13	MADO_A0088HZ	MADO_WP4_2	HLADRB1_341	13/05/2004	FALSE	none
14	MADO_A0088I0	MADO_WP4_1	HLADRB1_125	13/05/2004	FALSE	none
15	MADO_A0088I1	MADO_WP4_1	HLADRB1_125	13/05/2004	FALSE	none
16	MADO_A0088I2	MADO_WP4_2	HLADRB1_125	13/05/2004	FALSE	none
17	MADO_A0088I3	MADO_WP4_2	HLADRB1_125	13/05/2004	FALSE	none
18	MADO_A0088I4	MADO_WP4_1	HLADRB1_308	13/05/2004	FALSE	none
19	MADO_A0088I5	MADO_WP4_1	HLADRB1_308	13/05/2004	FALSE	none
20	MADO_A0088I6	MADO_WP4_2	HLADRB1_308	13/05/2004	FALSE	none
21	MADO_A0088I7	MADO_WP4_2	HLADRB1_308	13/05/2004	FALSE	none
22	MADO_A0088J4	MADO_WP4_3	HLADRB1_125	24/05/2004	FALSE	none
23	MADO_A0088J5	MADO_WP4_3	HLADRB1_125	24/05/2004	FALSE	none
24	MADO_A0088J6	MADO_WP4_4	HLADRB1_125	24/05/2004	FALSE	none
25	MADO_A0088J7	MADO_WP4_4	HLADRB1_125	24/05/2004	FALSE	none
26	MADO_A0088J8	MADO_WP4_3	HLADRB1_227	24/05/2004	FALSE	none
27	MADO_A0088J9	MADO_WP4_3	HLADRB1_227	24/05/2004	FALSE	none
28	MADO_A0088JA	MADO_WP4_4	HLADRB1_227	24/05/2004	FALSE	none
29	MADO_A0088JB	MADO_WP4_4	HLADRB1_227	24/05/2004	FALSE	none
30	MADO_A0088JC	MADO_WP4_3	HLADRB1_286	24/05/2004	FALSE	none
31	MADO_A0088JD	MADO_WP4_3	HLADRB1_286	24/05/2004	FALSE	none
32	MADO_A0088JE	MADO_WP4_4	HLADRB1_286	24/05/2004	FALSE	none
33	MADO_A0088JF	MADO_WP4_4	HLADRB1_286	24/05/2004	FALSE	none
34	MADO_A0088JG	MADO_WP4_3	HLADRB1_341	24/05/2004	FALSE	none

Sheet1 Sheet2

Ready NUM

Figure 14: Screenshot of the file "board.xls"

The command line "mado2helixir –g [target barcodes] [filename.hxr]" combines all these information to one hxr.-file. This resulting file can then be read and analyzed with the helixir tool. Helixir uses the input to identify peaks in the spectra and assign microhaplotypes to the peaks. The identified microhaplotypes are written into a table. All DNA samples are analyzed at least four times (two layouts and two target preparations) to assure getting at least one result per DNA sample. Sometimes it happens that spectra of the same DNA sample and the same marker show different results. This is mostly due to junk peaks or noise peaks. In these cases different spectra can be compared and results can be corrected. The

example (figure 15) shows a screenshot of sample analysis. At the top field of the screen the spectrum of a specific spot on a target is shown.
Helixir allows quick and easy quality control and correction of the genotypes. For example sometimes spectra are not easy to read. In these cases they can be validated by comparing with the other spectra. This is the case in the examples for the DNA samples A00CO2D and A00CO75. They are marked with a red spot showing a white cross. Here the software identified discordant microhaplotypes.

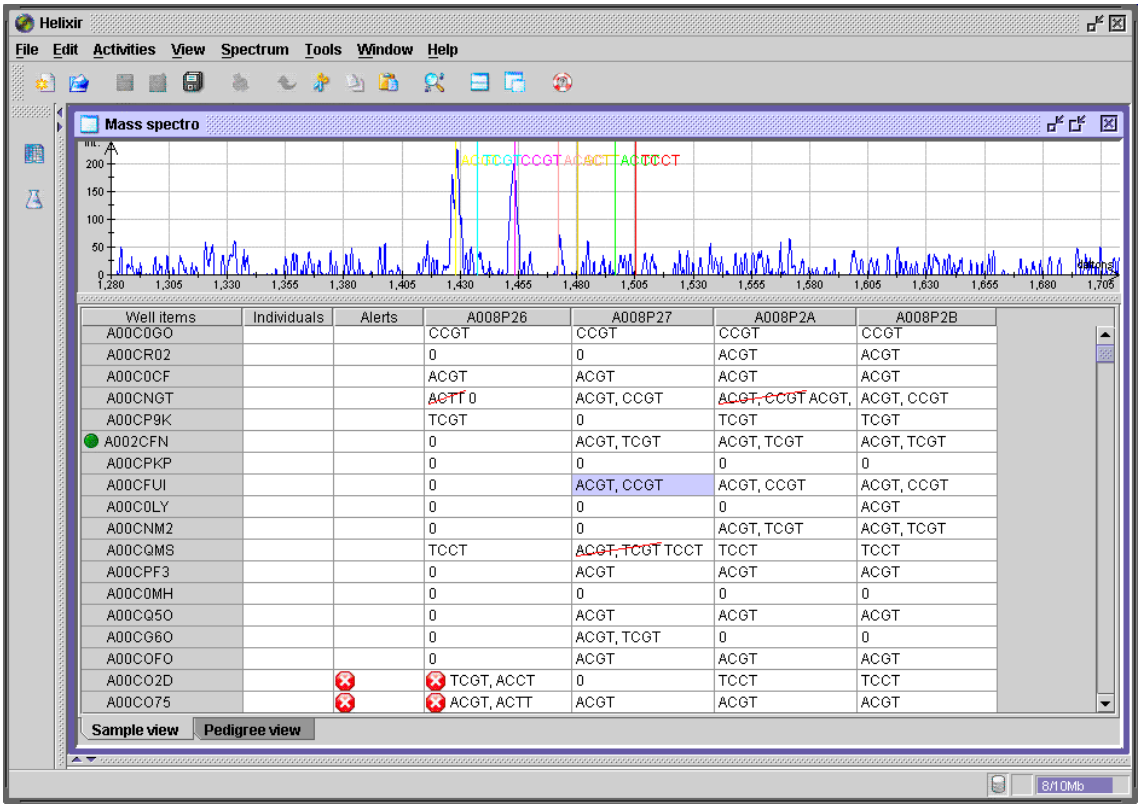


Figure 15: Screenshot of HELIXIR; The coloured bars indicate the masses of possible peaks, and they are labelled with their corresponding microhaplotypes. The table under the spectra displays in the very left column the barcodes of the individuals. The last four columns from the right side are the different targets, and indicated by their barcodes in the head row (e.g. A008P26). Each sample was prepared twice onto two different targets. This decreases the possibility of losing data. Further the results of "original" and "reorganized" layouts are joined and can be compared.

2.14.5 "HLAfamilies" – transforming microhaplotypes into HLA types

Verified microhaplotypes for the different markers are combined by this algorithm. This combining aligns the microhaplotypes. The resulting combinations are compared with a reference file, which contains all known alleles and microhaplotype combinations. An example of this lookup table is show in the

appendix (see section 5.6). HLA alleles represented by a combination of microhaplotypes are extracted and listed in an output file. The output was presented in two ways. First a simple list of the detected alleles was given. Second a list of the possible allele pairs was prepared. The second way has the advantage of giving more information. The pairing was done by respecting all detected heterozygote microhaplotyping results. For example, a sample is heterozygote for marker HLADRB1_197r1, HLADRB1_299f1 and HLADRB1_308r1. (see table 16)

HLADR_ 122r2	HLADRB _125r1	HLADRB _196f1	HLADRB _197r1	HLADRB _227f1	HLADRB _261f1	HLADRB _286r1	HLADRB _299f1	HLADRB _308r1	HLADRB _341f1
AACAT	ATGA	GAGT	ACGT/ CCGT	AGTA	CCGA	GACC	AGAG/ AGAA	AGGT/ CGGT	GGGT

Table 16: Example of a result of a DNA sample

With these genotyping results eight microhaplotype combinations are possible. These are exemplarily presented in table 17.

	122	125	196	197	227	261	286	299	308	341
Allele1	AACAT	ATGA	GAGT	ACGT	AGTA	CCGA	GACC	AGAG	AGGT	GGGT
Allele2	AACAT	ATGA	GAGT	CCGT	AGTA	CCGA	GACC	AGAG	AGGT	GGGT
Allele3	AACAT	ATGA	GAGT	ACGT	AGTA	CCGA	GACC	AGAA	AGGT	GGGT
Allele4	AACAT	ATGA	GAGT	ACGT	AGTA	CCGA	GACC	AGAG	CGGT	GGGT
Allele5	AACAT	ATGA	GAGT	CCGT	AGTA	CCGA	GACC	AGAA	AGGT	GGGT
Allele6	AACAT	ATGA	GAGT	CCGT	AGTA	CCGA	GACC	AGAG	CGGT	GGGT
Allele7	AACAT	ATGA	GAGT	ACGT	AGTA	CCGA	GACC	AGAA	CGGT	GGGT
Allele8	AACAT	ATGA	GAGT	CCGT	AGTA	CCGA	GACC	AGAA	CGGT	GGGT

Table 17: Example of microhaplotype combinations of Table 16

These eight alleles can build only 4 pairs, which are Allele1/Allele8, Allele4/Allele5, Allele3/Allele6 and Allele2/Allele7. Only in those allele pairs all three microhaplotype positions are heterozygote. Some microhaplotype combinations do not exist in the reference table, which not inevitably implicates that they do not exist at all. It is also possible that an unknown HLA allele is present. In these cases the pairs are completed with question marks. Since donors who carry an unknown HLA allele are generally of great interest, a determination

of likelihood for the discovered pairs should help to determine the importance of the samples.

The determination of the likelihood is based on the allele frequencies of the HLA alleles. These frequencies are available at the public database http://www.allelefrequencies.net[108]. This database was recently created and is not yet very precise. However, it presents an idea about the dimensions of allele frequencies of HLA alleles. As more samples are added this database will become more accurate, and so will the likelihood calculation.

The used frequencies for HLA-DRB1 alleles are listed in Table 18. Alleles which are not in this list have a frequency of 0 %. The frequencies presented in these tables are calculated for the populations of Western Europe. The sums of the frequencies are not 100%. These differences are due to rounding errors.

The frequencies for HLA-A and HLA-B are listed in the appendix (see 5.4).

Allele	Frequencies	Allele	Frequencies	Allele	Frequencies
DRB1*0701	15,72	DRB1*0103	1,26	DRB1*0810	0,06
DRB1*1501	12,32	DRB1*0407	1,05	DRB1*0410	0,04
DRB1*0301	10,99	DRB1*1001	1,02	DRB1*0416	0,04
DRB1*0101	6,59	DRB1*1103	1,01	DRB1*1503	0,04
DRB1*1101	6,56	DRB1*1502	0,94	DRB1*1406	0,04
DRB1*1301	5,69	DRB1*0901	0,79	DRB1*1402	0,03
DRB1*0401	5,18	DRB1*1305	0,38	DRB1*1116	0,02
DRB1*1104	4,73	DRB1*0408	0,38	DRB1*1306	0,02
DRB1*1302	3,71	DRB1*0803	0,29	DRB1*1310	0,02
DRB1*0404	2,87	DRB1*0804	0,29	DRB1*0106	0,02
DRB1*1401	2,72	DRB1*1602	0,23	DRB1*0414	0,02
DRB1*0102	2,47	DRB1*0406	0,22	DRB1*1407	0,02
DRB1*0801	1,86	DRB1*0304	0,17	DRB1*0411	0,02
DRB1*1601	1,69	DRB1*0305	0,14	DRB1*0417	0,02
DRB1*0403	1,58	DRB1*0802	0,13	DRB1*1417	0,02
DRB1*1303	1,55	DRB1*1404	0,12	DRB1*1423	0,02
DRB1*1201	1,40	DRB1*0806	0,10	DRB1*1433	0,02
DRB1*0402	1,30	DRB1*1202	0,09	DRB1*1109	0,01
DRB1*0405	1,28	DRB1*0302	0,09	DRB1*1408	0,01
DRB1*1102	1,27	DRB1*0805	0,06	DRB1*1403	0,01

Table 18: Frequencies of HLA-DRB1 alleles, not presented alleles have frequencies of 0% in Western European populations. Red coloured alleles were defined as frequent alleles for this project. (from www.allelefrequencies.net)

[108] Middleton, D. et al.; 2003, Tissue Antigens, Vol. 61: 403 - 407

The likelihood calculation is done as exemplarily explained with the individual 1333-14 from the CEPH panel.

Individual	Allele 1	Allele 2
1333-14	HLA-DRB1*0801	HLA-DRB1*1001
	HLA-DRB1*0804	HLA-DRB1*1001
	HLA-DRB1*0802	HLA-DRB1*1001
	HLA-DRB1*0806	HLA-DRB1*1001
	HLA-DRB1*0807	HLA-DRB1*1001
	HLA-DRB1*0826	HLA-DRB1*1001
	HLA-DRB1*0811	HLA-DRB1*1001
	HLA-DRB1*0805	?
	HLA-DRB1*0813	?
	HLA-DRB1*0824	?

Table 19: Example of result output

As described the frequencies of the alleles in the "general" population is factored in. Since multiplication with 0 is not possible the frequencies for those cases were defined as $1*10^{-6}$ (or 0,0001 %). The frequencies of unknown alleles (represented by a question mark in the result table) were defined as $1*10^{-7}$ (or 0,00001%). These products were compared by normalization. Therefore all resulting factors were summed. Finally all products are related to the sum, and the outcome corresponds to the likelihood that this allele combination represents the real presented HLA type of the individual. Table 20 shows an example for likelihood design.

Allele 1	Frequencies	Allele 2	Frequencies	Products of Frequencies	Likelihood
DRB1*0801	1,86	DRB1*1001	1,02	1,8972	0,781502735
DRB1*0804	0,29	DRB1*1001	1,02	0,2958	0,121847201
DRB1*0802	0,13	DRB1*1001	1,02	0,1326	0,054621159
DRB1*0806	0,1	DRB1*1001	1,02	0,102	0,042016276
DRB1*0807	0,00001	DRB1*1001	1,02	0,0000102	4,20163E-06
DRB1*0826	0,00001	DRB1*1001	1,02	0,0000102	4,20163E-06
DRB1*0811	0,00001	DRB1*1001	1,02	0,0000102	4,20163E-06
DRB1*0805	0,06	?	0,000001	0,00000006	2,47155E-08
DRB1*0813	0,00001	?	0,000001	1E-11	4,11924E-12

DRB1*0824	0,00001	?	0,000001	1E-11	4,11924E-12
				Σ = 2,42763066	

Table 20: Example for likelihood design.

Finally two files are presented. The first file contains all possible HLA allele combinations, for the measured DNA sample. Further the likelihood that this combination is really present in the analysed donor is established. This file was stored in a database for further use by the registries. A second files created at the same time contains only allele combination of an analyzed DNA, which together have a likelihood of 99 %. The main disadvantage is that valuable information could get lost, for instance if there is a possibility of new HLA types.

Individual	**Allele 1**	**Allele 2**		**Likelihood**
1333_14	DRB1*0801	DRB1*1001	r r	0.810890418006865
	DRB1*0804	DRB1*1001	r r	0.112205759343874
	DRB1*0802	DRB1*1001	r r	0.0412337692763193
	DRB1*0806	DRB1*1001	r r	0.035528391682563

Table 21: Example of an output of results reduced to 99% likelihood.

To avoid the loss of information an alternative way to present the estimated HLA types is provided. For this the Allele Code of the National Marrow Donor Program (NMDP)[109] was used to simplify the result output. This at regular intervals updated list of allele codes has been developed for cases where alleles are not unambiguously distinguishable. In the example of individual 1333-14 these are the HLA alleles DRB1*0801/0802/0804/0806/0807/0811/0826 as one parental allele. For these HLA allele combinations so far no Allele Code exists, but for DRB1*0801/0802/0804/0806/0807/0811 the Allele Code is DRB1*08MRZ. Further the Allele Code DRB1*08AHN is for the alleles DRB1*0805/0824. Therefore the output can be summarized as in Table 22. This allele coding is most commonly used by Transplant Centre Coordinators.

[109] www.nmdpresearch.org

Individual	Allele 1	Allele 2
1333-14	HLA-DRB1*08MRZ	HLA-DRB1*1001
	HLA-DRB1*0826	HLA-DRB1*1001
	HLA-DRB1*08AHN	?
	HLA-DRB1*0813	?

Table 22: Summarized output of a screening result for Individual 1333-14

Finally both ways of result presentation were provided in the database of the MADO project.