Results

Explanatory remarks

In the following chapters several novel procedures using MALDI-MS for genotyping single nucleotide polymorphisms termed the GOOD assays are shown. All of these procedures are based on the charge-state concept and therefore do not require any purification prior to MALDI analysis. They are done by simple addition of reagent solutions, thermocycling and incubations. As the GOOD assays were in continuous development several of its stages and enhancements are presented herein.

These methods do not need any separation or purification procedures and are - at least in some cases - implemented into a fully automated process. The standard GOOD assay for positive and negative ion mode is presented in several variations and for different applications. Thereafter the simplified GOOD assay is shown, which could be considered a culmination of procedures using primer extension for the generation of charge-tagged and allele-specific products. Subsequently it is demonstrated that also ligases readily accept the DNA backbone modifications required for charge-tagging of DNA. Finally, a procedure using a flap-endonuclease for the generation of charge-tagged and allele-specific products is shown. This might be an interesting addition to the GOOD assay technology.

Overview of the standard GOOD assay

The first developed version of the GOOD assay requires a PCR, a shrimp alkaline phosphatase digestion of unmodified nucleotides remaining from the PCR, a primer extension with a positive charge-tag carrying primer and phosphorothioate (dideoxy-) nucleotides, a phosphodiesterase II digestion for the removal of the unmodified DNA part of the extension primers and an alkylation step for the charge neutralisation of the DNA phosphorothioate backbone of the resulting product.[124,125] These products are then

measured by MALDI-MS with a sensitivity ca. 100-fold higher than with comparable unmodified DNA products. While first only a commercially available amino-modified T was used to attach positive charge-tags [124], the assay became fully applicable by the introduction of novel amino-modified G^{NH2}, A^{NH2}, C^{NH2} and U^{NH2}.[125] By the use of a novel DNA polymerase the sequence of the GOOD assay was shortened from 5 steps to 4 steps.[126] The principle of the procedure is shown in figure 3.1.

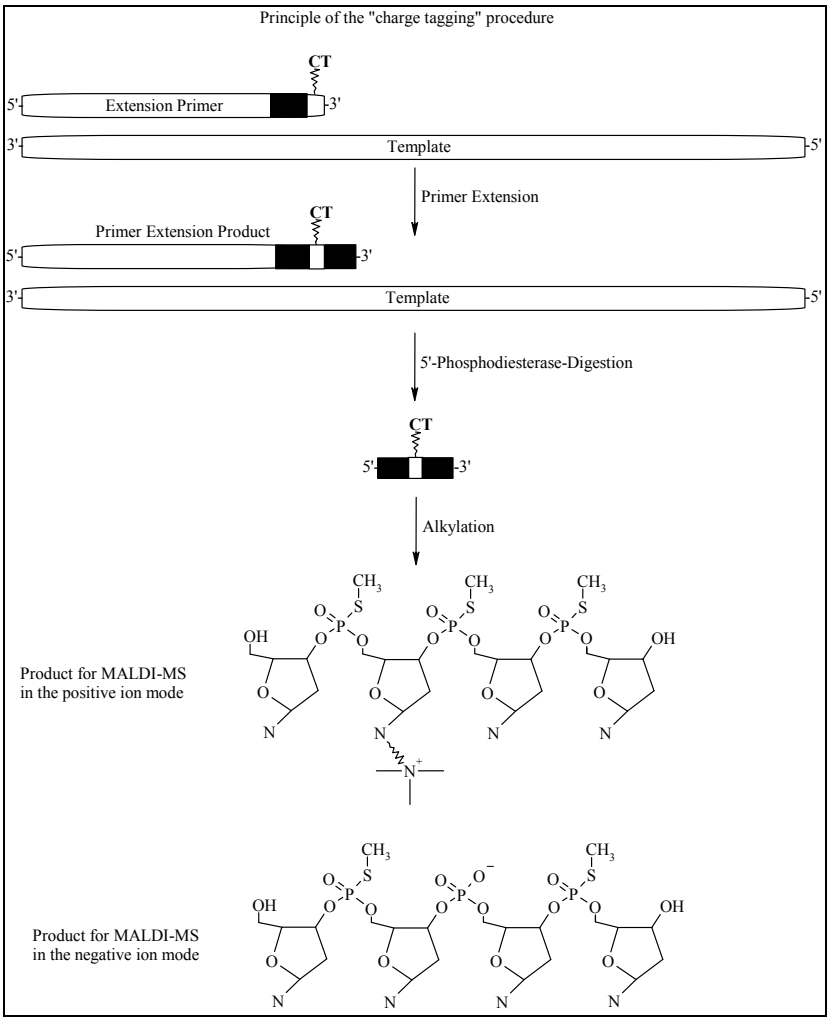

Figure 3.1. Scheme of the first two developed versions of the GOOD assay for positive and negative ion mode detection. N stands for nucleobases, while a quaternary ammonium ion is indicated by N^+. The primer extension can be executed with deoxynucleotides and/or dideoxynucleotides.

For the first developed GOOD assay for negative ion mode detection, extension primers contain a phosphate bridge at the 3'-ends, followed by phosphorothioates to the

phosphate. These primers are extended with α-S-ddNTPs (respective products of this variation of the GOOD assay are shown in figure 3.1).[124] The molecular biological protocol is similar to the GOOD assay in the positive ion mode version and universally applicable. Later it turned out that by introduction of two or three phosphorothioates masses could be shifted.[126] With more than three phosphorothioates product signals in the mass spectrometer became small and assays were not sufficiently stable.

Several enhancements of the GOOD assay were developed later (figures 3.2 and 3.3).[126] For positive ion mode detection extension primers contain phosphorothioate linkages at the 3'-end and a quaternary ammonium on one of the nucleobases. Surprisingly, these primers could not only be extended by α-S-ddNTPs but also by ddNTPs without significantly loosing detection sensitivity. Thus also zwitterionic, backbone-modified DNA is detectable by MALDI with very high sensitivity. This does not correspond entirely with the mentioned charge-state principle.

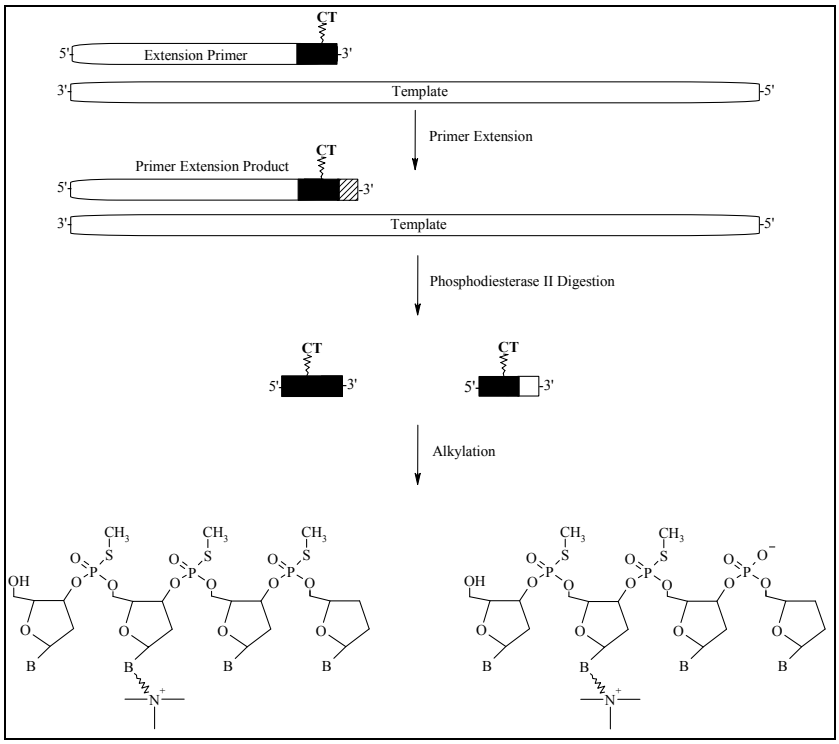

Figure 3.2. Two different variations of the GOOD assay for positive ion mode detection. B stands for a nucleobase. Phosphorothioates that resist 5'-phosphodiesterase digestion are highlighted in black. The hatchet part can optionally consist of a phosphorothioate or a phosphate linkage.

If the positively charge-tagged primer is elongated with α-S-ddNTPs, the resulting product after phosphodiesterase digestion and alkylation contains a single positive charge as is described above, while extension with ddNTPs results in zwitterions containing a negatively charged phosphate group and a positively charged quaternary ammonium ion (figure 3.2). Zwitterions like this are generally measured with a sensitivity ca. 2-fold lower than positively charge-tagged DNA molecules. These products (figure 3.2) are suitable for positive ion mode detection but hardly detectable in the negative ion mode.

The bandwidth of the negative charge-tag approach was enlarged by the use of primers containing only phosphorothioates at their 3'-ends and extension with ddNTPs.[126] The resulting products for negative ion mode detection carry phosphorothioate linkages that are charge-neutral after alkylation and one phosphate deriving from the ddNTP (figure 3.3). GOOD assays for negative ion mode detection do not require an extra charge-tagging of extension primers because the fixed charge derives from a phosphate in the oligonucleotide or an extended nucleotide.

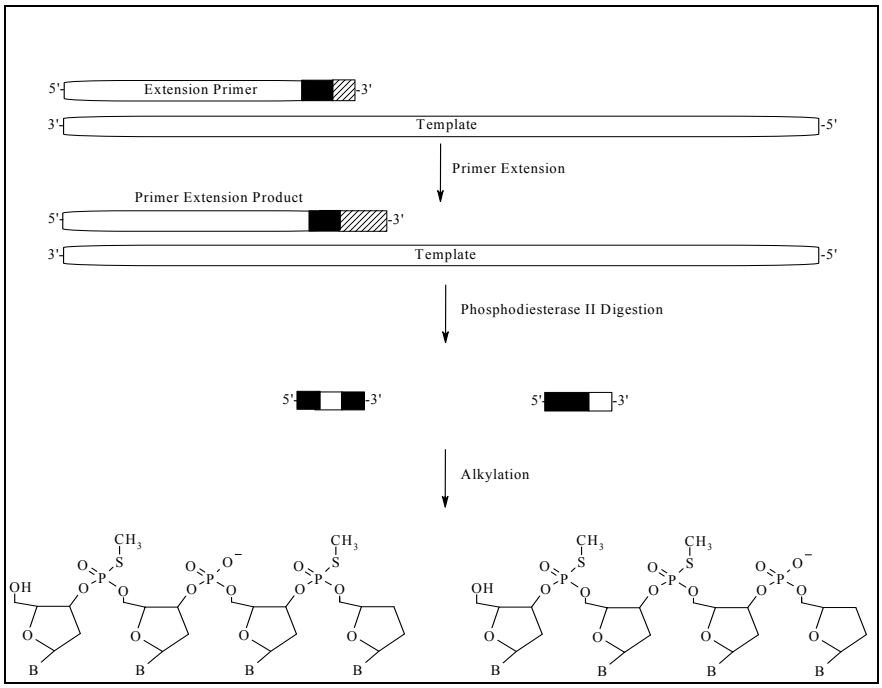

Figure 3.3. Two different variations of the GOOD assay for negative ion mode detection. B stands for a nucleobase. Phosphorothioates that resist 5'-phosphodiesterase digestion are highlighted in black. Again the hatchet part can optionally consist of a phosphorothioate or a phosphate linkage.

Usually, "regular" DNA is analysed in the negative ion or more seldomly in the positive ion mode. Nevertheless, unmodified DNA is usually detectable by MALDI-MS in both ion modes with only small differences. By applying the charge-tag approach it is possible to analyse positively and negatively charge-tagged DNA molecules specifically on the same sample using for positively charge-tagged molecules the positive ion mode and for negatively charge-tagged molecules the negative ion mode.[126] The multiplex preparation of positively and negatively charge-tagged DNA products of two different SNPs in one reaction sequence and sequential analysis on one sample preparation by switching sequentially the ion mode is a unique feature in MALDI-MS.

The robustness of the GOOD assay was large enough to work with crude DNA extracted from Proteinase K digested tissue samples.[125] A template extraction method was introduced that allows genotyping from tissue that was taken while placing an ear-tag on an animal.

Applications of the GOOD assay in the positive ion mode version

For the GOOD assay in the positive ion mode version four novel amino-modified phosphoramidates for A^{NH2}, U^{NH2}, G^{NH2}, and C^{NH2} (figure 2.1) were introduced into oligonucleotide synthesis and charge-tagged. The GOOD assay was established for genotyping SNP 129 in the human prion protein gene, which is implicated in prion diseases.[127] For the PCR of the prion protein gene, human DNA purified from blood samples was used. In the case of a PCR with bovine DNA, the procedure started with a DNA template obtained by a Proteinase K digestion of tissue taken from the animal's ear without any purification. Applying the same PCR conditions and materials, an amplification of a stretch of the human and the bovine prion protein gene containing the SNP 129 was achieved. The extension primer contained an A^{CT} and was implemented successfully even in the case of human DNA where this primer contained one mismatch at position 11 compared to the bovine sequence. Figure 3.4 shows the analysis of heterozygous and homozygous human DNA at SNP 129.

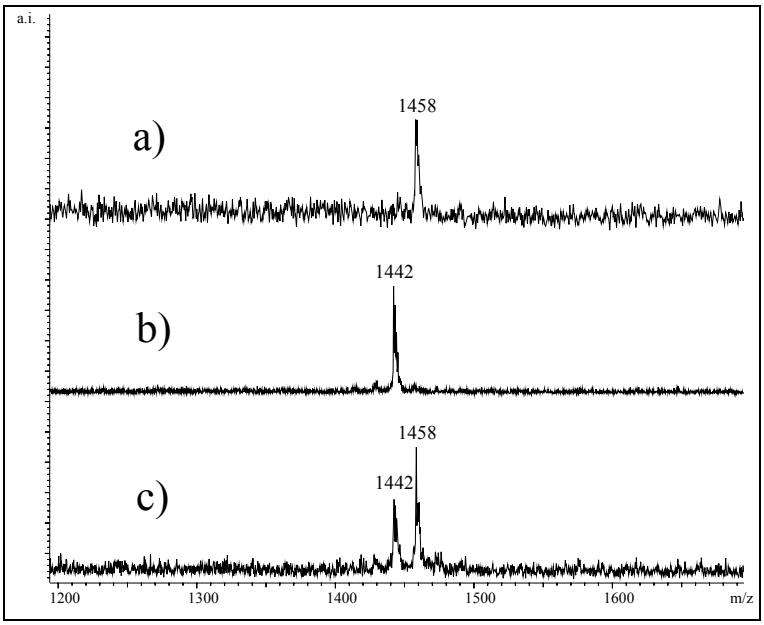

Figure 3.4. In trace a) the MALDI-MS analysis of an individual homozygous for G at the SNP 129 of the prion protein gene is shown. The resulting product of the GOOD assay ($G_{pt}A^{CT}{}_{pt}G_{pt}G$) has the mass of 1458 Da. In trace b) the analysis of an individual homozygous for A with the resulting product $G_{pt}A^{CT}{}_{pt}G_{pt}A$ and the corresponding mass 1442 Da is displayed. In trace c) the analysis of heterozygous DNA is shown. As it can be seen from the signal pattern isotopic resolution was achieved.

In Figure 3.5 the spectrum of the analysis of DNA from a cow homozygous for the A allele at SNP 129 is displayed.

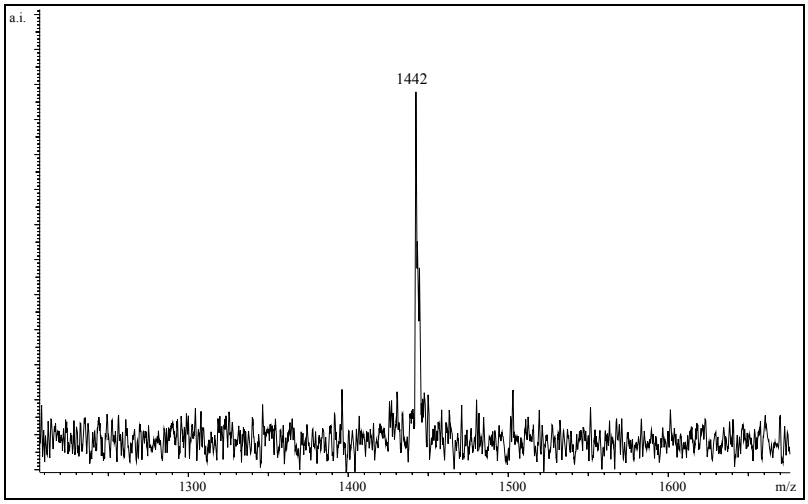

Figure 3.5. The GOOD assay is applied to SNP 129 of the prion protein gene using bovine DNA. The assay started with tissue samples that were only Proteinase K digested but not purified.

8 human and 4 cattle DNAs were used in this study and genotypes were confirmed by DNA sequencing. The quality of spectra was similar to those where purified human DNA was used as template for the GOOD assay.As there is no publication of a SNP in the bovine genome at position 129 of the prion gene, subsequently only cow DNA homozygous for A was found.

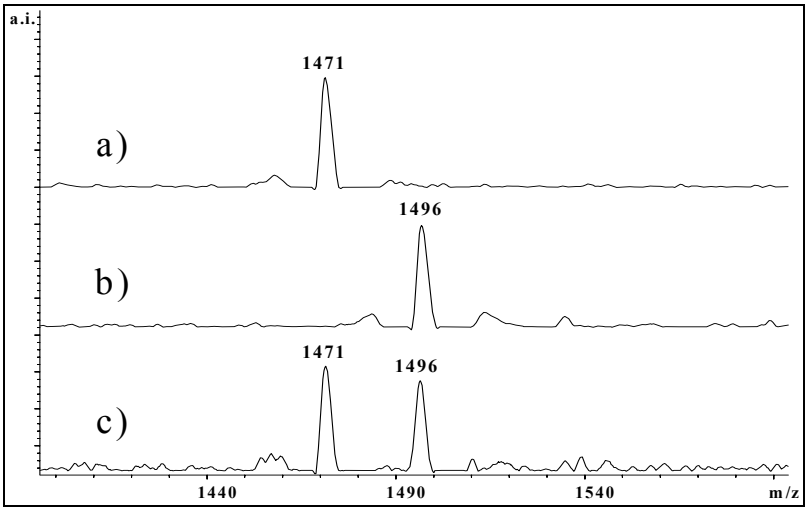

Figure 3.6. MALDI-MS spectra of genotyping SNP A457C in the CAV gene. Products of the assays are $A_{pt}G^{CT}{}_{pt}T_{pt}G$ (1496 Da) and $A_{pt}G^{CT}{}_{pt}T_{pt}T$ m/z (1471 Da). The three spectra show a homozygous individual for either allele (traces a) and b)) and a heterozygous individual (trace c)).

Figure 3.6 shows the analysis of SNPA457C in the Caveolin 1 (CAV 1) gene, which is a candidate gene for cardiovascular disease.[128] The GOOD assay in this case was performed with a primer containing a G^{CT}. 96 human DNAs were studied and correct SNP genotypes were confirmed by DNA sequencing. If a GOOD assay was not working, in most cases the sequencing reaction also did not give satisfactory results. As a control the PCR was verified by agarose gel electrophoresis and it turned out that this reaction was not efficient enough to provide template for the subsequent sequencing or the primer extension reaction.

Figure 3.7 displays the analysis of SNP 33730 in the angiotensin converting enzyme, which is a candidate gene for cardiovascular disease and hypertension.[129] For primer extension a primer containing a C^{CT} was used. 96 DNAs were examined and the correct results were confirmed by DNA sequencing. A success rate of 90 % was obtained. The

results of the GOOD assay matched completely with DNA sequencing. At 1090 m/z residual primer of the extension reaction was found. Signals deriving from digested residual primer were observed quite often. They can be used as internal controls for the phosphodiesterase digestion, the alkylation and for internal calibration.

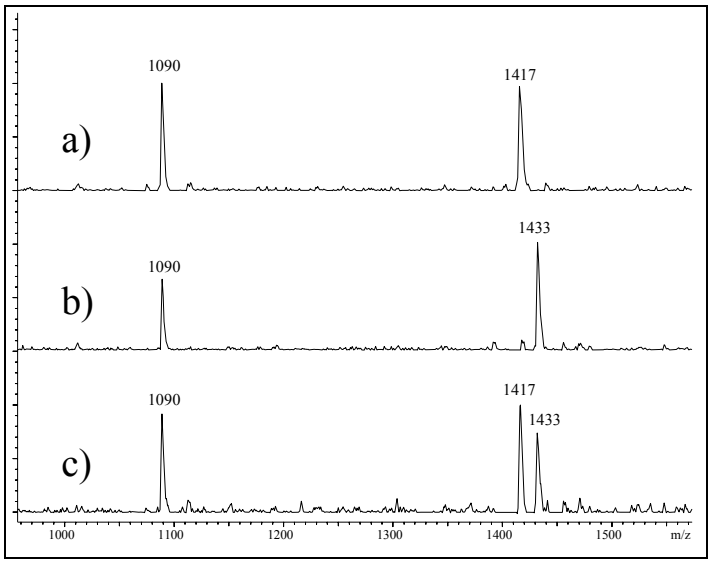

Figure 3.7. MALDI-MS spectra of the analysis of SNP 33710 in the angiotensin converting enzyme. Products of the assays are $T_{pt}C^{CT}{}_{pt}C_{pt}A$ (1417 Da) and $T_{pt}C^{CT}{}_{pt}C_{pt}G$ (1433 Da). The three spectra show a homozygous individual for either allele (traces a) and b)) and a heterozygous individual (trace c)).

In figure 3.8 the analysis of SNP R670G in the platelet/endothelial cell adhesion molecule (PECAM) gene is shown, which also is a candidate gene for cardiovascular disease.[130] A primer containing an U^{CT} was applied. Also in this case 96 DNAs were analysed and the results were confirmed by DNA sequencing. Both reactions gave corresponding results. Negative results (5) were obtained due to PCR failure. Therefore DNA sequencing was not feasible either.

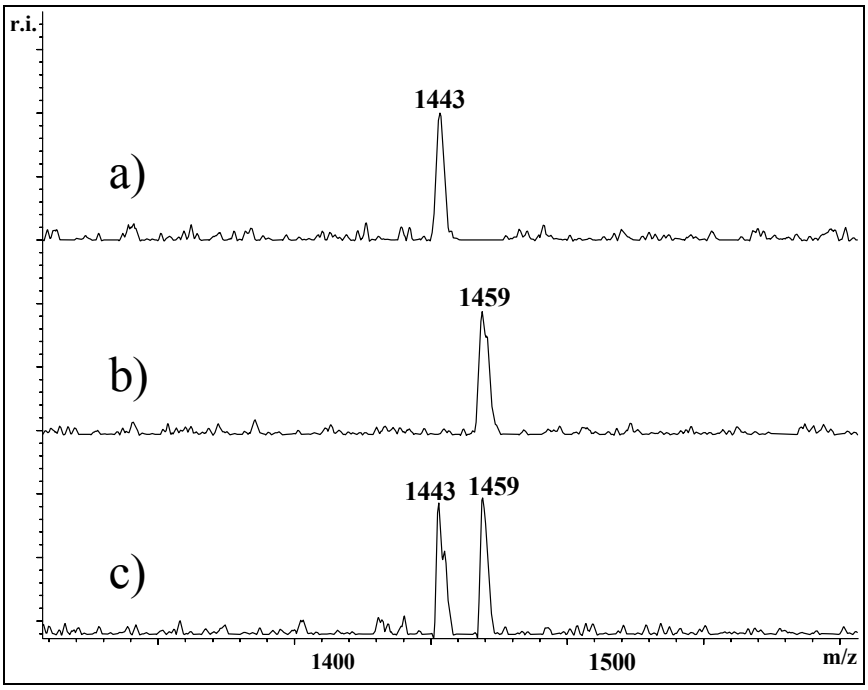

Figure 3.8. MALDI-MS spectra of genotyping of the SNP R670G in the PECAM gene. The products of the GOOD assay were $G_{pt}U^{CT}{}_{pt}C_{pt}A$ and $G_{pt}U^{CT}{}_{pt}C_{pt}G$ with masses 1443 Da and 1459 Da. In traces a) and b) the respective analysis of homozygous patient DNA is shown, while in trace c) heterozygous DNA was analysed.

In figure 3.9 spectra of four variants of the GOOD assay for positive ion mode detection are displayed using a positively charge-tagged primer and α-S-ddNTPs or respective ddNTPs for the generation of allele-specific products of SNP G58A in the PECAM gene. In each experiment heterozygous human mix DNA was used. As is highlighted in table 3.1, trace a) shows the analysis of products $G_{pt}A^{CT}{}_{pt}T_{pt}A$ and $G_{pt}A^{CT}{}_{pt}T_{pt}G$ with corresponding masses 1480 Da and 1496 Da. Therefore the primer was extended with α-S-ddATPs and α-S-ddGTPs. In trace b) the analysis of products $G_{pt}A^{CT}{}_{pt}TA$ and $G_{pt}A^{CT}{}_{pt}TG$ with masses 1450 Da and 1466 Da is displayed. In this case the primer was elongated with regular ddATPs and ddGTPs. For trace c) the primer extension was done with ddGTPs and α-S-ddATPs resulting in oligomers $G_{pt}A^{CT}{}_{pt}TG$ and $G_{pt}A^{CT}{}_{pt}T_{pt}A$ with masses 1466 Da and 1480 Da. In the case of trace d) the primer was extended with ddATPs and α-S-ddGTPs with masses 1450 Da and 1496 Da.

Trace	Primer sequence	Elongation substrate	Product	m/z	Charge state
a)	ATGTTCCGAGAAGAACAG$_{pt}$A$^{CT}_{pt}$T	α-S-ddGTP/ α-S-ddATP	G$_{pt}$A$^{CT}_{pt}$T$_{pt}$G G$_{pt}$A$^{CT}_{pt}$T$_{pt}$A	1496 1480	1+ 1+
b)	ATGTTCCGAGAAGAACAG$_{pt}$A$^{CT}_{pt}$T	ddGTP/ ddATP	G$_{pt}$A$^{CT}_{pt}$TG G$_{pt}$A$^{CT}_{pt}$TA	1466 1450	Zwitterion Zwitterion
c)	ATGTTCCGAGAAGAACAG$_{pt}$A$^{CT}_{pt}$T	ddGTP/ α-S-ddATP	G$_{pt}$A$^{CT}_{pt}$TG G$_{pt}$A$^{CT}_{pt}$T$_{pt}$A	1466 1480	Zwitterion 1+
d)	ATGTTCCGAGAAGAACAG$_{pt}$A$^{CT}_{pt}$T	α-S-ddGTP/ ddATP	G$_{pt}$A$^{CT}_{pt}$T$_{pt}$G G$_{pt}$A$^{CT}_{pt}$TA	1496 1450	1+ Zwitterion

Table 3.1.

All of these DNA oligomers were easily measured in the positive ion mode. Interestingly, these molecules were hardly detected in the negative ion mode. Signals were detected with at most 10 % of the signal to noise ratio of spectra obtained in the positive ion mode. All spectra of the GOOD assay for positive ion mode detection exhibited the beginning of isotopic resolution and a more than sufficient signal to noise ratio for easy allele calling. In the spectra shown in figure 3.9 zwitterionic products were measured with equal sensitivity as regularly charge-tagged DNA. However, from experience these molecules are generally detectable with a lower signal to noise ratio or sensitivity compared to regularly charge-tagged DNA molecules (ca. 1:2).

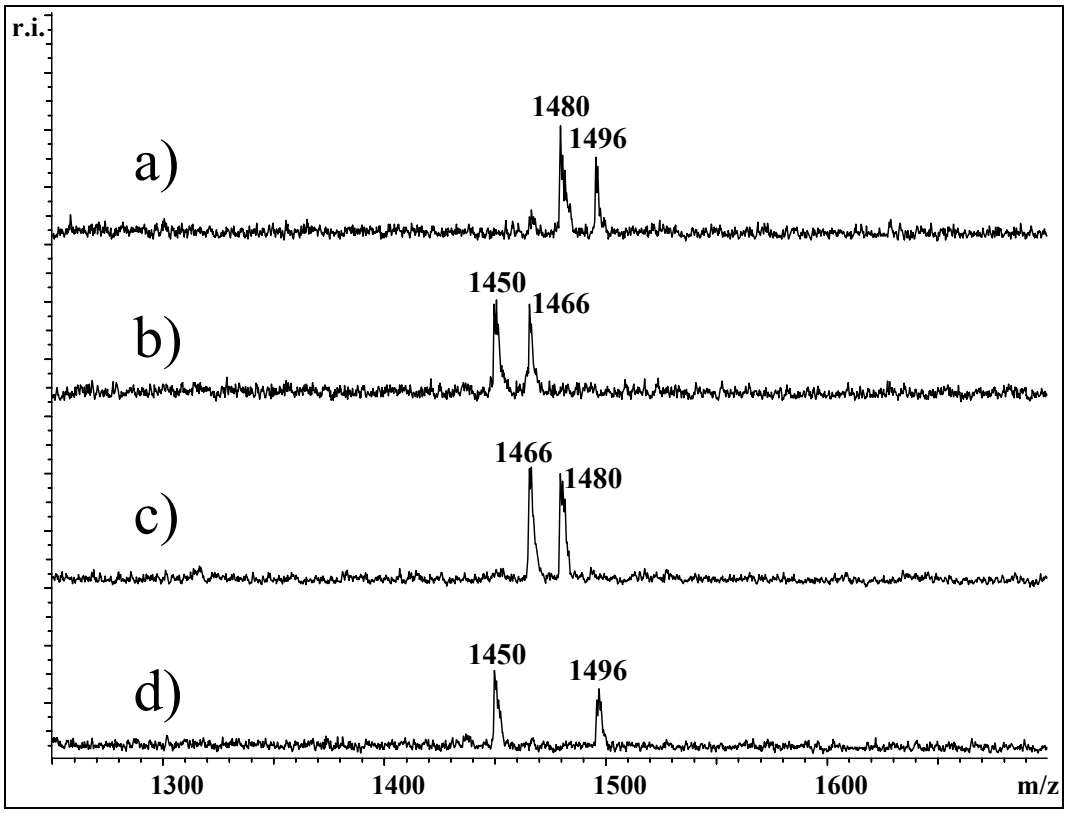

Figure 3.9. Four variations of the GOOD assay for positive ion mode detection were executed for SNP G58A in the PECAM gene. Trace a) shows the analysis of products $G_{pt}A^{CT}{}_{pt}T_{pt}A$ and $G_{pt}A^{CT}{}_{pt}T_{pt}G$ with corresponding masses 1480 Da and 1496 Da. In this case the extension primer was elongated with α-S-ddATPs and α-S-ddGTPs. Trace b) shows the analysis of products $G_{pt}A^{CT}{}_{pt}TA$ and $G_{pt}A^{CT}{}_{pt}TG$ with masses 1450 Da and 1466 Da. The primer was extended with regular ddATPs and ddGTPs. In the case of trace c) the primer extension was performed with ddGTPs and α-S-ddATPs resulting in products $G_{pt}A^{CT}{}_{pt}TG$ and $G_{pt}A^{CT}{}_{pt}T_{pt}A$ with masses 1466 Da and 1480 Da. For trace d) the primer was extended using ddATPs and α-S-ddGTPs resulting in products $G_{pt}A^{CT}{}_{pt}TA$ and $G_{pt}A^{CT}{}_{pt}T_{pt}G$ with masses 1450 Da and 1496 Da.

Applications of the GOOD assay in the negative ion mode version

For negative ion detection three different polymorphisms of the human gene for the granulocyte-macrophage colony stimulating factor (GM-CSF) were analysed: a C/A transversion at position 99, a C/T transition at position 345 and a C/T transition at position 417 of the generated PCR product. GM-CSF is a growth factor with a proliferative stimulus for bone marrow neutrophil stem cell precursors and plays a

crucial role in the regulation of the neutrophil-mediated inflammatory response.[131,132] The products after primer extension, phosphodiesterase II digestion and alkylation were 4-mers. They contained a single negative charge deriving from the phosphate bridge between nucleoside 1 and 2 from the 3'-end of the extension primer.

The different polymorphisms were analysed individually (figure 3.10).

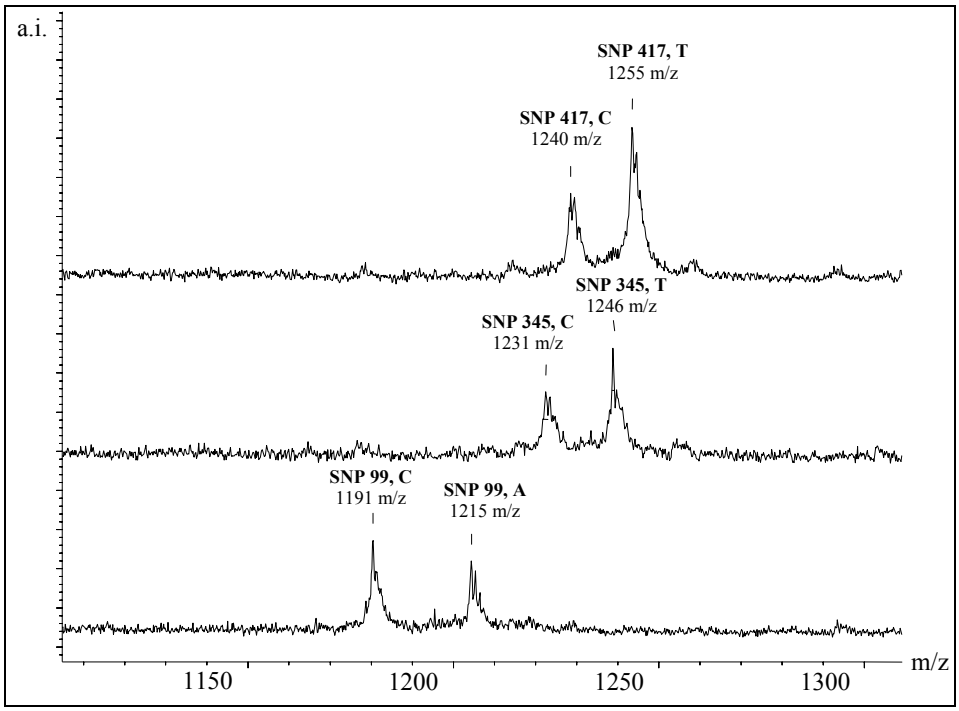

Figure 3.10. The GOOD Assay in negative ion mode applied to polymorphisms in position 99, 345 and 417 of GM-CSF with individuals that are heterozygous. The signals corresponding to the two alleles are easily assigned and distinguished.

The products for position 99 with the sequences $T_{pt}GC_{pt}C$ and $T_{pt}GC_{pt}A$ have corresponding masses 1191 Da and 1215 Da, the products for position 345 with the sequences $G_{pt}TG_{pt}C$ and $G_{pt}TG_{pt}T$ have corresponding masses 1231 Da and 1246 Da, while the products for position 417 with the sequences $A_{pt}GG_{pt}C$ and $A_{pt}GG_{pt}T$ have corresponding masses 1240 Da and 1255 Da. The alleles of all polymorphisms can be distinguished easily both in individual (figure 3.10) and multiplex analysis (figure 3.12). As a control the DNAs used were sequenced for confirmation of genotypes. The mass

spectrometric analysis matches with the DNA sequences in all cases. Fifteen patient DNAs were used for this study.

In figure 3.11 spectra of three different possibilities to obtain allele-specific products by the GOOD assay for negative ion mode detection using primer 2 (table 3.2) with α-S-ddNTPs, primer 3 with α-S-ddNTPs, and primer 1 with ddNTPs, are demonstrated.

Primer	Primer sequence	Extension substrates	DNA Polymerase	Product	m/z
1	ATGTTCCGAGAAGAACAG$_{pt}$A$_{pt}$T	ddGTP/ ddATP	Tma 31 FS	G$_{pt}$A$_{pt}$TG G$_{pt}$A$_{pt}$TA	1257 1241
2	ATGTTCCGAGAAGAACAG$_{pt}$AT	α-S-ddGTP/ α-S-ddATP	Tma 31 FS or Thermosequenase	G$_{pt}$AT$_{pt}$G G$_{pt}$AT$_{pt}$A	1257 1241
3	ATGTTCCGAGAAGAACA$_{pt}$G$_{pt}$AT	α-S-ddGTP/ α-S-ddATP	Tma 31 FS or Thermosequenase	A$_{pt}$G$_{pt}$AT$_{pt}$G A$_{pt}$G$_{pt}$AT$_{pt}$A	1600 1584

Table 3.2

Efficient extension of primer 1 was only achievable using Tma 31 FS DNA polymerase while with primers 2 and 3 this DNA polymerase and Thermosequenase yielded equal extension efficiencies. Primer 1 contained two phosphorothioate bridges at its 3'-end and was extended with ddGTPs and ddATPs. The respective spectrum is shown in trace a). For the approach shown in trace b) primer 2 and α-S-ddGTPs and α-S-ddATPs were used. The measured products in trace a) were G$_{pt}$A$_{pt}$TA and G$_{pt}$A$_{pt}$TG with respective masses 1241 Da and 1257 Da while the detected products of trace b) were G$_{pt}$AT$_{pt}$A and G$_{pt}$AT$_{pt}$G with the same masses 1241 Da and 1257 Da. The signal sensitivity of these products and resulting signal to noise rations lie in a similar range (approximately 10:1). By using primer 3 containing one more phosphorothioate bridge

than primer 2, the product masses were shifted as it is demonstated in trace c). Respective products $A_{pt}G_{pt}AT_{pt}A$ and $A_{pt}G_{pt}AT_{pt}G$ with masses 1584 Da and 1600 Da were detected with a similar signal sensitivity and signal to noise ratio.

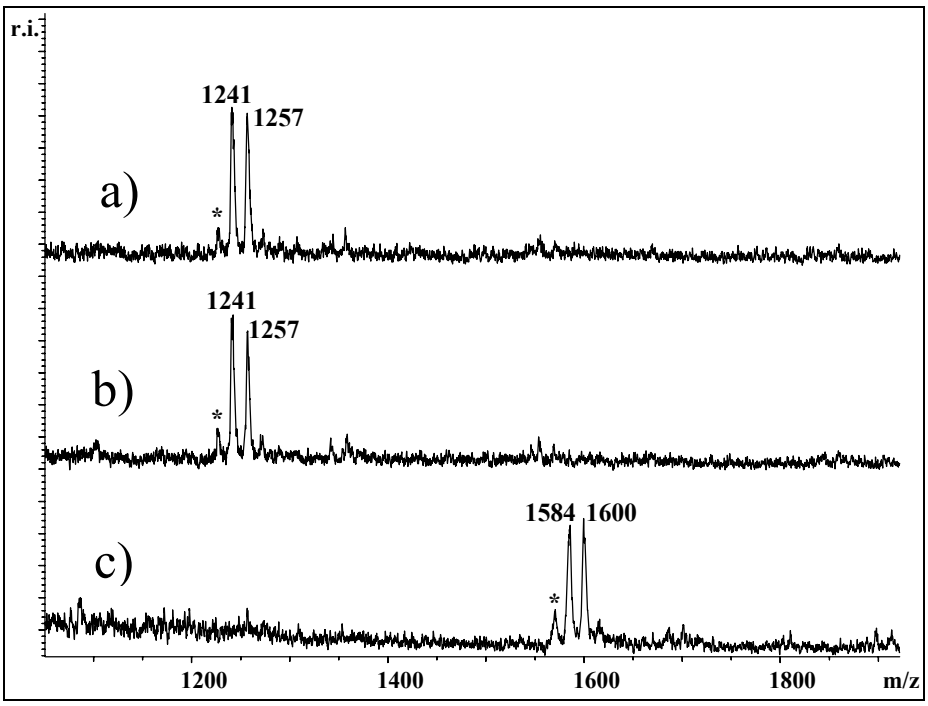

Figure 3.11. Three variations of the GOOD assay for negative ion mode detection are presented on SNP G58A in the PECAM gene. In the case of trace a) primer 1 was extended with ddATPs and ddGTPs resulting in products $G_{pt}A_{pt}TA$ and $G_{pt}A_{pt}TG$ with masses 1241 Da and 1257 Da while for trace b) primer 2 was elongated with α-ddATPs and α-ddGTPs resulting in products $G_{pt}AT_{pt}A$ and $G_{pt}AT_{pt}A$ having the same masses. In the case of trace c) primer 3 containing one phosphorothioate linkage more than primer 2 was used resulting in products $A_{pt}G_{pt}AT_{pt}A$ and $A_{pt}G_{pt}AT_{pt}G$ with masses 1584 Da and 1600 Da. Signals indicated with an asterisk in this figure were under-alkylated byproducts that were sometimes observed using the negative ion mode detection of the mass spectrometer.

Automation of the standard GOOD assay

The aim of this work was to provide efficient procedures for the analysis of SNPs. One of the main objectives therefore was, that the method could be applied to commercially available pipetting robots, as an everyday user will not be interested in

applying a technology for which he has to invest in the development of automation. The volume and thereby the amount of materials for the GOOD assay was reduced to a volume starting with 3 µl PCR and ending with 28 µl total volume.

Automation protocols were applied successfully on a BasePlate robot. This is a liquid- handling robot equipped with a 96-tip head. 384 samples were prepared in one run with four robot movements for each of the three liquid dispensings. Microtiter plates were sealed and manually transferred to thermocyclers for the PCR and primer extension reaction step, or an incubator set for the phosphodiesterase digestion. With the BasePlate robot a high-throughput device for SNP genotyping was established in combination with automatic measurement on a Bruker Reflex III and a Bruker Biflex III mass spectrometer, both equipped with a SCOUT 384^{TM} ion source, and on a Bruker Autoflex time-of-flight mass spectrometer, which was constructed for high-throughput applications. High reproducibility in automatic measurements was observed, accumulating on average only 10 laser shots per spectrum. Spectra were recorded and alleles called automatically using the GenoTools SNP manager software. The quality of the spectra posed no problem for the allele-calling software.

Further details about the GOOD assay

The multiplexibility of the GOOD assay was studied and generally a factor of 3 was easily achieved on the basis of one PCR product and extension reactions with three primers as is shown for 3 SNPs in GM-CSF (figure 3.12). The ability to multiplex reactions is of great importance for reducing the cost per SNP genotype and analysis time. Nevertheless, the efficiency of enzymatic reactions (PCR and primer extension) in a multiplex assay depended strongly on the SNPs that had to be combined. The surrounding DNA sequences had a significant influence on the quality of the PCR reaction and the primer extension.

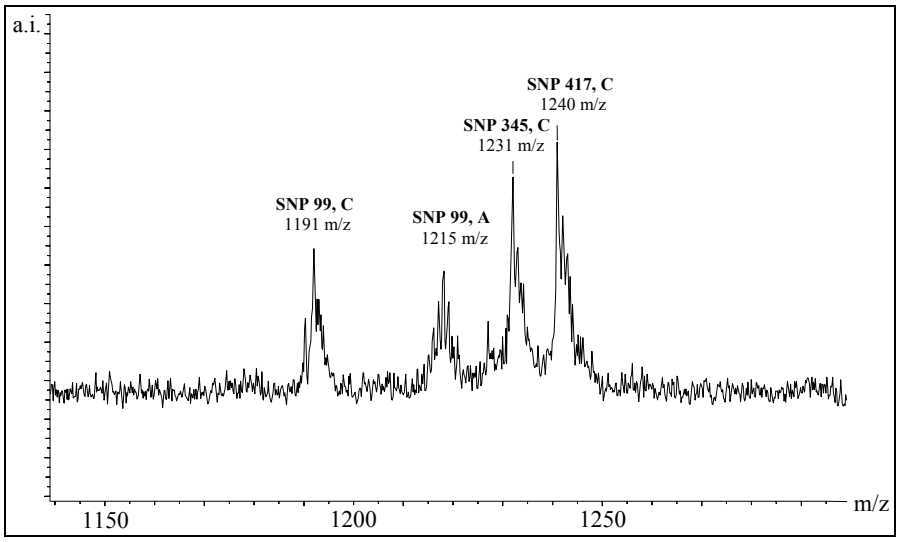

Figure 3.12. Multiplexed genotyping by the GOOD Assay in negative ion mode of an individual that is heterozygous for A and T at position 99, homozygous for C at position 345 and homozygous for C in position 417 in GM-CSF.

As was shown by several examples of the GOOD assay in the positive ion mode version, amino-modified phosphoramidites A^{NH2}, G^{NH2}, C^{NH2}, and U^{NH2} were successfully integrated into extension primers.

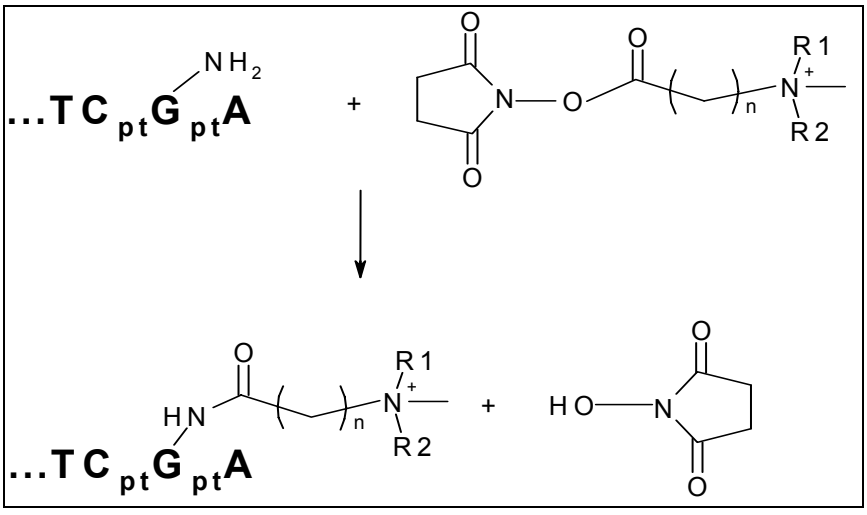

Figure 3.13. The charge-tag reaction of an amino-modified primer (3'-sequence in bold) and a charge-tag reagent (usually 6-trimethylammoniumhexyryl-N-hydroxy-succinimidylester). For increasing the potential of multiplexing different charge-tag reagents can be used.

The option for multiplexing was thereby increased by the addition of charge tags with different masses. Usually, 6-trimethylammoniumhexyryl-N-hydroxy-succinimidylester is used for charge-tagging. Three additional charge-tags (figure 3.13), 6-trimethylammoniumpentyryl-N-hydroxy-succinimidylester (14 Da lighter), 6-dimethyl-ethylammoniumhexyryl-N-hydroxy-succinimidylester (14 Da heavier) and 6-diethyl-methylammoniumhexyryl-N-hydroxy-succinimidylester (28 Da heavier) synthesized by Dr Kurt Berlin were tested successfully. These reagents can be used for shifting masses of products of the GOOD assays.

It was found that for subsequent reactions of the initial PCR a template abundance of 20 fmol could be sufficient for optimised GOOD assays. A 1:50 dilution of a PCR that contained around 1 pmol amplification product was utilised for the following steps and yielded a sufficient amount of product for detection. This information may be valuable particularly for miniaturisation and for multiplexing of the GOOD assay.

The influence of the use of phosphorothioates concerning efficient hybridisation as well as primer extension was studied. A typical primer for GOOD assays containing two phosphorothioate linkages has four possible stereo-configurations. Stereoisomers of a charge-tagged primer utilised for the analysis of SNPA457C in the CAV gene were separated by Dr Noah P. Christian (CNG) using ion-exchange HPLC (figure 3.14).

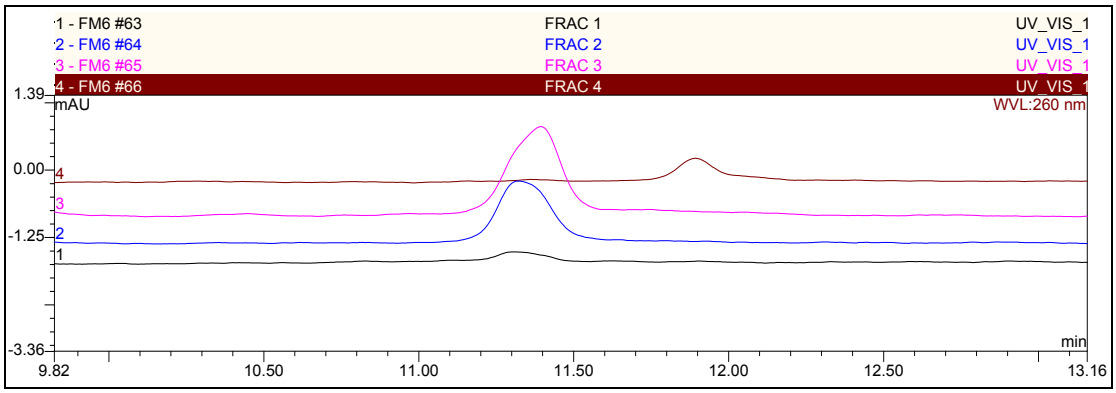

Figure 3.14. Chromatogram of the ion-exchange HPLC. Separation of stereoisomers of the extension primer for the analysis of SNPA457C in the CAV gene was performed. Four fractions were obtained. This picture was kindly provided by Dr Noah P. Christian.

The aim of this experiment was to find out if the extension reaction would be more efficient if primers containing only phosphorothioate linkages having a more suitable stereo-configuration were used. Oligonucleotides having stereocenters with a S_P configuration are known to hybridise significantly worse and therefore they require much lower annealing temperatures, which is unfavourable for efficient primer extension reactions.[133]

Four fractions of the HPLC experiment and the respective charge-tagged primer before the separation were tested. The same reaction conditions used for the experiment shown in figure 3.6 were applied.

The corresponding spectra are displayed in figure 3.15.

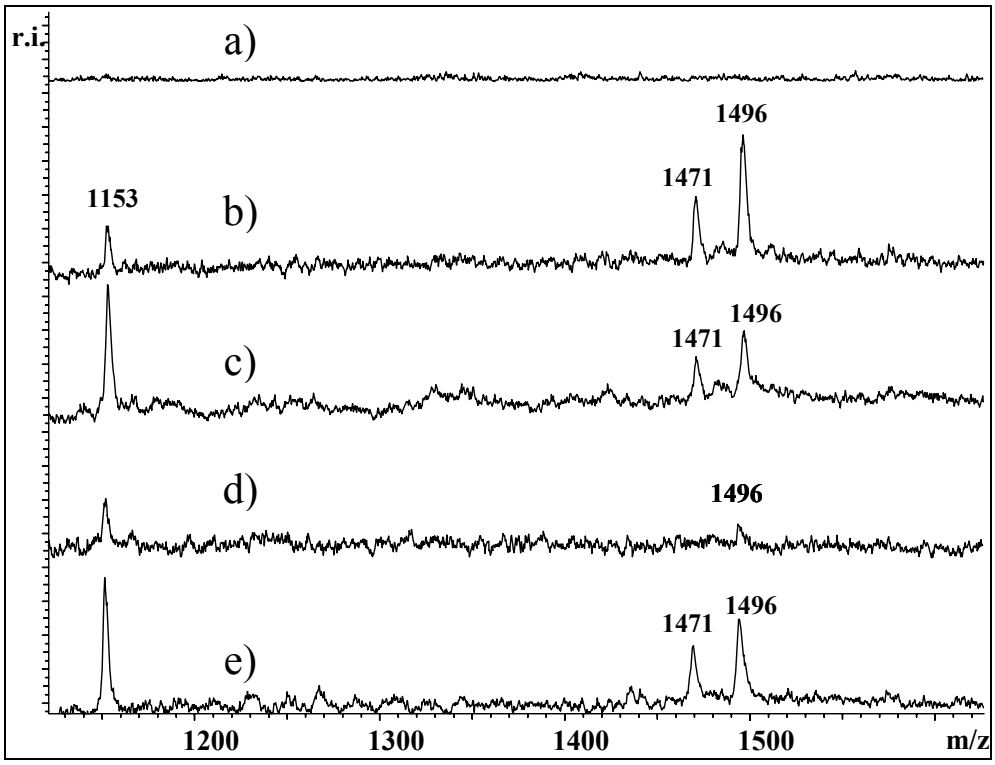

Figure 3.15. For traces a) to d) phosphorothioate containing extension primers of fractions 1 to 4 of the HPLC separation shown in figure 3.14 were used. For trace e) the respective primer before HPLC separation was used.

As is shown fraction 1 did not contain any primer. By UV analysis at 260 nm no DNA product was detectable. The signal in the chromatogram might stem from

impurities. The use of primers having significantly more of the "good" configuration was more advantageous than taking the mixture as can be seen by comparison of traces b) and e). In trace b) less rest primer and higher product signals were observed. Thus primer extension was more efficient. With primers of fraction 3, which contained partly primers of fraction 2, less efficient primer extension could be done (trace c)), while by use of primers of fraction 4 no elongation was feasible (trace d)).

Probably fraction 4 only contained primers with an unfavourable configuration. The absolute stereo-configuration of the oligonucleotides was not further determined. Trace e) shows a spectrum where the respective primer before HPLC separation was used.

The primers for the extension reaction were sometimes problematic for the following phosphodiesterase II digestion. Particularly if primers contained sequence motifs such as two following cytosines or for building hairpin structures, digestion turned out to be much less efficient. Nevertheless, for the majority of assays that were developed this problem did not occur.

The GOOD assay was connected successfully with a PCR system optimised for denaturing HPLC (dHPLC), a method for SNP discovery.[134] More importantly, the connection of different PCRs to the GOOD assay, particularly PCR conditions that were used for DNA sequencing, principally posed no problem. Nevertheless certain reagents could disturb the GOOD assay. Particularly, detergents used for enzyme buffers like Tween 20 or Nonidet P-40 significantly affected the procedure leading to bad crystallisation during MALDI sample preparation and therefore to spectra of reduced quality, while Triton X 100 is not harmful for the assay. Other disturbing reagents were thiol-containing mercaptoethanol or DTT also commonly used in buffers for enzymes, which led to suppressed matrices during sample preparation impeding a good desorption.

The reduction of dNTPs for the PCR and the use of a novel DNA polymerase (Tma 31 FS) led to a reaction sequence going directly from PCR to primer extension without needing shrimp alkaline phosphatase digestion thus reducing the number of reagent addition of the GOOD assays from 5 to 4. This holds for each variation of the GOOD assay presented herein. GOOD assays executed with Thermosequenase for the primer extension but without using shrimp alkaline phosphatase yielded in addition to regular

allele-specific products unspecific products. The Tma 31 FS DNA polymerase is described in more detail in the chapter of the simplified GOOD assay.

The use of oligonucleotides for primer extension containing three phosphorothioates resulted in signals that were significantly weaker and assays were no longer stable. Primers containing more than three phosphorothioates were not suitable for the GOOD assay. No extension products were detected by MALDI-MS, which is discussed below.

The unique feature of the charge-state concept to measure on the same sample and with high discrimination positively charge-tagged DNA molecules exclusively in the positive ion mode and negatively charge-tagged DNA products only in the negative ion mode was implemented in a molecular biological procedure for the generation of allele-specific products. The analysis of allele-specific products of SNPs G58A and R670G in the PECAM gene that were generated in a duplex reaction, prepared on the same sample and measured first in the positive and then in the negative ion mode is shown in figure 3.16.

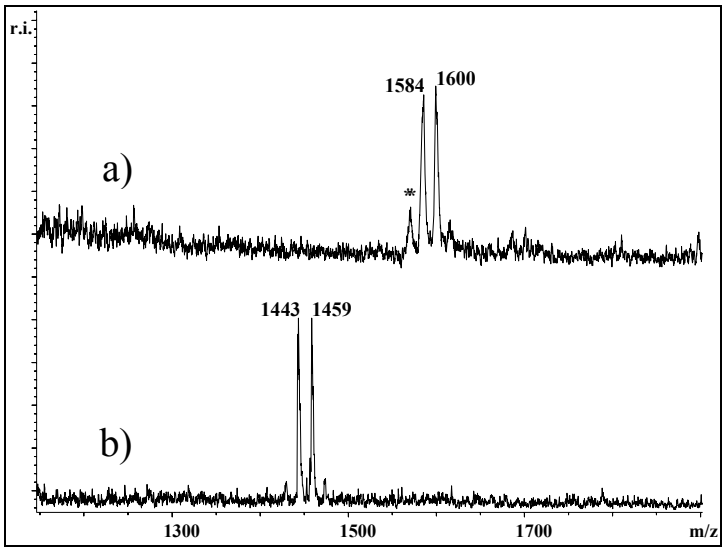

Figure 3.16. Allele-specific products of SNPs R670G and G58A in the PECAM gene were generated in a duplex reaction. SNP G58A was first analysed in the negative ion mode (trace a)), and then SNP R670G was analysed in the positive ion mode (trace b)) on the same spot. These two typical spectra show that positively charge-tagged products are slightly superior for MALDI-analysis than negatively charge-tagged DNA molecules. The signal to noise ratio in the positive ion mode is about twofold higher than in the negative ion mode. The signal indicated with an asterisk in this figure derived from under-alkylated byproducts that were detected in the negative ion mode.

The products of SNP R670G were positively charge-tagged oligonucleotides and only detectable using the positive ion mode of the mass spectrometer while the products of the SNP G58A were negatively charge-tagged and only detectable in the negative ion mode. The products for SNP R670G were $G_{pt}T^{CT}{}_{pt}C_{pt}A$ and $G_{pt}T^{CT}{}_{pt}C_{pt}G$ with masses 1443 Da and 1459 Da and the products for SNP G58A were $A_{pt}G_{pt}AT_{pt}A$ and $A_{pt}G_{pt}AT_{pt}G$ with masses 1584 Da and 1600 Da.

Novel insights concerning DNA charge-tagging

The surprising results showing zwitterionic, non-regularly charge-tagged molecules being adapted for very sensitive MALDI analysis in the positive ion mode evoked further experiments with synthetic DNA oligomers.[114,115] As the following results show, there is still a protonation/deprotonation involved in the ionisation process dissenting from recent literature of the charge-state concept.

For the following measurements a series of MALDI analyses was performed on oligomers listed in table 3.3. All of these molecules were alkylated as for GOOD assays but 1,000 pmol oligomer were used. After the reaction and dilution in 40 % acetonitrile a solution containing approximately 15 fmol/µl DNA product was obtained. 0.5 µl was applied onto the MALDI target. Thus the measurements were performed close to the sensitivity limit.[107] In table 3.3 the main signals having at least a signal to noise ratio of 5 were noted although there were sometimes peaks deriving from over- or under-alkylation and other satellite peaks. The "T-series" were 4-mers with T-nucleobases and the "G-series" 4-mers with G-nucleobases. One of each was a positively charge-tagged, one a zwitterionic and one a negatively charge-tagged oligomer. Furthermore a double-positively and single-negatively charge-tagged oligomer (2PosCT1NegCTMoT), a double-positively charge-tagged oligomer (DoubleCTModelT) and a double-positively and double-negatively charge-tagged oligomer (DoubleZwitterMoT) were measured on α-cyano-4-hydroxy-cinamic acid methyl ester.

Oligomer	Net charge	Positive ion mode	Negative ion mode
T Model CT +	1+	1440	1437
T Model Zwitter	0	1409	1407
T Model CT -	1-	No signal	1213
G Model CT +	1+	1553	1550
G Model Zwitter	0	1522	1520
G Model CT -	1-	No signal	1313
2PosCT1NegCTMoT	1+	2595	2589
DoubleCTModelT	2+	2624	No signal
DoubleZwitterMoT	0	2577	2572

Table 3.3. The m/z values of the main signals for detection in the positive and in the negative ion mode of the mass spectrometer are noted.

All of these model molecules were measured in the positive and in the negative ion mode. The single positively charge-tagged molecules were detected in the positive ion mode and with a significantly inferior sensitivity (1:5) in the negative ion mode. The sensitivity was deduced from peak heights and signal to noise ratios. The single negatively charge-tagged molecules were only detectable in the negative ion mode. The same result was obtained with the double-positively and single-negatively charge-tagged oligomer.

The zwitterions were measured in both ion modes with a higher sensitivity in the positive ion mode (the ratio was at least 2:1). Zwitterions were analysed in the positive ion mode with sensitivity approximately 50 % lower than positively charge-tagged equivalents. Surprisingly, the double-positively charge-tagged oligomer (DoubleCTModelT) was only detected at 2624 m/z (z = 1!) and no signal was seen at 1312 m/z. Following a restrictive view of the charge-state model only a product at 1312 m/z would have been expected.

All of the mentioned model oligonucleotides were also analysed using α-cyano-4-methoxy-cinamic acid methyl ester. The crystallisation of this matrix derivative seemed to be quite bad. None of the molecules used here was detectable. Therefore the ionisation process of charge-tagged DNA during MALDI analysis cannot be elucidated finally.

The simplified GOOD assay

The introduction of primers containing methylphosphonates that are charge-neutral replaced phosphorothioates for the simplified GOOD assay.[135] Thereby the potentially toxic methylation reaction and problems like over- and under-alkylation were omitted.

The simplified GOOD assay starts with a PCR. Following this, primer extension yields allele-specific products using ddNTPs and/or α-S-ddNTPs. The extension primers contain methylphosphonate bridges at the 3'-end that like phosphorothioates resist the digestion of phosphodiesterase II [136], which is used in the third step to get rid of the unmodified part of the extension primer. The resulting products for MALDI analysis carry methylphosphonate linkages that are charge-neutral and one negatively charged linkage deriving from a ddNTP or a α-S-ddNTP. Thus the molecules are negatively charge-tagged. Crucial to the simplified GOOD assay is the application of the novel DNA polymerase isolated from *Thermotoga maritima* termed Tma 31 FS. The GOOD assay procedure was reduced to 3 steps including PCR, primer extension and phosphodiesterase digestion. The principle of the simplified GOOD assay is shown in figure 3.17.

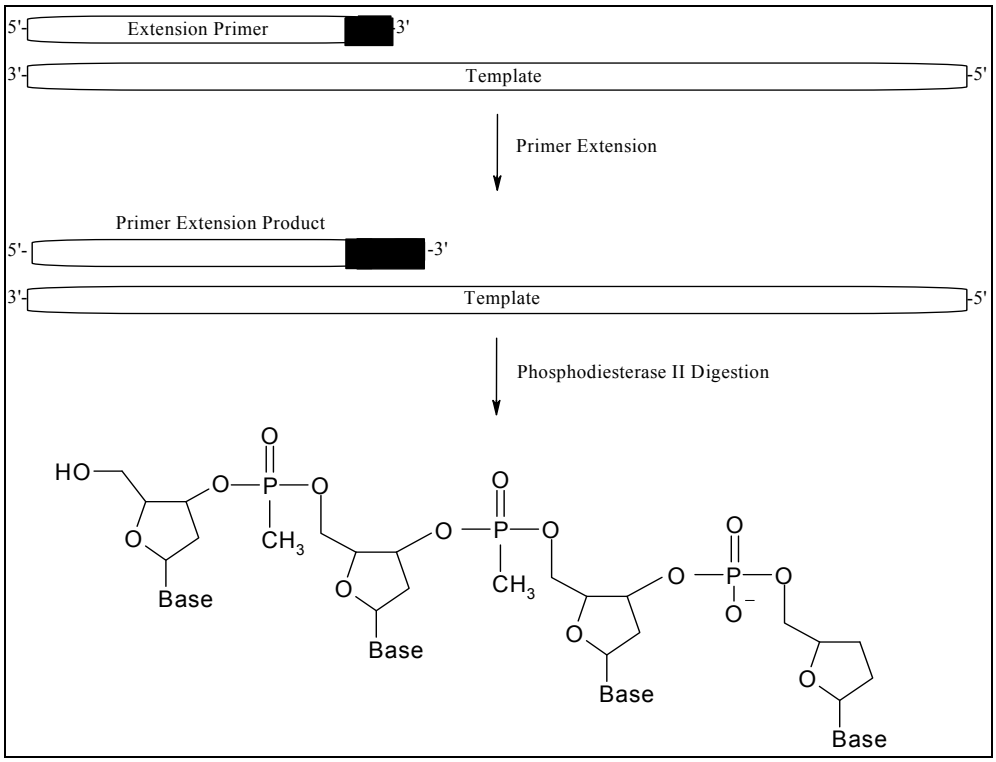

Figure 3.17. The principle of the simplified GOOD assay. The procedure starts with a PCR. The allele-specific step consists of a primer extension reaction with ddNTPs and of oligonucleotides containing methylphosphonates at their 3'-ends (indicated in black). The unmodified part of the primer is digested by phosphodieterase II. The methylphosphonate linkages inhibit complete digestion. The resulting product contains a backbone with one negative charge deriving from the phosphate group of the extended ddNTPs. Thus the product is negatively charge-tagged. Samples are diluted and transferred onto a MALDI target for analysis.

Initially, primer extension was evaluated using synthetic templates with primers containing methylphosphonates at their 3'-ends, as is shown with templates 1 and 2 in figure 3.18. Tma 31 FS DNA polymerase elongates primers containing methylphosphonates with ddNTPs allele-specifically. The titration of dNTPs to ddNTPs showed that with equimolar dNTPs to ddNTPs the Tma 31 FS DNA polymerase incorporated ddNTPs with an at least 5-fold preference over dNTPs. If only dNTPs were used, significantly weaker signals of extension products were observed under the described reaction conditions. Assuming the worst case that none of the dNTPs of the PCR were consumed, the ratio of ddNTPs to dNTPs in the primer extension reaction is 5:1 in the complete assay. This provides at least a 25-fold preference of ddNTPs in the primer extension reaction.

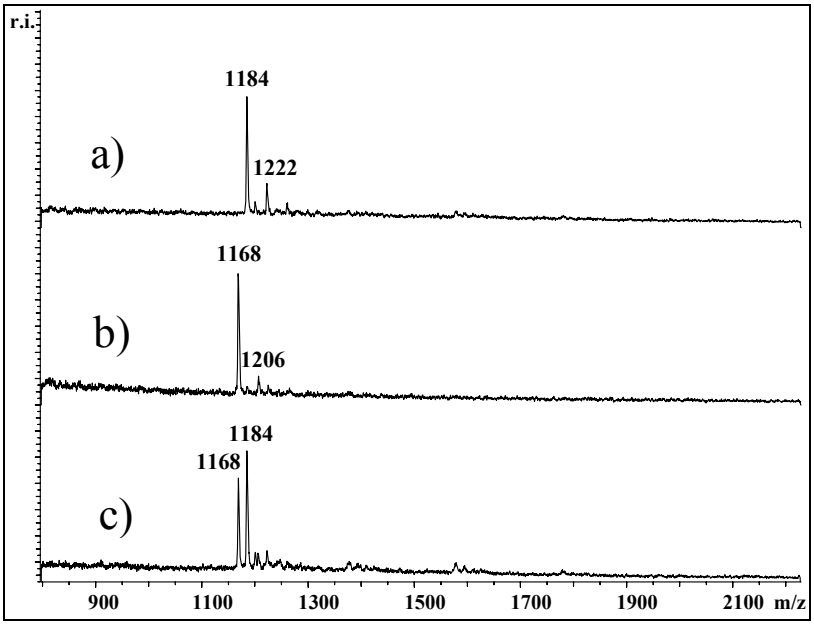

Figure 3.18. 5'-ACTGTTTGCCCGCCAGmpTmpT was used as primer for an extension with ddGTP and ddATP at a synthetic template. Two templates differing only in the adjacent position 3' of the primer in C or T. The respective products after phosphodiesterase II digestion are GmpTmpT[G/A] with respective masses, 1184 Da and 1168 Da. Trace a) shows the analysis of the primer extension using template 1 with the allele C while in b) the template 2 with the allele T was used. In trace c) the analysis of a primer extension where both templates were used is shown. At 1206 and 1222 m/z signals of potassium adducts of the respective products were observed.

Tma 31 FS DNA polymerase was compared to Thermosequenase, as was used in the standard GOOD assay, Taq DNA polymerase and Deep Vent DNA polymerase. Thermosequenase and Taq DNA polymerase extended primers containing methylphosphonates very inefficiently. Only Deep Vent DNA polymerase showed a marginally acceptable performance for primer extension, but was by far inferior to Tma 31 FS DNA polymerase. Therefore Tma 31 FS DNA polymerase was chosen for the simplified GOOD assays.

The phosphodiesterase II digestion is inhibited by the methylphosphonate linkages in the extension primers. To check the efficiency of the digestion, mixtures of the primer extension were used that had not been thermocycled and were digested under assay conditions. Resulting spectra of these probes did not contain any signals in the mass range of the products. In the case of inadequate reaction conditions for the digestion (for

example at pH higher than 9) the phosphodiesterase stopped one phosphate linkage short of the 3'-methylphosphonate nucleotide. Products containing four bases, two methylphosphonate linkages, and one phosphate linkage 5' of the methylphosphonate linkages deriving from the primer were generated. These products were conditioned as products of the assay and could disturb the mass spectrometric analysis. Care had to be taken to lower the pH for the phosphodiesterase II digestion.

Methylphosphonate linkages are stable under the used reaction conditions. Therefore signals with similar sensitivity and resolution to corresponding compounds of the GOOD assay containing alkylated phosphorothioates and charge-tags were observed. Sometimes as is shown in figure 3.18 small peaks due to potassium adducts of the products were observed. This mainly occurred if signal to noise ratios higher than 40:1 were achieved.

Applications of the simplified GOOD assay

The simplified GOOD assay was applied to genotyping SNPs in candidate genes for cardiovascular disease and hypertension such as the β-2-adrenergic receptor gene [137,138] and the PECAM gene. The analysis of SNPs 298 and 325 in the β-2-adrenergic receptor gene are displayed in figures 3.19 and 3.20 SNP 298 is a C/T polymorphism, while SNP 325 is a G/A polymorphism.

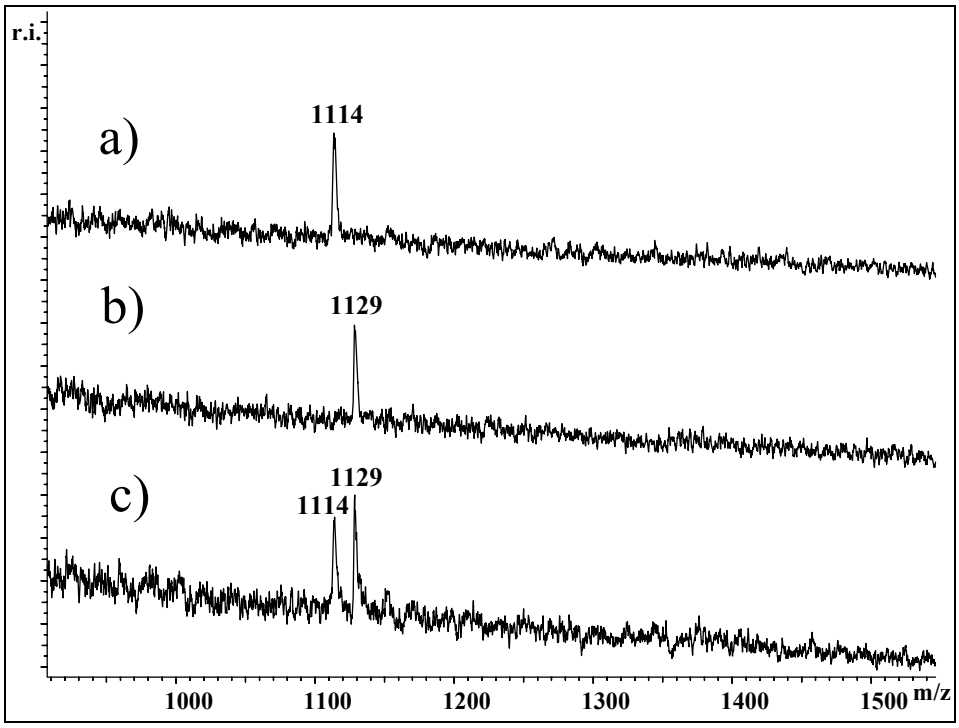

Figure 3.19. For SNP position 298 of the β-2-adrenergic receptor gene the primer 5'-CCCGCCGTGGGTCCGmpCmpC was extended with ddTTP and ddCTP. The respective products after phosphodiesterase II digestion are GmpCmpC[C/T] and have masses 1114 Da in case of incorporation of ddCTPs and 1129 Da in case of elongation with ddTTPs. Spectra of the MALDI analysis of DNA homozygous for C (trace a), T (trace b), and heterozygous DNA (trace c) are shown.

Human DNA was PCR amplified to obtain a sufficient amount of template for the primer extension reaction. Primer extension followed by phosphodiesterase II digestion was performed to generate allele-specific and negatively charge-tagged DNA products that were analysed by MALDI-MS without using purification. Efficient and reliable incorporation during primer extension of all four ddNTPs opposite according bases was observed. The correct analysis of genotypes was confirmed by DNA sequencing and the standard GOOD assay for positive ion mode detection using 20 different patient DNAs.

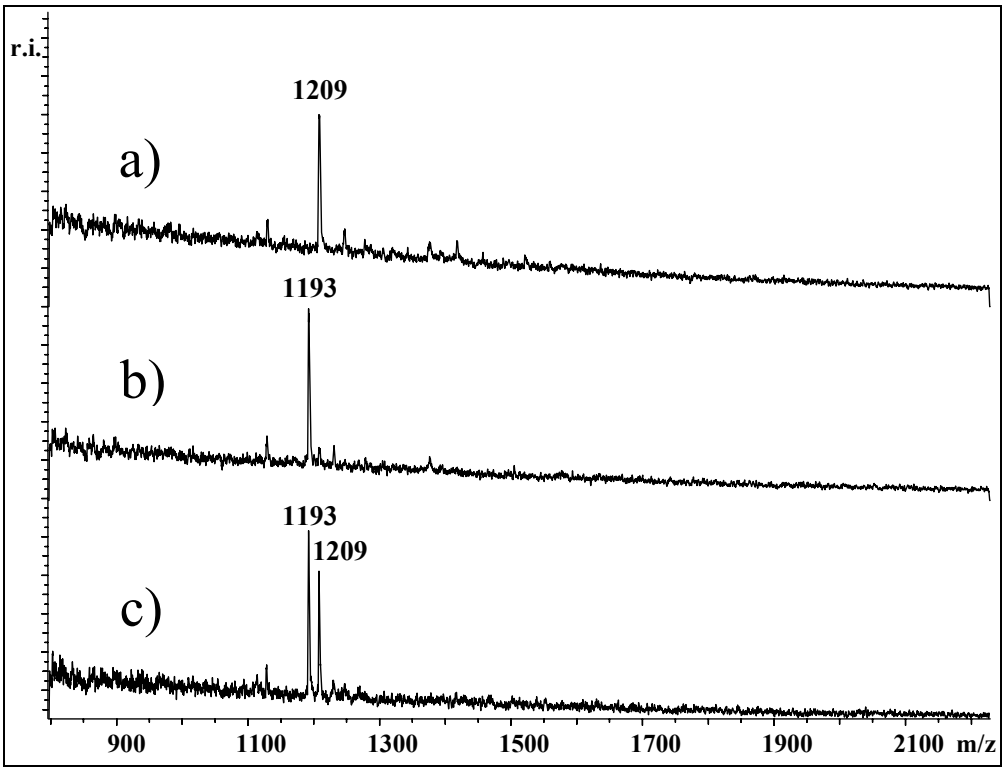

Figure 3.20. For SNP position 325 of the β-2-adrenergic receptor gene the primer 5'-CGCGCAGTCTGGCAGmpGmpT was extended with ddGTP and ddATP. Products of the simplified GOOD assay GmpGmpT[G/A] have masses 1209 Da for extension with ddGTP and 1193 Da for elongation with ddATP. The MALDI analysis of DNA homozygous for A (trace a), G (trace b), and the analysis of heterozygous DNA (trace c) is shown.

The simplified GOOD assay was adapted to 384 microtiter plates using a BasePlate robot. Thereby analogously to the GOOD assay the easy automation of the procedure was demonstrated on the SNP G58A in the PECAM gene that is a G/A polymorphism. In this case the reaction volume was reduced starting with a 3 µl PCR and ending with a volume of 7 µl after phosphodiesterase II digestion. α-Cyano-4-hydroxy-cinnamic acid methyl ester turned out to be the ideal matrix for the analysis of products of the standard GOOD assay. It was also well suited for the analysis of the products of this assay. Also with the simplified GOOD assay high reproducibility in automatic measurements was obtained, accumulating on average only 10 laser shots per spectrum. Even so, spectra with signal to noise ratios higher than 20:1 and isotopic resolution were observed. As for GOOD assays using primers with phosphorothioates spectra were recorded and alleles called automatically using the GenoTools software. The quality of the spectra

posed no problem for the allele-calling software. Three typical results of the 384 are shown in figure 3.21. In more than 90 % of preparations, results were obtained on first pass, which is in a similar range compared to the standard GOOD assay. The observed genotype frequencies were already in good agreement with predictions under the assumption of Hardy-Weinberg equilibrium. As in the standard GOOD assay missing results were obtained because of inefficient PCRs probably because of insufficient integrity of DNA templates.

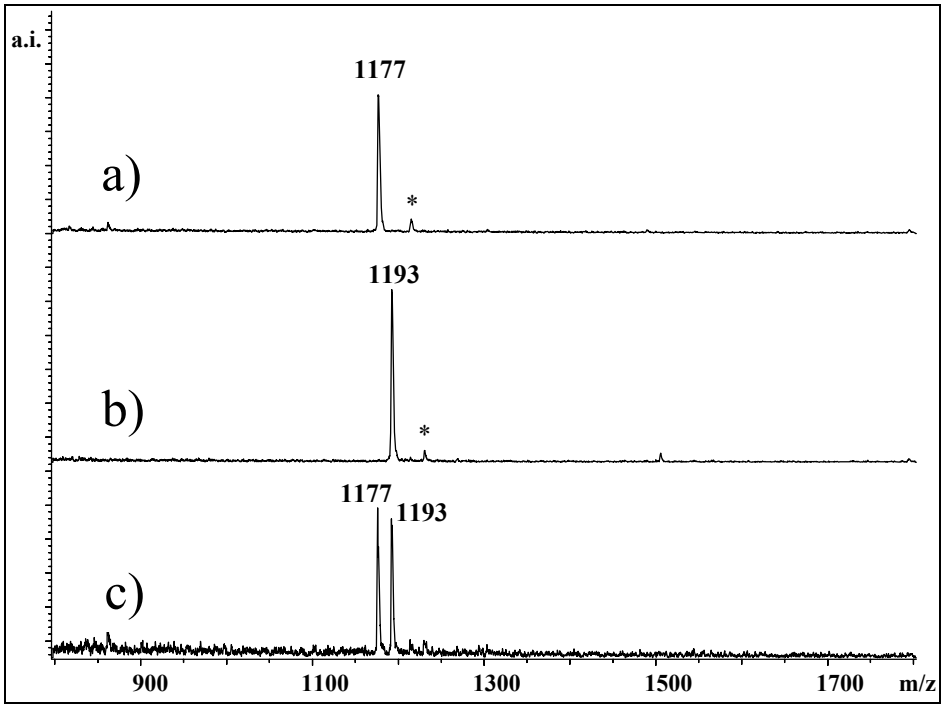

Figure 3.21. For the SNP G58A of the PECAM gene primer 5'-ATGTTCCGAGAAGAACAGmpAmpT was used. Respective products of the assay were GmpAmpT[G/A] with masses 1193 Da and 1177 Da. At 1231 Da and 1215 Da very small potassium adduct signals indicated with an asterisk were observed. Trace a) shows the analysis of a DNA homozygous for A, while in trace b) the analysis of DNA homozygous for G is shown. In c) the analysis of heterozygous DNA is displayed. The assay was done fully automatically starting with a 3 µl PCR in a 384 microtiter plate. The final volume of the assay was 7 µl. The sample was diluted and transferred by a robot onto a 384 MTP MALDI target plate. One plate was measured automatically needing on the average 10 laser shots per analysis.

The simplified GOOD assay demonstrated in figure 3.21 was performed with a primer containing two methylphosphonates at its 3'-end. Furthermore, respective results

were obtained with a primer containing three methylphosphonates instead of two thereby shifting masses of allele-specific products at 311 Da from 1177 Da to 1488 Da and from 1193 Da to 1504 Da. Respective spectra are shown in figure 3.22. The signal intensities and signal-to-noise ratios were lower compared to the experiment shown in figure 3.21 but still of good quality.

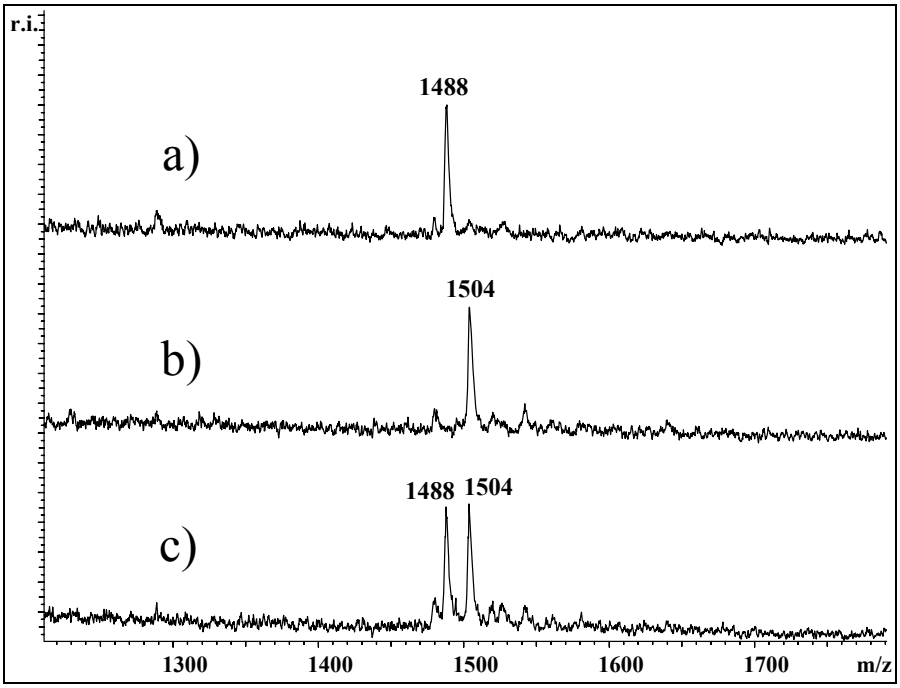

Figure 3.22. The simplified GOOD assay was done for SNP G58A of the PECAM gene using an extension primer (5'-ATGTTCCGAGAAGAACAmpGmpAmpT) with three methylphosphonate linkages. Respective products of the assay were AmpGmpAmpT[G/A] with masses 1504 Da and 1488 Da. Trace a) shows the analysis of a DNA homozygous for A, while in trace b) the analysis of DNA homozygous for G is shown. In trace c) the analysis of heterozygous DNA is displayed.

α-S-ddNTPs in place of ddNTPs can be used for primer extension under these conditions with comparable efficiency. As is shown in figure 3.23, a combination of ddNTPs and α-S-ddNTPs allows the separation of signals of two alleles beyond than the natural mass differences of nucleobases. This has, as for GOOD assays using extension primers with phosphorothioates, interesting implications for multiplexing because it adds a degree of freedom.

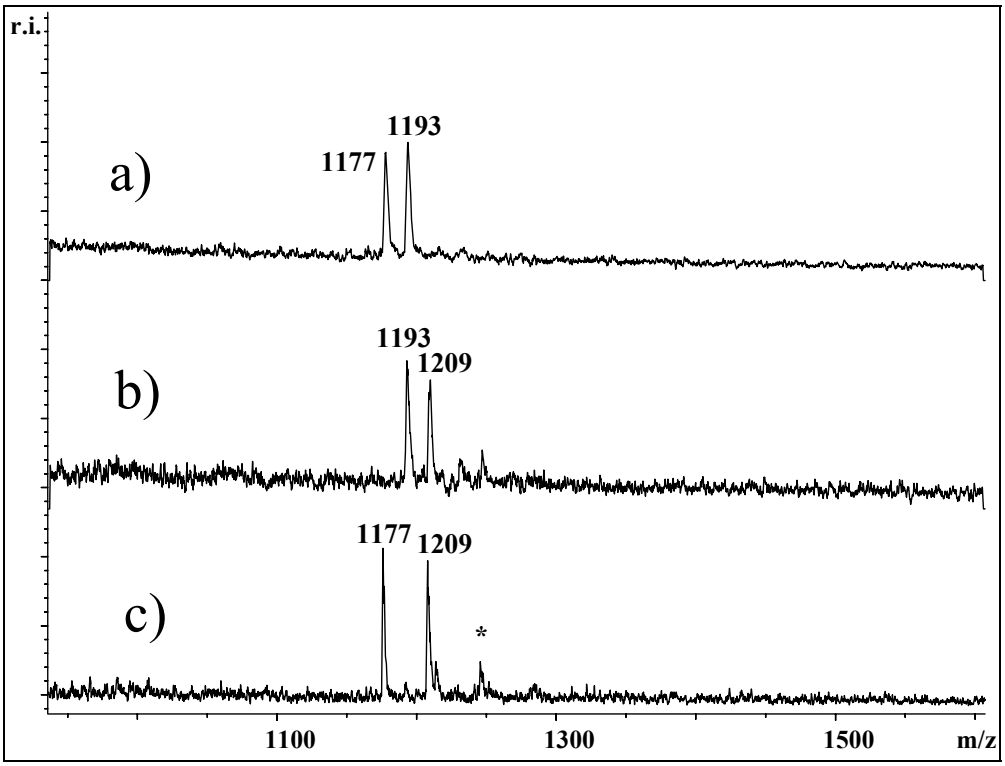

Figure 3.23. Alternatively, α-S-ddNTPs can be used as substrates for primer extension. The analysis of patient DNA heterozygous at SNP G58A using ddGTP and ddATP is shown in trace a) (respective masses of products are 1193 Da and 1177 Da), and the same experiment using α-S-ddGTP and α-S-ddATP is shown in trace b) (respective masses 1209 Da and 1193 Da). In order to shift masses ddATP and α-S-ddGTP were used in the experiment displayed in trace c) (the respective masses are 1177 Da and 1209 Da). Spectra of experiments done with ddNTPs or α-S-ddNTPs show similar quality.

In table 3.4 the performance of four DNA polymerases for the different GOOD assays is highlighted. The Tma 31 FS - that is a novel, engineered DNA polymerase - is useful for each variation of the GOOD assay. This enzyme has an increased affinity towards dideoxynucleotides compared to the other three commercially available polymerases. Taq polymerase, a common polymerase for PCR, was not suitable for simplified GOOD assays. No satisfactory MALDI spectra were obtained. Thermosequenase, a rather expensive enzyme, is a proper polymerase for the GOOD assay in the positive ion mode and one version of the negative ion mode. Deep Vent polymerase is a DNA polymerase that lacks, as the Tma 31 FS DNA polymerase, a proofreading function. This enzyme is suitable for all variations of the GOOD assay. In particular for the simplified GOOD assay the Tma 31 FS gave significantly better

results in terms of MALDI peak intensities and signal-to-noise ratios than all other tested DNA polymerases.

	GOODpositive	GOODnegative[a]	GOODnegative[b]	SGA
Taq	-	-	-	-
Thermosequenase	+	+	-	-
Deep Vent	+	+	(+)	(+)
Tma 31 FS	+	+	+	+

Table 3.4. The applicability of four different DNA polymerases for GOOD assays. GOODnegative[a] stands for a GOOD assay with an extension primer containing a negatively charged phosphate linkage and GOODnegative[b] stands for a GOOD assay where the negative charge is introduced during primer extension by the negatively charged phosphate bridge of a dideoxynucleotide. SGA stands for simplified GOOD assay. "+" means that results of an assay with sharp signals had a signal to noise ratio higher than 5, "(+)" indicates weak signals with signal to noise ratios equal or lower than 4, "-" means no that no signals were obtained.

Ligation of DNA backbone modified oligonucleotides

A conceivable way to produce allele-specific and charge-tagged DNA molecules by means of a ligase is shown in figure 3.24. Jurinke et al. presented a ligation assay combined with MALDI analysis using a modified procedure of a commercial available LCR (Ligase Chain Reaction) kit.[139] Allele-specific amplification of a cloned DNA template is performed by a ligase. A similar approach was evaluated here using oligonucleotides containing phosphorothioates at their adjacent 5' and 3' ends.

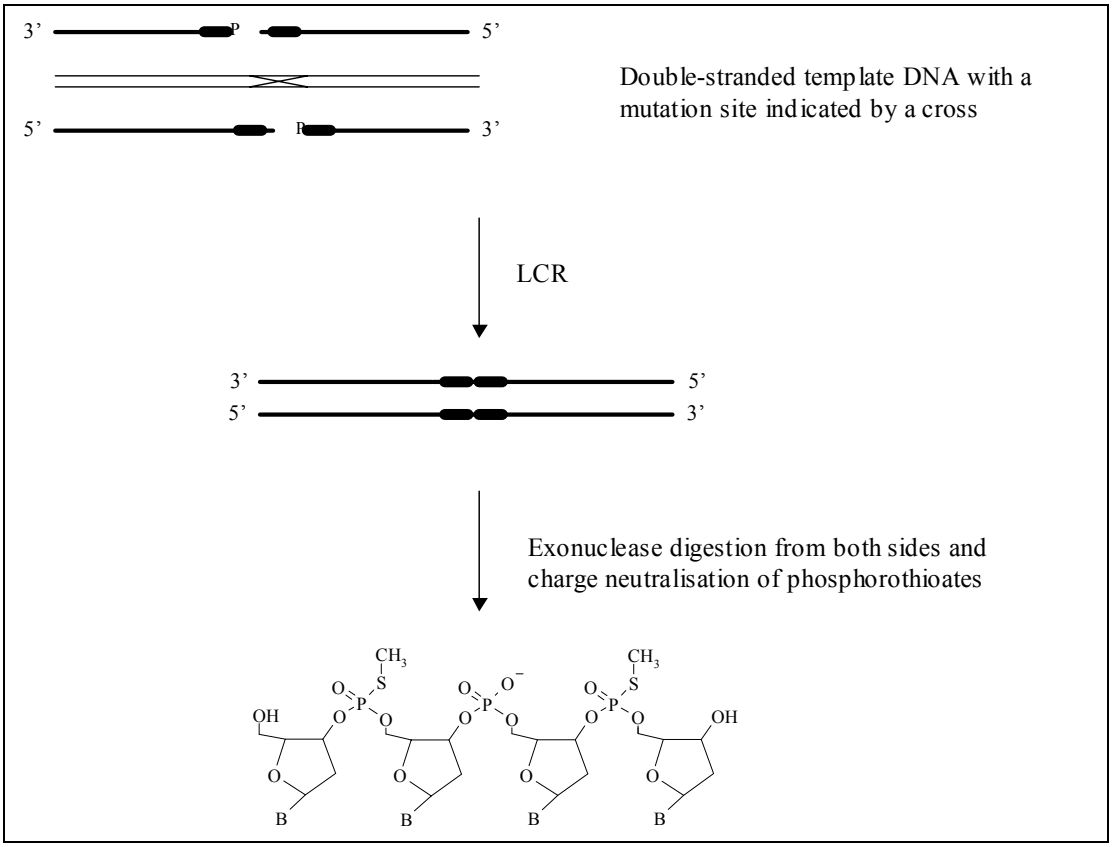

Figure 3.24. A conceivable way to produce charge-tagged DNA by a ligase reaction. Four oligonucleotides containing phosphorothioates adjacent to the linkage site are used to amplify allele-specifically a DNA template such as a PCR product. Afterwards a removal of the unmodified part of ligated products is performed. As for GOOD assays using phosphorothioates an alkylation step is required to generate negatively charge-tagged DNA.

As is shown in figure 3.25 ligases readily accept this DNA backbone modification. LCRs worked if phosphorothioates were integrated at the 5'-ends at the ligation site of two of the four employed oligonucleotides or vice versa if phosphorothioates were integrated at the 3'-ends of the ligation site of two of the four oligonucleotides. Furthermore, LCR was feasible even if all four oligonucleotides contained a phosphorothioate at a position next to the ligation site. Polyacrylamide gels were used for the separation of products and ethidium bromide staining for UV-detection.

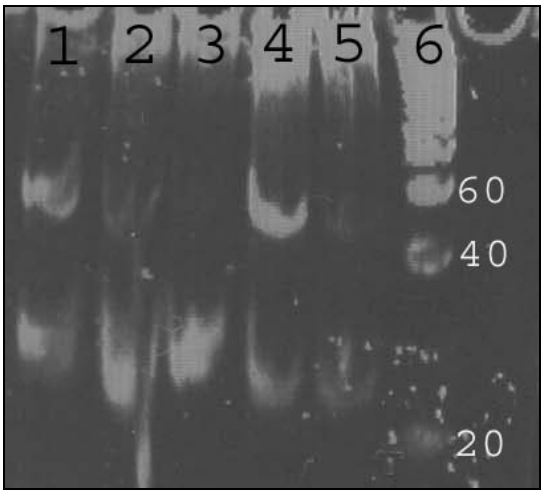

Figure 3.25. Polyacrylamide gel of a LCR experiment using unmodified oligonucleotides and oligonucleotides containing phosphorothioates. The standard ranges from 20 bp to 60 bp in increments of 20 bp (lane 6). The product size of the LCR is 50 bp. More details of the experiment are described in the text.

For lane 1 (figure 3.25) a wildtype template was amplified by unmodified oligonucleotides of a LCR kit showing allele-specificity. Corresponding products of a size of ca. 50 bp were observed. For the experiment of lane 4 oligonucleotides containing phosphorothioates were used. A similar result to common LCR was obtained. For lane 3 no template and only residual phosphorothioate-containing oligonucleotides were used. Only oligonucleotides (ca. 25 bases) were detectable. For lane 2 unmodified oligonucleotides and the mutant template were utilised. A shadow of amplification product is discernable. For lane 5 oligonucleotides containing phosphorothioates and the mutant template were used and therefore no amplification took place. Strikingly, the efficiency of the LCR seems to be higher using modified oligonucleotides.

For the experiment shown in figure 3.26 two synthetic templates and their complementary sequences were used. A template and its complementary sequence were incubated together with the four phosphorothioate-modified oligonucleotides employed above. In lane 2 both wildtype sequences, in lane 3 one wildtype and one mutant, in lane 4 two mutant and in lane 5 the reverse configuration of oligonucleotides of lane 3 were applied. As for lane 4 no wildtype sequence was used and thus no LCR

amplification took place. The LCR could be applied on single or double stranded DNA as is shown for lanes 2, 3 and 5.

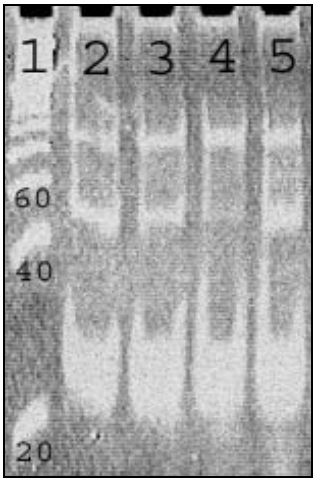

Figure 3.26. LCR with phosphorothioate-containing oligonucleotides using four possible template configurations. In lane 1 a DNA marker was applied. Sizes are indicated in base pairs (bp). The product of the LCR has a size of 50 bp. At ca. 90 bp the double-stranded synthetic DNA templates are discernable.

The next step was to establish exonuclease digestion from both ends (5‘ and 3’). As the whole procedure should terminate in a single tube reaction sequence as was done for GOOD assays, purification of products of the ligase reaction and subsequent adjusting of the best buffer conditions for exonuclease reactions was undesirable. It turned out that the combination of two exonuclease reactions (5’ and 3’) - serially or in parallel - was very difficult. A series of experiments for digestion of synthetic oligonucleotides mimicking products of the LCR and using different exonucleases was done in ligase buffer or in water. In figure 3.27 an experiment is shown in which the digestion was performed in ligase buffer using consecutively phosphodiesterase I and II. A single stranded synthetic DNA containing phosphorothioates and one phosphate linkage between them was used and digested from both sides.

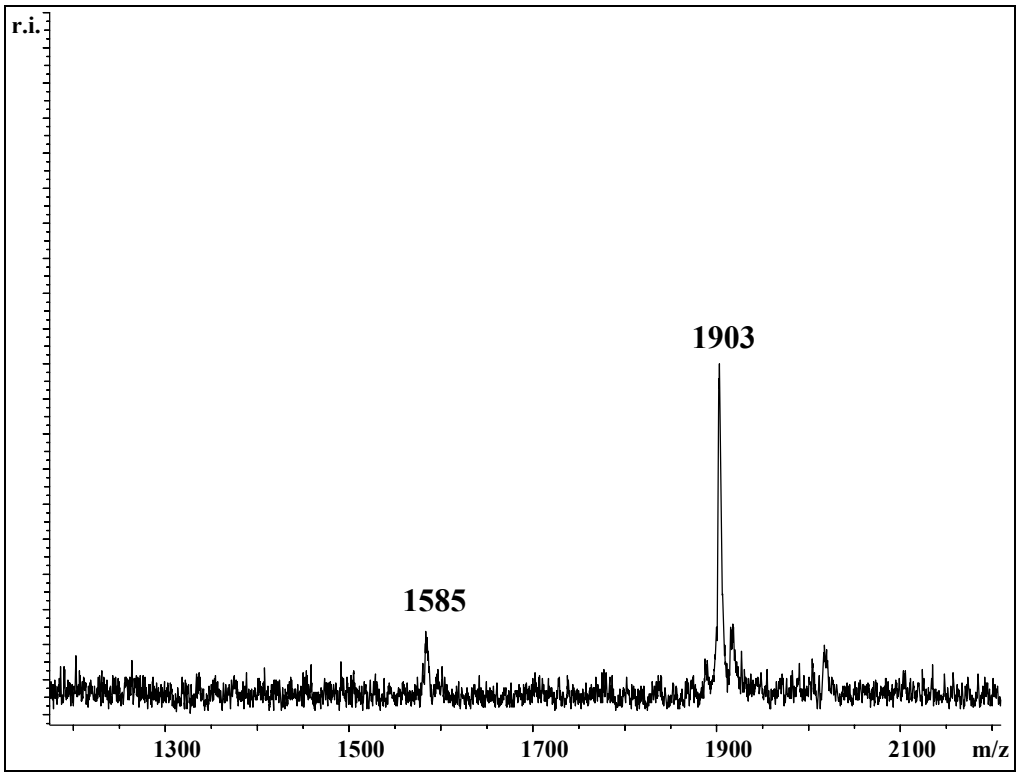

Figure 3.27. Digestion of a synthetic standard oligonucleotide from both sides. 60 pmol digestion standard were used to obtain sufficient signal quality of the resulting product ($G_{pt}C_{pt}AA_{pt}A_{pt}C$, 1903 Da). The byproduct detected at 1585 m/z corresponds to sequence $G_{pt}C_{pt}AA_{pt}A$ proving the quite aggressive digestion of phosphodiesterase I even at phopsphorothioate linkages.

The digestion was successful even if an equimolar amount of a complementary DNA strand of the digestion standard was incubated. Negatively charge-tagged DNA products were detected by MALDI-MS. Acceptable results for MALDI analysis were obtained if more than 60 pmol of digestion standard were used. More than 100 laser shots had to be accumulated. Signals became significantly weaker if less digestion standard was used. No signals were detected if less than 10 pmol were used. The approach applied for synthetic templates did not work using LCR products and subsequent MALDI analysis. Nevertheless, as is shown in figure 3.28 the double stranded LCR product was digested. Digestion products such as those of the reaction shown in lane 3 of figure 3.28 were alkylated and prepared for MALDI analysis. No expected signals were observed because of insufficient product.

Thus the chosen digestion procedure was not efficient enough to generate product for MALDI-MS. These negative results are discussed below. Furthermore it is explained why the ligase approach was not followed up.

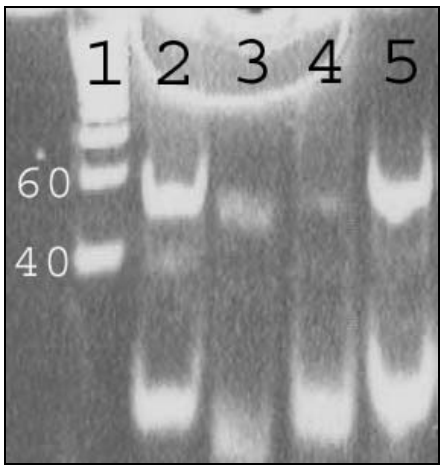

Figure 3.28. Polyacrylamide gel of LCR products that were digested under the reaction conditions of the experiment shown in figure 3.27. In lane 1 the DNA marker and respective sizes are shown. For lane 2 an undigested LCR was applied, while for lane 3 the LCR was digested by phophodiesterase I and II. For the digestion shown in lane 4 only phophodiesterase I was used, while for the experiment of lane 5 only phophodiesterase II was applied.

Several enzymes such as the T7 gene 6 exonuclease were tested to replace phosphodiesterase II but results were even worse, which is not shown in detail in this work. Both enzymes are single-strand specific, while phosphodiesterase I is active on double- and single-strand DNA. Another promising digestion enzyme, the Bal 31, an exonuclease that digests short double stranded DNA from both ends, was also too inefficient for generating enough charge-tagged DNA for MALDI analysis. Truncated digestions in the case of Bal 31 were observed.

The FEN-GOOD approach

In this chapter experiments are shown that combine Invader technology with charge-tagging for rendering MALDI analysis of DNA amenable without using purification (figure 3.29). The FEN-GOOD approach is based on the generation of allele-specific products using flap-endonucleases, which are abbreviated by "FEN". The flaps are modified in such a way that resulting DNA oligomers are charge-tagged analogously to GOOD assays using primer extension.

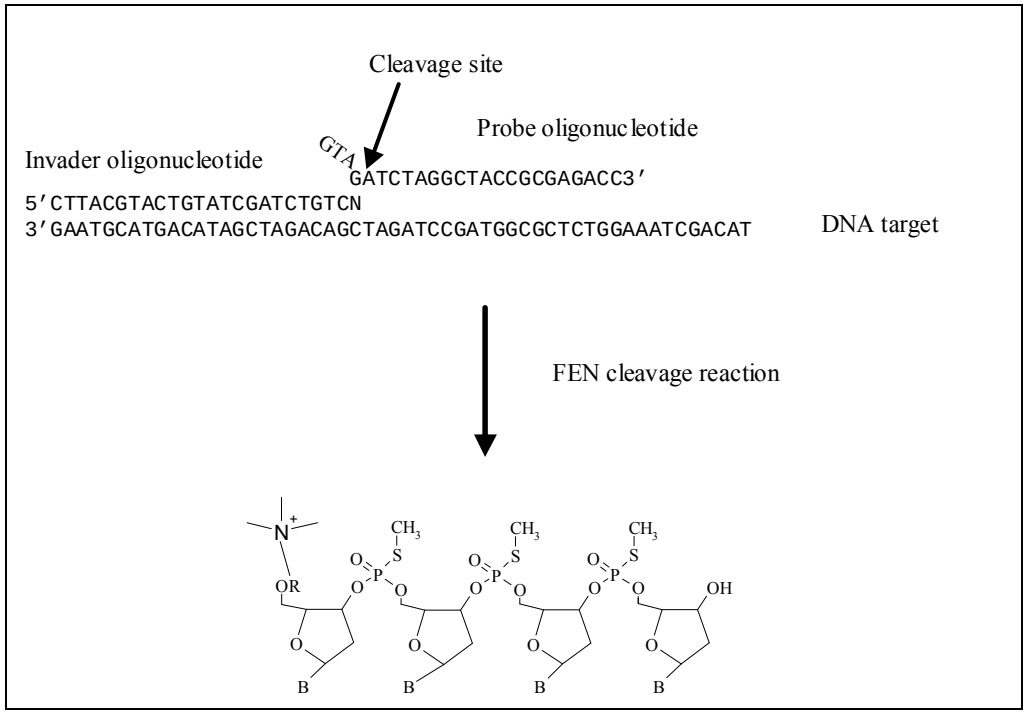

Figure 3.29. The principle of the FEN-GOOD assay. FEN stands for flap-endonuclease.

A clone of a flap-endonuclease deriving from *Methanoccus janaschii* was kindly provided by Dr James G. Wetmur. This enzyme was expressed and purified as was described.[123] The flap-endonuclease readily accepted phosphorothioates and positive charge-tags or methylphosphonates and a negative charge-tag (a phosphate linkage) at the flap of the probe molecules. As was demonstrated in other applications of the Invader assay ca. 200 ng of flap endonuclease were used. Nevertheless because of the proceeding PCR, buffer conditions varied from original protocols. As is shown in figure

3.30 sufficient allele-specificity could be achieved on synthetic templates. In contrast to GOOD assays where usually 5-20 laser shots are needed for MALDI analysis, the approaches shown here require at least 100 laser shots. Unusual difficulties during the MALDI desorption process even of purified samples of Invader assays using unmodified DNA and a dried-droplet sample preparation were described.[104] Also in the experiments shown here striking spot-to-spot variation was observed.

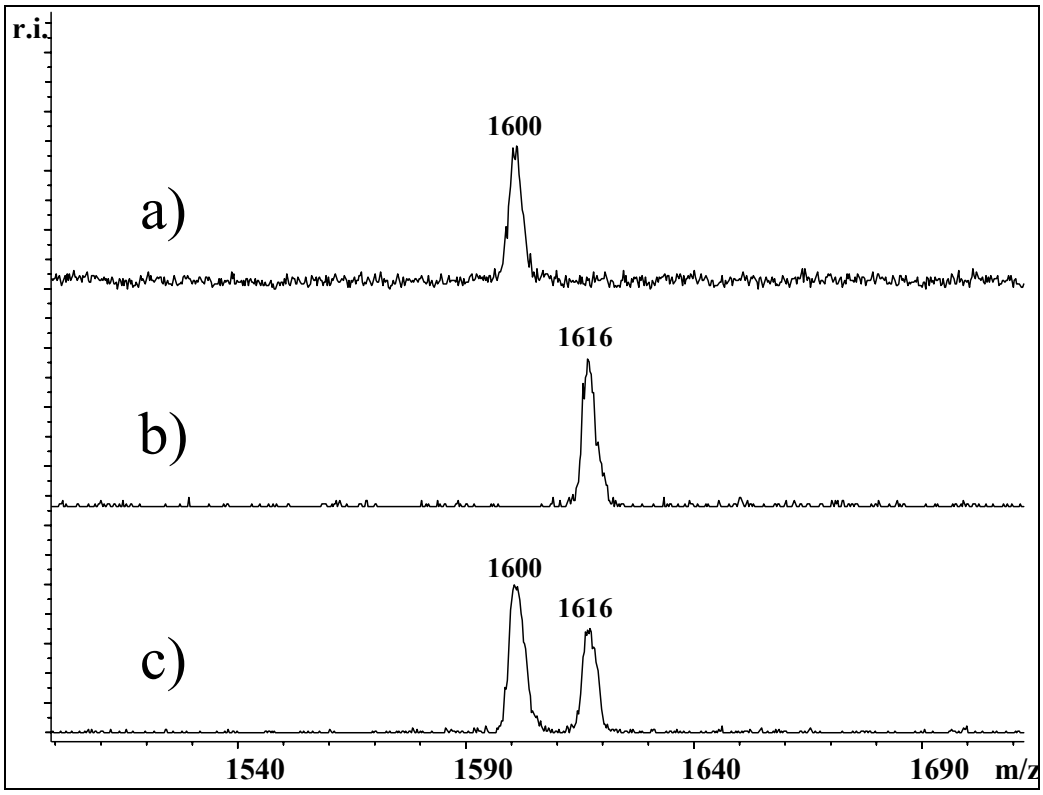

Figure 3.30. The flap endonuclease reaction was tested for allele-specificity on synthetic templates. If template 2 was used only the corresponding charge-tagged flap DNA deriving from probe oligonucleotide 2 could be detected by MALDI-MS (trace a)). In the case of template 1 that contained a base exchange at the observed position, only the corresponding flap deriving from probe oligonucleotide 1 was detected (trace b)). For the experiment of trace c) both templates were mixed to imitate heterozygosity. 5'-(C6-Aminolink)-TptGptCGTTGTGCCACGCGGTTGGGAATGTA was used as probe 1 and 5'-(C6-Aminolink)-TptGptCATTGTGCCACGCGGTTGGGAATGTA as probe 2. Products of this experiment were CT-(C6-Aminolink)-TptGptCG (1616 m/z) and 5'-(C6-Aminolink)-TptGptCA (1600 m/z). As the same base composition for the overhang of probe oligonucleotides was used the mass difference of 16 derives from an A in probe oligonucleotide 2 and a G in probe oligonucleotide. Measurements were performed in the positive ion mode. For these spectra 300 laser shots were accumulated.

If template 1 was mixed with both probe oligomers, only the respective flap deriving from probe 1 was detected. Corresponding results were obtained if template 2 was used. If probe oligonucleotide 1 was incubated with template 2 or template 1 was incubated with probe 2 no flap molecules were detected by MALDI-MS. While for figure 3.30 probe oligonucleotides containing phosphorothioates and positive charge-tags were used, for experiments in figure 3.31 probe oligonucleotides containing methylphosphonates and a negatively charged phosphate linkage were taken.

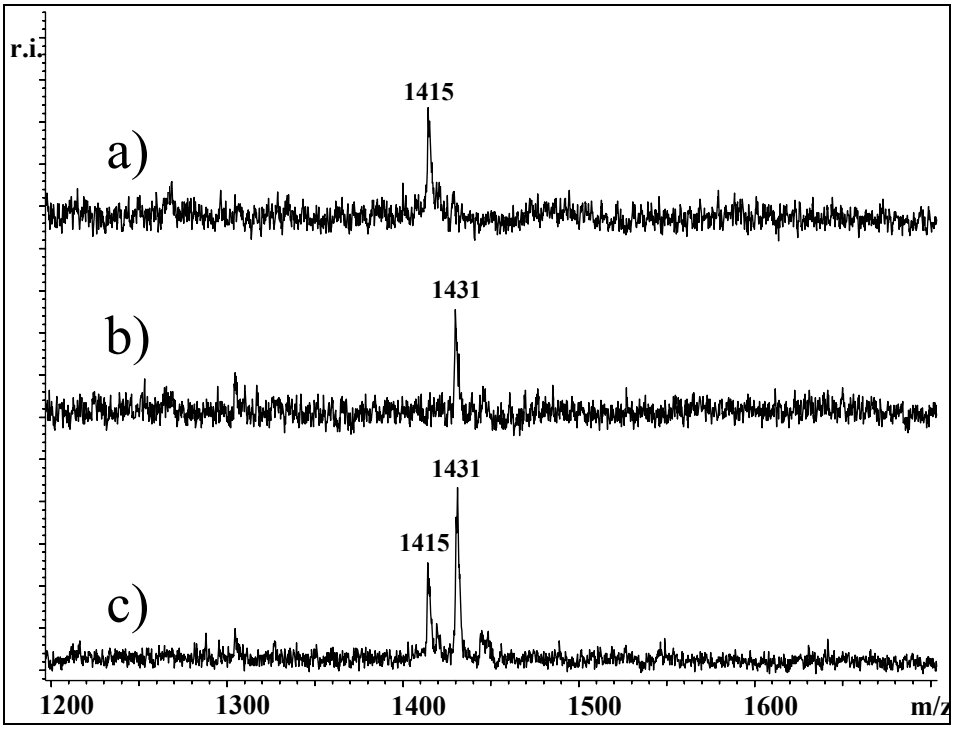

Figure 3.31. Corresponding experiments to figure 3.30 were done using methylphosphonates as linkages in the flap. If template 2 was used, only the corresponding charge-tagged flap DNA oligomer deriving from probe oligonucleotide 2 could be detected by MALDI-MS (trace a)). In the case of template 1 that contained a base exchange at the observed position, selectively the corresponding flap molecule deriving from probe oligonucleotide 1 was detected (trace b)). For the experiment for trace c) both templates were used to imitate heterozygosity. 5'-CmpTmpCmpCATTGTGCCACGCGGTTGGGAATGTA was used as probe 1 and 5'-CmpTmpCmpCGTTGTGCCACGCGGTTGGGAATGTA as probe 2. Respective products of this experiment were 5'-CmpTmpCmpCA (1415 m/z) and 5'-CmpTmpCmpCG (1431 m/z). Measurements were performed in the negative ion mode. For these spectra 300 laser shots were accumulated.

The isolated flap endonuclease deriving from *Methanococcus janaschii* is not applied in common Invader assays, probably because of too low specificity.[140,141] Homologous enzymes from *Archaeoglobus fulgidus* or engineered variants of the flap-endonuclease from *Thermus thermophilus* are preferred. Unfortunately these enzymes were not available for this work. For example, low specificity of the FEN from *Methanococcus janaschii* was observed in the experiment shown in figures 3.31 and 3.32.

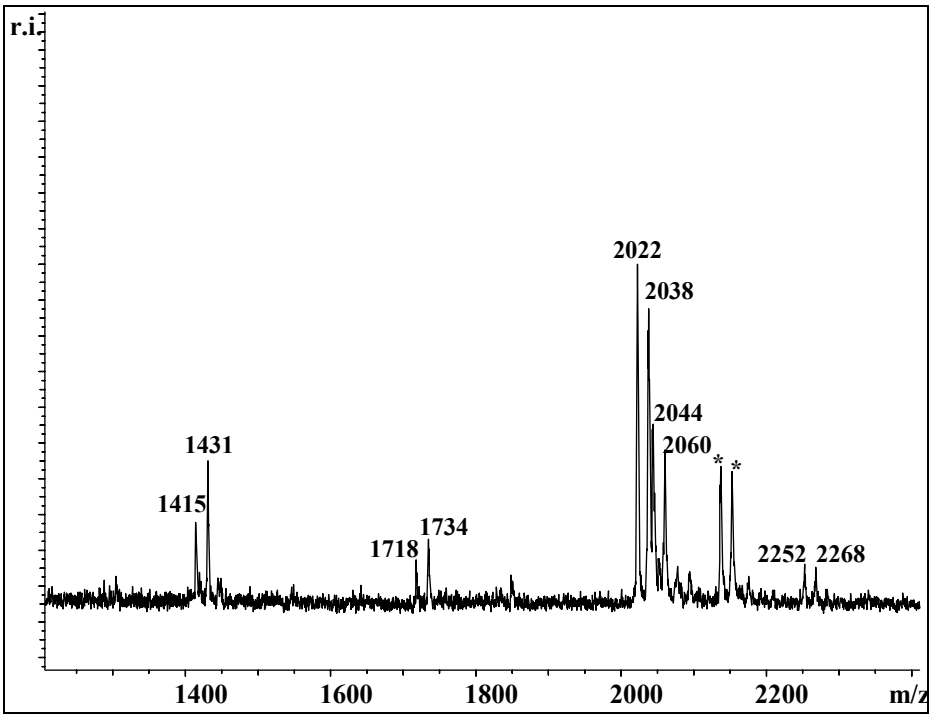

Figure 3.32. A larger view of the spectrum of trace c) in figure 3.31. The striking signals at 2022 and 2038 m/z derive from unspecific digestion of the flap endonuclease leading to products 5'-CmpTmpCmpCATT and 5'-CmpTmpCmpCGTT. Although these molecules are three times negatively charged, they were measured surprisingly well, probably because of high abundance. Signals at 2044 and 2060 m/z were observed due to sodium adducts. Signals at 1718 and 1734 m/z correspond with twofold negatively charged products 5'-CmpTmpCmpCAT and 5'-CmpTmpCmpCAT and signals at 2252 and 2268 m/z with fourfold negatively charged products 5'-CmpTmpCmpCATTG and 5'-CmpTmpCmpCATTG. Signals marked by an asterisk are non-identified side-products. The same panoply of side products was observed in larger spectra of traces a) and b) in figure 3.31.

A possible reaction sequence for SNP genotyping presented here consists of a PCR, followed by a flap-endonuclease reaction for the generation of allele-specific products

and an alkylation/phase separation of the flap of the used probe molecules. These contain phosphorothioates and a positive charge-tag that is attached at an amino-linker at the 5'-end (figure 3.29).[142] A stretch of DNA containing SNPs was PCR-amplified and by the cleavage reaction of the flap-endonuclease, products were obtained for alkylation. The whole procedure was performed in a single tube and applied to genotyping SNP 390 in the β-2-adrenergic receptor gene. A typical spectrum of these approaches is shown in figure 3.33.

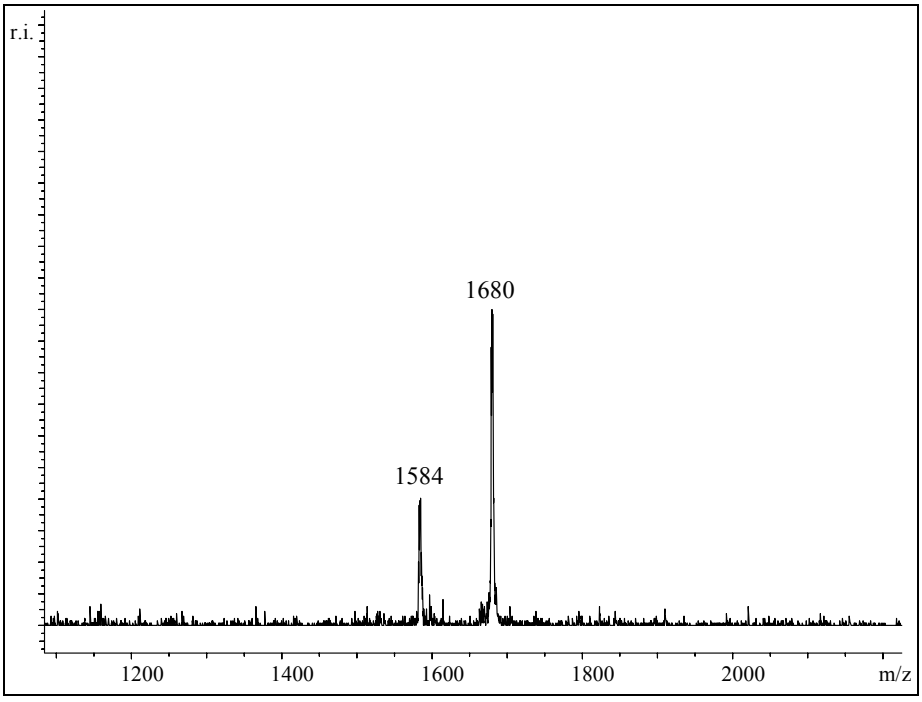

Figure 3.33. Analysis of SNP 390 in the β-2-adrenergic receptor gene. Oligonucleotide 5'-(C6-Aminolink)-GptGptGGAAGCCATGCGCCGGACCACGACGT was used as probe 1 and 5'-(C6-Aminolink)-CptCptGAAAGCCATGCGCCGGACCACGACGT as probe 2. Respective products of this experiment were CT-(C6-Aminolink)-GptGptGptG (1680 m/z) and 5'-(C6-Aminolink)-CptCptGptA (1584 m/z). Heterozygous DNA was analysed.

Invader assays using flap endonuclease deriving from *Methanococcus janaschii* were not sufficiently allele-specific, which is confirmed by recent literature.[140,141] In cases of homozygous DNA (verified by DNA sequencing and the standard GOOD assay) signals deriving from both "flaps" were obtained. However if no DNA or PCR template were present in the flap-endonuclease reaction no signals were observed.