Materials and Methods

Materials

Unmodified oligonucleotides and oligonucleotides containing amino-modified nucleobases were synthesized by an in-house synthesis service or by MWG (Ebersberg, Germany) and were all purified using oligonucleotide-purification cartridges, HPLC or HPSF. Amino-modified phosphoramidites were obtained from Bruker Saxonia (Leipzig, Germany) and GlenResearch (Sterling, VA). Oligonucleotides containing methylphosphonates were from Eurogentec (Liège, Belgium) and PAGE purified. Oligonucleotides with 5'-amino-linkers were also obtained from Eurogentec and FPLC purified. An "Expedite" DNA synthesizer was purchased from Perseptive Biosystems (Framingham, MA) and oligonucleotide-purification cartridges were obtained from Eurogentec (Liège, Belgium). A HPLC was purchased from Dionex (Jouy-en-Jonas, France).

For sealing an Automated Laboratory Plate Sealer (ALPS 300) from ABgene (Villebon sur Yvette, France) was tested. Standard plastic laboratory material was obtained from Eppendorf (Eppendorf, Germany) and ABgene (Villebon-sur-Yvette, France).

Taq DNA polymerase, Tsc DNA ligase thermostable, dNTPs, AEBSF and phosphodiesterase II (calf spleen) were purchased from Roche Molecular Diagnostics (Mannheim, Germany). α-S-dNTPs and α-S-ddNTPs were provided by Amersham (Little Chalfont, UK) and Biolog (Bremen, Germany). Platinum™ Taq DNA polymerase high fidelity (TPHF) and Platinum Taq DNA polymerase were purchased from Gibco BRL Life Technologies (Karlsruhe, Germany). Thermosequenase, phosphodiesterase I (snake venom), Proteinase K and shrimp alkaline phosphatase were purchased from Amersham Buchler (Braunschweig, Germany). Tma 31 FS DNA polymerase was kindly provided by Roche Molecular Systems (Alameda, CA). Deep Vent DNA polymerase was obtained from New England Biolabs (Frankfurt am Main, Germany). Ampligase Thermostable DNA ligase was purchased from Biozym (Hess.

Oldendorf, Germany). Standard reagents were obtained from Aldrich (Steinheim, Germany) and from Merck (Darmstadt, Germany). Heterozygous human mixed DNA was purchased from Promega (Charbonnieres, France). A LCR kit was obtained from Stratagene (La Jolla, USA). A DNA purification kit was purchased from Qiagen (Hilden, Germany). PCR Low Ladder set, LB medium, Ampicillin, Chloramphenicol, IPTG and Benzamidine were purchased from Sigma (St. Quentin Fallavier, France). Centricons were obtained from Amicon (Beverly, CA). Silver staining kits were purchased from BioRad (Munich, Germany). Coomassie reagent kits for protein staining and Bradford assays were obtained from Pierce (Bezons, France). An UV spectrometer from Pharmacia Biotech (Orsay, France) was used. A Centrifuge 5415 D and a Gradient Thermocycler were purchased from Eppendorf (Eppendorf, Germany). Additionally, a Primus Thermocycler from MWG Biotech (Ebersberg, Germany) was utilised. A BasePlate robot from The Automation Partnership (Cambridge, UK) was used for automatic liquid-handling. The MALDI matrix for the GOOD assay (α-cyano-4-hydroxy-cinnamic acid methyl ester) was synthesized in-house and is also available from Bruker Saxonia Analytik (Leipzig, Germany). A Bruker Biflex III, a Bruker Reflex II, a Bruker Reflex III time-of-flight mass spectrometer equipped with a SCOUT 384^{TM} ion source, and an Autoflex from Bruker Daltonik (Bremen, Germany) were used. DNA sequencing was done on an ABI PRISM 3700 DNA sequencer from PE Applied Biosystems (Foster City, CA).

Standard procedures

Standard molecular biological procedures such as determination of DNA concentration, agarose and polyacrylamide gel-electrophoresis, gel staining and analysis were done according to Sambrook et al. [116]

Preparation of bovine DNA

1 g of tissue samples from cow ears extracted by a Biopsytak from Biopsytec (Berlin, Germany) was mixed with 20 µl of a buffer containing 50 mM Tris-HCl, 20 mM NaCl, 1 mM EDTA and 1 % SDS, pH 8. 1 µl Proteinase K (20 mg/ml) was added and the reaction was incubated for 30 min at 55 °C. Afterwards the reaction was stopped by heat denaturation (100 °C for 5 min). Then 200 µl distilled water was added. 1 µl of the solution was used for the following PCR without further purification.

Preparation of human DNA

Genomic DNA was extracted from full blood according to the procedure described in Sambrook et al.[116] The concentration was generally adjusted to 5 ng/µl.

DNA sequencing for confirmation of results of the GOOD assay

PCR fragments were purified using a Qiagen PCR purification kit. Cycle sequencing was performed according to the BigDye terminator protocol from PE Applied Biosystems using AmpliTaq DNA polymerase FS. Purified PCR products (1-1.5 µl) were added to 1 µl of sense or antisense primer (10 µM) and 3 µl of BigDye terminator mix (PE Applied Biosystems). The linear amplification consisted of an initial denaturation step for 2 min at 96°C, 25 cycles of 10 s of denaturation at 96°C, a 5 s annealing step (55–61°C) and a 4 min extension step at 60°C. The reaction products were purified and separated on an Applied Biosystem Sequencer 3700. Resulting sequence ladders were analysed by CNG-software termed “Genalys”.

Oligonucleotide synthesis with amino-modified nucleobases

Recently, Seela et al. described the synthesis of various 7-(ω-aminoalk-1-ynyl) functionalized 7-deazapurine nucleosides and their conversion into the corresponding β-cyanoethyl phosphoramidites.[117-119] In the case of 5-(alk-1-ynyl) modified pyrimidine nucleosides only marginal informations are available.[120] The syntheses of 3-phthalimido-1-propynyl functionalised β-cyanoethyl phosphoramidites 1, 3 and 4 (figure 2.1) were developed by Dr Thomas Wenzel (Bruker Saxonia, Leipzig, Germany). 1 is abbreviated as A^{NH2}, 2 as G^{NH2}, 3 as C^{NH2} and 4 as U^{NH2}. The same nomenclature is maintained after deprotection of these compounds.

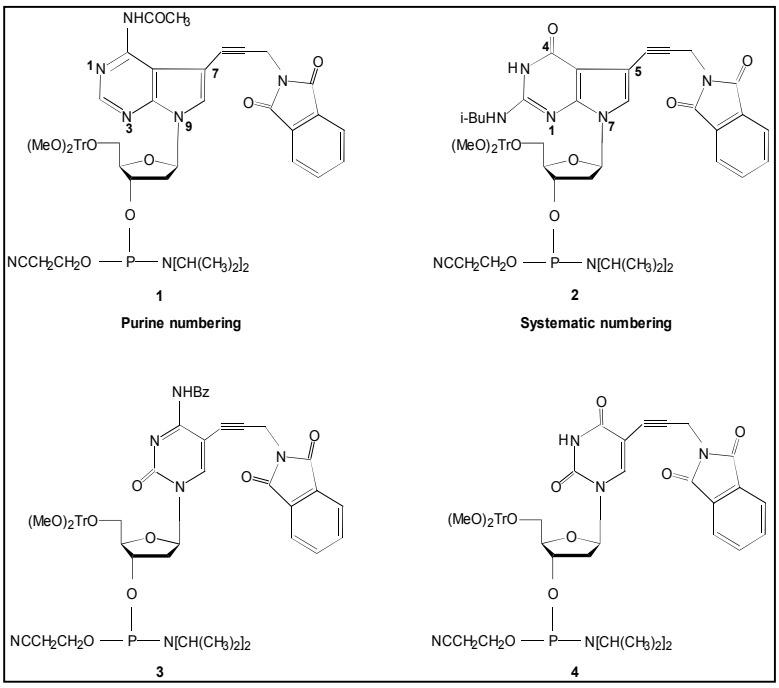

Figure 2.1. Novel β-cyanoethyl phosphoramidites used for the amino-modified oligonucleotides for primer extension in the GOOD assay.

The β-cyanoethyl phosphoramidites 1-4 (figure 2.1) were employed in solid-phase synthesis of oligonucleotides. The coupling yield of the modified DNA-building block, as determined by monitoring the 4,4'-dimethoxytrityl cation at 498 nm, was always higher than 96%. After deprotection with concentrated aqueous NH_3 solution (8h at

60°), the 5'-$(MeO)_2$Tr protected oligomers were purified, detritylated, and desalted on oligonucleotide-purification cartridges. Molecular masses of synthesized oligonucleotides were determined by MALDI-MS. A saturated solution of hydroxypicolinic acid in 1:1 (v/v) acetonitrile and 0.1 M diammonium citrate was used as matrix. 0.4 µl of the matrix and 0.2 µl of the purified oligonucleotide were transferred onto the MALDI target using the dried-droplet sample preparation method.

Positive charge-tagging of oligonucleotides containing amino-modified primers

The amino functionality of the synthesized primers were used for attaching a positive charge tag (6-trimethylammoniumhexyryl-N-hydroxy-succinimidylester) according to Gut et al. and Bartlett-Jones et al. T^{NH2} is converted to T^{CT}, U^{NH2} is converted to U^{CT}, A^{NH2} to A^{CT}, C^{NH2} to C^{CT}, and G^{NH2} to G^{CT}. T^{NH2} is an amino modifier C6 dT obtained from Glen Research (Sterling, USA).

The primers containing the aminofunctionality were dissolved in 1 % TE-buffer at 500 pmol/µl. 30 µl of this solution were mixed with 1.5 µl 2 M triethylammoniumhydrogencarbonate (pH 8.0) and 24 µl fresh 1 % 6-trimethylammoniumhexyryl-N-hydroxy-succinimidyl ester, 6-trimethylammonium-pentyryl-N-hydroxy-succinimidylester or 6-dimethyl-ethylammoniumhexyryl-N-hydroxy-succinimidylester diluted in distilled water. For a 1 % solution of 6-diethyl-methylammoniumhexyryl-N-hydroxy-succinimidylester 30 % acetonitrile was used as solvent. The reaction mixture was incubated for 30 min at 0 °C. Afterwards the reaction mixture was lyophilised and resuspended in 15 µl 300 mM ammoniumacetate and 60 µl ethanol. The precipitation of the oligonucleotide was completed by incubation at -20 °C for 1 h. After microcentrifugation with an Eppendorf Centrifuge 5415 D at 13.000 rpm for 2 min the precipitate was washed twice with 60 µl 80 % ethanol and resuspended in 30 µl bidistilled water. The concentration was measured by UV-spectroscopy and the quality of charge tagging was routinely verified by MALDI-MS as described in the preceeding chapter. Usually, no residual starting material was observed, from which

was concluded that more than 95 % of the amino-modified oligonucleotide were converted into charge-tagged primers.

Standard GOOD assay protocol for positive ion mode MALDI-MS detection

PCR: 20 ng human genomic DNA was used as template for PCR. 5 pmol of the forward (5'-CAGCCCCATGGTGGTGGC) and 5 pmol of the reverse (5'-GGTTGGGGTAACGGTGC) primer, 40 mM Trisbase, 32 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgCl_2$, 200 µM dNTPs and 0.2 U Taq-DNA-polymerase were mixed in a resulting 10 µl volume. The reaction was denatured 1 min at 94 °C, then thermocycled 15 s at 94 °C, 30 s at 56 °C and 1 min at 68 °C 35 times.

SNP R670G in the platelet/endothelial cell adhesion molecule (PECAM) gene: 7.5 pmol of the forward (5'-AAATCAGGCTTGACTTTGTTAG) and 7.5 pmol of the reverse (5'-TCATTAAGAGAGGTGGGAAAT) primer were mixed together with 1 µl Platinum Taq polymerase (10X) buffer (pH 8.9), 0.2 µl $MgCl_2$ (50 mM), 100 µM dNTPs, 20 ng human genomic DNA and 0.75 U of Platinum Taq DNA-polymerase in a 10 µl volume. The reaction was denatured for 4 min at 94 °C, then thermocycled for 30 s at 94 °C, for 45 s at 60 °C and 30 s at 72 °C, repeating 30 times. Afterwards the reaction was incubated for 4 min at 72 °C.

SNP A457C in the Caveolin 1 (CAV 1) gene: 1 µl of human genomic DNA (5 ng), 2.5 pmol of the forward (5'-GGGAAGAATTCCAGGGTATG) and 2.5 pmol of the reverse (5'-TTGAAAAGGAAAATGTGAAAA) primer were mixed together with 0.3 µl Platinum Taq polymerase (10X) buffer (pH 8.9), 0.06 µl $MgCl_2$ (50 mM), 100 µM dNTPs, and 0.1 U of Platinum Taq DNA-polymerase in a 3 µl volume and covered with 3 µl oil. The reaction was denatured for 4 min at 94 °C, then thermocycled for 30 s at 94 °C, for 45 s at 54 °C and 30 s at 72 °C, repeating 30 times. Afterwards the reaction was incubated for 4 min at 72 °C.

Angiotensin converting enzyme (ACE) gene: 2.5 pmol each of the forward primer (5'-GACAAGCTGAGTGTGCAGGA) and 2.5 pmol of the reverse primer (5'-GCCAACATGATTAAACCCC) were mixed together with 5 ng genomic DNA, 0.3 µl of TPHF (10X) buffer, 0.06 µl of $MgSO_4$ (50mM), 50 µM dNTPs and 0.05 U TPHF DNA polymerase in a 3 µl volume and covered with 3 µl oil. The reaction was denatured for 4 min at 94 °C, then thermocycled for 30 s at 94 °C, for 45 s at 65 °C and 30 s at 72 °C, repeating 30 times.

Shrimp alkaline phosphatase (SAP) digestion: For a 10 µl PCR 0.5 µl (1 U/µl) of shrimp alkaline phosphatase were added to the PCR reaction and incubated for 1 h at 37 °C. In the case of a 3 µl PCR 0.25 µl SAP and 1.75 µl Tris-HCl (200mM, pH 8.0) were added. The SAP was denatured for 10 min at 90 °C. By replacing the Thermosequenase with TMA 31 FS DNA polymerase for the primer extension the shrimp alkaline phosphatase digestion can be omitted.

Primer extension reaction: This primers contained phosphorothioate bridges (pt) and charge-tags (CT). For the SNP 129 in the prion protein gene 5'-$GGGGCCTTGGTGGCT_{pt}A^{CT}{}_{pt}C$ was used as primer for the extension reaction. This primer contains two phosphorothioates, between T and A^{CT} and between A^{CT} and C. It has one mismatch in the case of the human prion gene. At position 11 there is a T in the primer sequence instead of a C but it also works very well for primer extension. 25 pmol of the extension primer was added together with 2 mM $MgCl_2$, 0.2 mM $MnCl_2$, 100 µM α-S-ddNTPs and 2 U Tma 31 FS DNA polymerase. The reaction volume was increased to 10 µl by the addition of water. This reaction mix was added to the preceding PCR. An initial denaturing step 2 min at 95 °C was used, followed by 35 cycles of 10 s at 95 °C, 30 s at 58 °C and 15 s at 72 °C.

For the analysis of SNP CAV 1 A457C $CTTAAACAGTGGGAAAAGA_{pt}G^{CT}{}_{pt}T$ was used as primer. 5 pmol of the extension primer was added together with 2 mM $MgCl_2$, 0.2 mM $MnCl_2$, 100 µM α-S-ddNTPs and 0.5 U Tma 31 FS DNA polymerase. The reaction volume was increased to 2 µl by the addition of water. This mixture was added to the preceding 3 µl PCR. An initial denaturing step 4 min at 94 °C was used, followed by 30 cycles of 15 s at 95 °C, 30 s at 56 °C and 30 s at 72 °C, and a final incubation at for 4 min 72 °C.

In the case of SNP 33730 in the angiotensin converting enzyme gene, 5 pmol of the primer (5‘-CATGTCCTCTAAATGGTTT$_{pt}$C$^{CT}{}_{pt}$C) was added using the same reaction conditions as for SNP CAV 1 A457C.

In the case of the SNP R670G in the PECAM gene 5'-AGGTCACAATGACGATG$_{pt}$U$^{CT}{}_{pt}$C was used as primer for the extension reaction employing the same conditions as for SNP CAV 1 A457C.

Primer Removal: For the digestion of products in 20 µl volume 1 µl of a 0.5 M acetic acid solution was added to the processed primer extension resulting in a reaction pH around 5.0. Then 3 µl of phosphodiesterase II that was previously dialysed against ammonium citrate (0.1 M, pH 6.0) were added and the reaction mix was incubated for 1 h at 37 °C. In the case of a 5 µl volume after primer extension 0.5 µl of 0.5 M acetic acid solution and 1.5 µl phosphodiesterase II were added.

Alkylation reaction: For a big volume (ca. 24 µl after primer removal) 45 µl of acetonitrile, 15 µl of triethylammonium bicarbonat solution (pH 8.5) and 14 µl of methyliodide were added, while for a small volume 15 µl of acetonitrile, 5 µl of triethylammonium bicarbonat solution (pH 8.5) and 5 µl of methyliodide were used. The reaction was incubated for 25 min at 40 °C. Afterwards for big volumes 20 µl and for small volumes 10 µl of bidistilled water were added. Upon cooling a biphasic system was obtained. The upper layer contained the charge-tagged DNA products while the lower layer contained some of the reagents that were added to stabilise the enzymes (i.e. detergents) used in this procedure. 10 µl of the upper layer were sampled and diluted in 22 µl of 40 % acetonitrile. This solution was directly used to transfer the samples onto the matrix.

Sample preparation for MALDI analysis: The α-cyano-4-hydroxy-cinnamic acid methyl ester matrix was prepared by spotting 0.5 µl of a 1.5 % solution in acetone onto the target and spotting 0.3 µl of a solution of the sample in 40 % acetonitrile on top of the dried matrix. 40 % acetonitrile dissolves the surface layer of the matrix allowing for a concentrated incorporation of the analytes into the matrix surface. By this preparation, a very thin and fine crystalline matrix layer is achieved.

Mass spectrometric analysis: Spectra were recorded on a Bruker Reflex III time-of-flight mass spectrometer equipped with a SCOUT 384^{TM} ion source and delayed extraction. Acceleration potentials typically chosen were 18 kV in both ion modes. For delayed extraction the acceleration potential was switched with a delay of 200 ns. Generally 5-20 spectra were averaged.

Standard GOOD assay for negative ion mode MALDI-MS detection and the GOOD assay by switching the ion mode

PCR:

Granulocyte-macrophage colony stimulating factor gene (GM-CSF): 5 pmol of the forward (5´-TGTGCACATGGTGGTCAT) and 5 pmol of the reverse (5´-GAATCTCCTGGCCCTTATC) primer were mixed with 1 µl of human genomic DNA (5 ng), 1 µl of TPHF (10X) buffer (pH 8.9), 0.5 mM $MgSO_4$, 200 µM dNTPs and 0.75 U of TPHF-DNA-polymerase in a 10 µl volume. The reaction for GM-CSF was denatured for 1 min at 94 °C, then thermocycled 15 s at 94 °C, 30 s at 56 °C and 1 min at 68 °C repeating 35 times.

SNP PECAM G58A: 7.5 pmol of the forward (5'-CATTTTGCATTTCTCTCCACC) and 7.5 pmol of the reverse primer (5'-GCAGGGCAGGTTCATAAATAAG) were mixed together with 1 µl Platinum Taq polymerase (10X) buffer (pH 8.9), 0.2 µl $MgCl_2$ (50 mM), 100 µM dNTPs, 20 ng human genomic DNA and 0.75 U of Platinum Taq DNA-polymerase in a 10 µl volume. The reaction was denatured for 4 min at 94 °C, then thermocycled for 30 s at 94 °C, for 45 s at 60 °C and 30 s at 72 °C, repeating 30 times. Afterwards the reaction was incubated for 4 min at 72 °C.

SNP PECAM R670G: 1 µl of human genomic DNA (5 ng), 7.5 pmol of the forward (5'-AAATCAGGCTTGACTTTGTTAG) and 7.5 pmol of the reverse (5'-TCATTAAGAGAGGTGGGAAAT) primer were used following exactly the same reaction conditions as for SNP G58A. For a duplex PCR of these two fragments the four primers were used adopting the same reaction conditions as in a singleplex PCR.

Shrimp alkaline phosphatase digestion: This reaction was done as described for the GOOD assay with positive ion mode detection. By replacing the Thermosequenase with the TMA 31 FS DNA polymerase this step can be omitted.

Primer extension reaction:

Primers for GM-CSF:

5´-CTTACTGGACTGAGGTT$_{pt}$GC (the SNP is located on position position 99 of the PCR product), extension with α-S-ddNTPs

5´-CCAGGAAGTCCAAACTG$_{pt}$TG (the SNP is located on position 345 of the PCR product), extension with α-S-ddNTPs

5´-GAGAGCCCTCAGGAA$_{pt}$GG (the SNP is located on position 417 of the PCR product), extension with α-S-ddNTPs

Primers for PECAM:

PECAM G58A (primer 1): 5'-ATGTTCCGAGAAGAACAG$_{pt}$A$^{CT}{}_{pt}$T, extension with α-S-ddNTPs or ddNTPs

PECAM G58A (primer 2): 5'-ATGTTCCGAGAAGAACAG$_{pt}$AT, extension with α-S-ddNTPs

PECAM G58A (primer 3): 5'-ATGTTCCGAGAAGAACA$_{pt}$G$_{pt}$AT, extension with α-S-ddNTPs

PECAM G58A (primer 4): 5'-ATGTTCCGAGAAGAACAG$_{pt}$A$_{pt}$T, extension with ddNTPs

PECAM R670G (primer 5): 5'-AGGTCACAATGACGATG$_{pt}$U$^{CT}{}_{pt}$C, extension with α-S-ddNTPs

25 pmol of the extension primers were added together with 40 mM Trisbase (pH 8.8), 2 mM $MgCl_2$, 0.2 mM $MnCl_2$, 100 µM α-S-ddNTPs or respective ddNTPs and 2 U Tma 31 FS DNA polymerase. The reaction volume was increased to 10 µl by the addition of water. This reaction mix was added to the preceding PCR. An initial denaturing step for 2 min at 95 °C was used followed by 35 cycles of 15 s at 95 °C, 45 s

at 58 °C and 30 s at 72 °C. For the duplex reaction 12.5 pmol of primer 2 and 12.5 pmol of primer 4 were used.

Primer digestion, alkylation and sample preparation and mass spectrometric analysis were done as described above.

MALDI analysis of synthetic DNA oligomers

List of used oligomers:

T-ModelZwitter : 5'-$T_{pt}U(NH_2)_{pt}TT$
T-ModelCT+ : 5'-$T_{pt}U(NH_2)_{pt}T_{pt}T$
T-ModelCT- : 5'-$T_{pt}T_{pt}TT$
G-ModelZwitter : 5'-$G_{pt}G(NH_2)_{pt}GG$
G-ModelCT+ : 5'-$G_{pt}G(NH_2)_{S}G_{pt}G$
G-ModelCT- : 5'-$G_{pt}G_{pt}GG$
DoubleCTModelT : 5'-$U(NH_2)T_{pt}T_{pt}T_{pt}T_{pt}U(NH2)_{pt}T$
2PosCT1NegCTMoT : 5'-$U(NH_2)_{pt}T_{pt}TT_{pt}T_{pt}U(NH2)_{pt}T$
DoubleZwitterMoT : 5'-$U(NH_2)_{pt}T_{pt}TTT_{pt}U(NH2)_{pt}T$

The DNA oligomers containing an amino-modified nucleobase were charge-tagged as is described above. All of these molecules were alkylated mixing 20 µl of the oligomer (50 pmol/µl), 45 µl of acetonitrile, 15 µl of triethylammonium bicarbonat solution (pH 8.5), and 14 µl of methyliodide. The reaction mix was incubated for 25 min at 40 °C. Afterwards 20 µl distilled water were added. Upon cooling a biphasic system was obtained. 20 µl of the upper layer were sampled and diluted in 40 µl of 40 % acetonitrile. The α-cyano-4-hydroxy-cinnamic acid methyl ester matrix or a derivative of this matrix methylated at the 4-hydroxy position (synthesized by Dr Kurt Berlin) were prepared by spotting 0.5 µl of a 1.5 % solution in acetone onto the target and spotting 0.5 µl of a solution of the sample in 40 % acetonitrile on top of the dried matrix.

Mass Spectra were recorded on a Bruker Reflex III time-of-flight mass spectrometer equipped with a SCOUT 384^{TM} ion source and delayed extraction. Acceleration potentials typically chosen were 18 kV in both ion modes. For delayed extraction the acceleration potential was switched with a delay of 200 ns. Generally 100 spectra were averaged.

The simplified GOOD assay

Synthetic templates:

Template 1:

5’-AGCCTGAATCCTGAGCTGAATTACATTCCCAACCGCGTGGCACAAC

AACTGGCGGGCAAACAGTCGTTGCTGATTGCTGAATGGCTGACT

Template 2:

5’-AGCCTGAATCCTGAGCTGAATTACATTCCCAACCGCGTGGCACAAT

AACTGGCGGGCAAACAGTCGTTGCTGATTGCTGAATGGCTGACT

Template 3:

5'-TTCCCAACCGCGTGGCACAAGAACTGGCGGGCAAACAGTCGTTGCTGA

TT

Template 4:

5'-TTCCCAACCGCGTGGCACTTAAACTGGCGGGCAAACAGTCGTTGCTGA

TT

Primer extension primers:

MP in this case stands for a methylphosphonate linkage.

Primer for the synthetic template: 5’-ACTGTTTGCCCGCCAG$_{mp}$T$_{mp}$T

Position 298: 5’-CCCGCCGTGGGTCCG$_{mp}$C$_{mp}$C

Position 325: 5'-CGCGCCAGTCTGGCAG$_{mp}$G$_{mp}$T

PECAM G58A: 5'-ATGTTCCGAGAAGAACAG$_{mp}$A$_{mp}$T

PECAM R670G: 5'-AGGTCACAATGACGATG$_{mp}$T$_{mp}$C

Primer extension on synthetic templates:

Templates 1, 2, 3, and 4 were used as targets for the primer extension reaction. The primer extension was performed with 20 pmol primer and 100 µM ddNTPs using the same buffer conditions as described for GOOD assays. The same thermocycling protocol was applied but using an annealing temperature of 55 °C. The primer digestion by means of phosphodiesterase II was done as described above. 1 µl of the final solution was diluted in 10 µl 40 % acetonitrile. The preparation for the MALDI-target was applied as described above for GOOD assays.

PCR:

β-2-Adrenergic receptor gene: 20 ng human genomic DNA was mixed with 3.5 pmol of the forward (5'-CTCGCGGCCCGCAGAGCC) and 3.5 pmol of the reverse primer (5'-GTTGGTGACCGTCTGCAGACGCTC), 40 mM Trisbase (pH = 8.8), 32 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgCl_2$, 40 µM dNTPs and 0.2 U Taq-DNA-polymerase in a 10 µl volume. The reaction was denatured for 2 min at 95 °C, then thermocycled 20 s at 95 °C, 30 s at 68 °C and 30 s at 72 °C, repeating the cycle 30 times.

SNP PECAM G58A: 2.5 pmol of the forward (5'-CATTTTGCATTTCTCTCCACC) and 2.5 pmol of the reverse primer (5'-GCAGGGCAGGTTCATAAATAAG) were mixed together with 5 ng genomic DNA, 0.3 µl Platinum^{TM} Taq DNA polymerase high fidelity (10X) buffer, 0.06 µl of $MgSO_4$ (50 mM), 20 µM dNTPs and 0.1 U Platinum^{TM} Taq DNA polymerase high fidelity in a 3 µl volume. The reaction was denatured for 4 min at 94 °C, then thermocycled for 30 s at 94 °C, for 45 s at 65 °C and 30 s at 72 °C, repeating 30 times.

Primer extension: For the primer extension on synthetic templates 1, 2, 3, and 4, 1 pmol and/or 1 pmol of a respective template for the simulation of heterozygous DNA

were mixed with 20 pmol extension primer (5'-ACTGTTTGCCCGCCAG$_{mp}$T$_{mp}$T), 40 mM Trisbase (pH = 8.8), 32 mM $(NH_4)_2SO_4$, 50 mM KCl, 6 mM $MgCl_2$, 0.2 mM $MnCl_2$, 100 µM ddNTPs and 2 U Tma 31 FS DNA polymerase, Deep Vent DNA polymerase, Thermosequenase or Taq DNA polymerase in a final volume of 20 µl. For the analysis of SNPs in the β-2-adrenergic receptor gene 20 pmol of the primer for SNP position 298 (5'-CCCGCCGTGGGTCCG$_{mp}$C$_{mp}$C) or 20 pmol of the primer for SNP position 325 (5'-CGCGCAGTCTGGCAG$_{mp}$G$_{mp}$T) were mixed with with 40 mM Trisbase (pH 8.8), 2 mM $MgCl_2$, 0.2 mM $MnCl_2$, 100 µM respective ddNTPs (or α-S-ddNTPs) and 2 U Tma 31 FS DNA polymerase. The reaction volume was increased to 10 µl by the addition of water and added to the preceding PCR. Initially, the reaction mix was denatured for 1 min at 95 °C, followed by 35 cycles of 10 s at 95 °C, 30 s at 58 °C and 15 s at 72 °C. In the case of the SNP G58A in the PECAM gene 5 pmol of the primer (5'-ATGTTCCGAGAAGAACAG$_{mp}$A$_{mp}$T) was added using the same reaction conditions as described above but the total reaction volume was reduced to 5 µl and subsequent steps adapted proportionally.

Primer digestion: In the case of a primer extension reaction volume of 20 µl, 1.2 µl of a 0.5 M acetic acid solution were added to the reaction resulting in a pH around 5. Then 3 µl of phosphodiesterase II, that was previously dialysed against ammonium citrate (0.1 M, pH 6.0), was added and the reaction was incubated for 1 h at 37 °C. For a 5 µl reaction volume, 0.4 µl of a 0.5 M acetic acid solution and 1.5 µl of the phosphodiesterase were added.

For small reaction volumes it was important that sealing of microtiter plates was performed accurately. In contrast to GOOD assays in a small volume no oil was used. Aluminium sealing was not appropriate for assays with such small volumes because of too much evaporation. Therefore semi-automatic heat-sealing with an Abgene system (Villebon sur Yvette, France) was preferred.

After the primer digestion 1 µl of the reaction solution was diluted in 12 µl 40 % acetonitrile. Sample preparation and mass spectrometric analysis were done according to standard GOOD assays.

Software used for the analysis of results of the GOOD assay

Standard software from Bruker Daltonik (Bremen, Germany) was used for MALDI analysis. For automatic measurements AutoXecute was utilised. The Genotools SNP manager[121] was used for automatic analysis of automatic measurements and can be used with BIFLEX/REFLEX III and Autoflex mass spectrometers from Bruker Daltonik (Bremen, Germany).

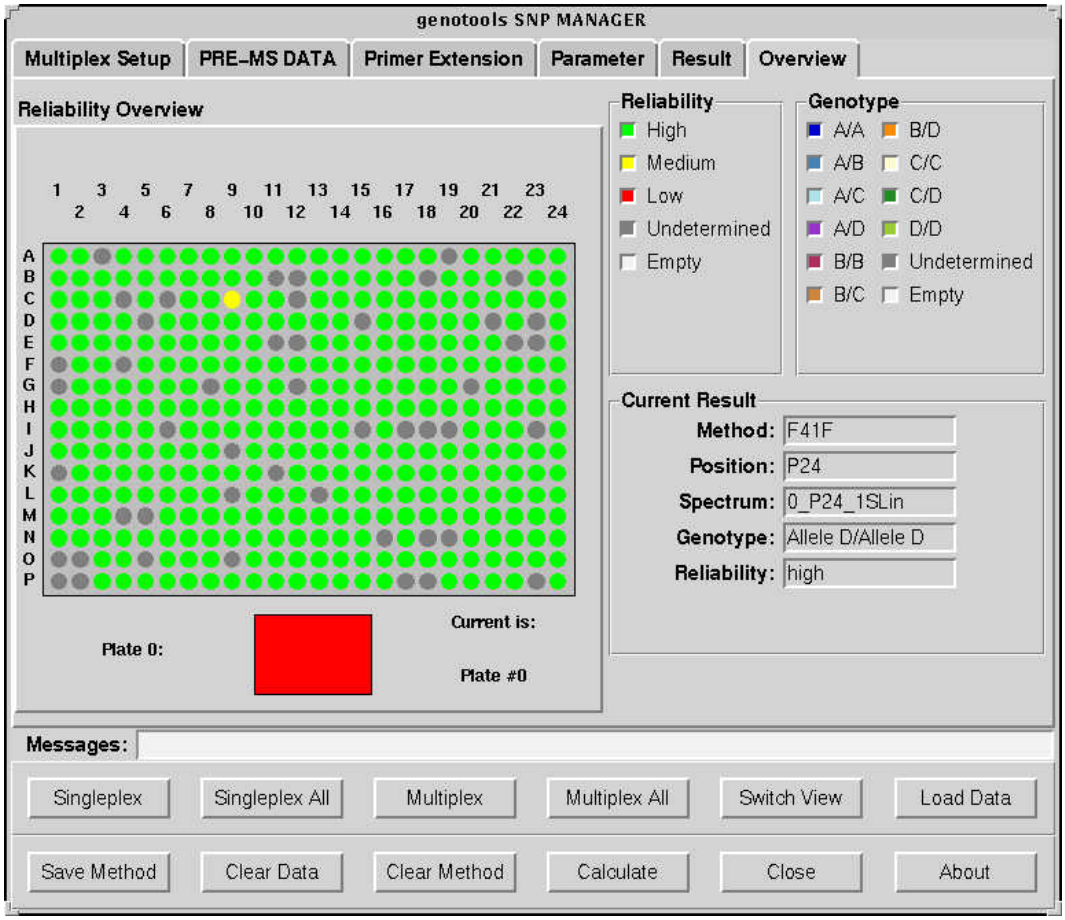

Figure 2.2. A result overview of an analysis of a GOOD assay on a 384-well plate target. Green points indicate spectra with high, yellow points with medium and red points with low reliability. Grey points are undetermined results due to low quality of spectra.

This software requires standard software for acquisition (XACQ 4.0., AUTOXECUTE 5.0.) and data processing (XMASS/XTOF 5.0.). It administers the data

of SNP assay conditions and calculates the molecular masses of the extension primers and the expected products. The Genotools SNP manager can define critical data processing settings for peak selection and calibration. The performed genotype analysis is displayed together with an evaluation of its quality deriving from the respective spectrum. Analysis can be done offline with previously generated data or online during data acquisition. Depending on the utilised set-up a single offline genotyping experiment requires 2-3 seconds. Therefore the analysis of a complete 384-well plate target takes 13-20 minutes. In figure 2.2 a result overview of the spectra acquired from a measurement on a 384-well plate target is shown. The reliability of results is displayed in a green/yellow/red colour code for each target position. Reliabilities are classified by the intensity of obtained signals and by the difference of detected and expected masses.

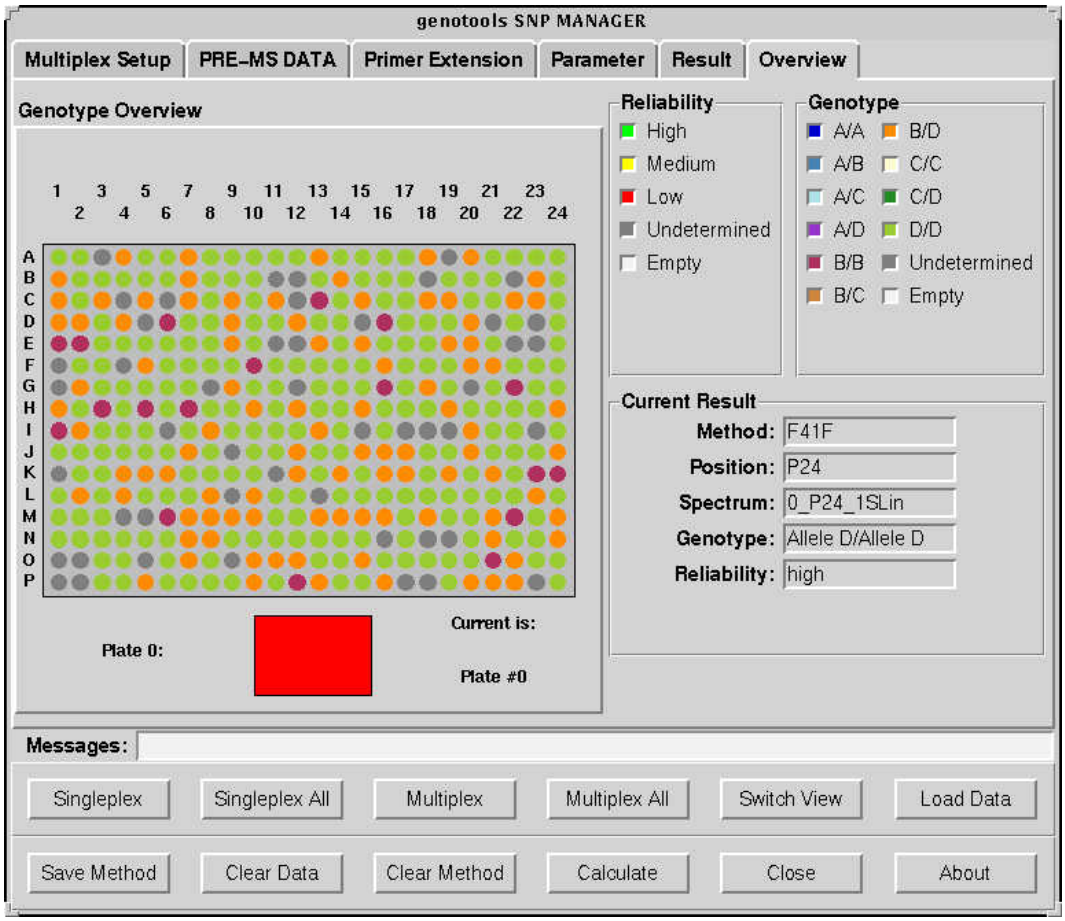

Figure 2.3. Genotype analysis of the results shown in figure 2.2. Again, grey points are undetermined results, while green points stand for homozygote D/D, orange points for heterozygotes B/D and violet points for homozygous B/B DNA. The corresponding biological alleles are thus coded A, B, C, and D.

Analysis results can be verified by a mouse click on the respective target position. The corresponding spectrum is loaded in the XMASS programme and the analysis data for the chosen active method are loaded in the Genotools SNP manager. The results for each analysis and an overview table for all spectra on a target are stored together in a result table. The result table is in ASCII format and can be easily processed using spreadsheet programmes or LIMS software. In figure 2.3, the overview of resulting genotypes of figure 2.2 is shown.

Design of GOOD assays is facilitated by the use of CNG-software termed "SNIP machine".[122] This software determines the best combination of primers for the PCR and the following primer extension reaction, considering annealing temperature, sense of the DNA oligomer, mass separation (in the range of 1,000 to 2,000 Da) and preparative cost. Because of the finite mass resolution of mass spectrometers a minimum mass separation of alleles is maintained. The SNIP Machine generates instructions for the required charge tag reagents and extension nucleotides. Its output can be forwarded to an oligonucleotide provider. With SNIP Machine the design of multiplex experiments is also facilitated. By the use of the provided computer software the routine development of the GOOD assays with respect to PCR, primer extension and phosphodiesterase II digestion is facilitated.

LCR on artificial templates

For LCRs a kit from Stratagene was used following the reaction conditions described in the instruction manual.

The following oligonuleotides were used:

Oligo A/N:

5'-TTGTGCCACGCGGTTGGGAATGTA, Phosphate at the 5'-end

Oligo A/PT:

5'-T_{pt}TGTGCCACGCGGTTGGGAATGTA, Phosphate at the 5'-end

Oligo B/N:

5’-AGCAACGACTGTTTGCCCGCCAGTTG

Oligo B/PT:

5’-AGCAACGACTGTTTGCCCGCCAGTT$_{pt}$G

Oligo C/N:

5’-TACATTCCCAACCGCGTGGCACAAC

Oligo C/PT:

5’-TACATTCCCAACCGCGTGGCACAA$_{pt}$C

Oligo D/N:

5’-AACTGGCGGGCAAACAGTCGTTGCT, Phosphate at the 5’-end

Oligo D/PT:

5’-A$_{pt}$ACTGGCGGGCAAACAGTCGTTGCT, Phosphate at the 5’-end

Template 1:

5'-AGCCTGAATCCTGAGCTGAATTACATTCCCAACCGCGTGGCACAACAA

CTGGCGGGCAAACAGTCGTTGCTGATTGCTGAATGGCTGACT

Template 2 (complementary to template 1):

5'-AGTCAGCCATTCAGCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTG

TGCCACGCGGTTGGGAATGTAATTCAGCTCAGGATTCAGGCT

For LCRs on synthetic templates 10 fmol of templates 1 and/or 2 were used. The template sequences as the templates of the kit derive from the lacI gene of E. coli.

The following thermocycling programme was used: First a heat denaturation for 4 min at 92 °C, and then annealing/ligation for 3 min at 60 °C was done. Afterwards 25 times thermocycles, executed for 20 sec at 92 °C and for 20 sec at 60 °C were performed.

Alternatively to the ligase reaction with the Pfu DNA ligase of the LCR kit, the Tsc DNA ligase thermostable and the Ampligase Thermostable DNA ligase were tested. The first one needs ATP as a cofactor; the two other ligases need NAD. While the two other buffers contain Nonidet P-40, the Ampligase buffer contains Triton X-100, which is more convenient for MALDI preparation. For the Tsc DNA ligase an alternative 10X buffer was prepared containing 200 mM Tris-HCl (pH 7.5), 200 mM KCl, 100 mM $MgCl_2$, 5 mM NAD and 10 mM DTT.

Gel-analysis was done using 10 % polyacrylamide gels in 1 X TBE (1:10 dilution of the buffer purchased by Sigma (St. Quentin Fallavier, France)) and ethidium bromide staining. The 20-mer base pair ladder (PCR Low Ladder set) was applied according to the supplier's recommendations.

Exonuclease digestion

Digestion Standard:

5'-GCGTGGCACAACAACTGGCGGG$_{pt}$C$_{pt}$AA$_{pt}$A$_{pt}$CAGTCGTTGCTGATTG

CTGAAT

Complementary oligonucleotide to the digestion standard:

5'-ATTCAGCAATCAGCAACGACTGTTTGCCCGCCAGTTGTTGTGCCACGC

0.6 µl (100 pmol/µl) of digestion standard was mixed with 18 µl water (and optionally 1 µl 10 X Ampligase buffer) and 1 µl phosphodiesterase I (0.39 U). The mixture was incubated for 1 h at 37 °C. Alternatively an equimolar amount of complementary DNA oligomer was added. Afterwards 2 µl of dialysed phosphodiesterase II that was also used for GOOD assays were added and the reaction mix was incubated for 1 h at 37 °C. If phosphodiesterase II was added to a ligase buffer containing medium, 0.75 µl of 0.5 M acetic acid was used to adjust the pH according to the GOOD assay protocols.

The subsequent alkylation reaction was performed as described for GOOD assays containing ca. 24 µl after phosphodiesterase digestion. MALDI analysis done similar but more than 100 laser shots had to be accumulated to obtain good spectra.

Expression and purification of Methanococcus jannaschii Flap Endonuclease

Cloning of this flap endonuclease was done according to Rao et al.[123] using a modified protocol from the Novagen pET system. The resulting BL21(DE3)pLysS clone (kindly provided by Dr James G. Wetmur) containing the pET20b+ M. jannaschii FEN expression vector was propagated at 37 °C in LB medium containing ampicillin (50 µg/ml) and chloramphenicol (25 µg/ml). Overnight cultures were diluted 1:50 into the same medium, grown to an A_{600} of roughly 0.5, then induced with 1 mM isopropyl-β-D-thio-galactopyranoside (IPTG), and grown for an additional 4 hours. Cells were collected, resuspended in protease inhibitor (1 mM AEBSF and 2.5 mM benzamidine), frozen and thawed to disrupt the envelopes, and clarified by microcentrifugation. The supernatants were made 1.5 M $(NH_4)_2SO_4$ by addition of a 3.8 M stock, heated to 75 °C for 15 min to denature thermolabile proteins, placed on ice for 30 minutes to aggregate the denatured proteins, and clarified for 15 min at 4 °C.

The crude FEN protein was diluted 6-fold with 20 mM Tris-HCl (pH 9.0), loaded onto a 1 ml Mono Q anion exchange column (Pharmacia), repeatedly washed with 20 mM Tris-HCl (pH 9.0), and eluted with 0.3 M NaCl in the same buffer. The resulting product was dialysed against 50 mM KCl, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0) using a Centricon 30 filter. Protein concentrations were determined by Bradford assays and compared with the complete absorbance spectra to determine an extinction coefficient and to verify removal of nucleic acids. The successful purification was confirmed by polyacrylamide gel electrophoresis and silver or Coomassie brilliant blue R staining.

FEN reaction on a synthetic template

Charge-tagging of oligonucleotides was done as described for the GOOD assay for positive ion mode detection.

1 µl 10X buffer (400 mM Trisbase (pH 8.8), 320 mM $(NH_4)_2SO_4$, 500 mM KCl) was mixed with 2 µl $MgCl_2$ (20 mM), 1 µl Template (10 pmol/µl), 1 µl Invader (20 pmol/µl), 1 µl Probe (20 pmol/µl), 200 ng FEN and water to a 10 µl volume. The reaction mixture was incubated for 2 h at 60 °C.

Template 1 and 2 utilised for the simplified GOOD assay were used as model templates for this procedure as well.

The following oligonucleotide was used as an invader:

5’-AATCAGCAACGACTGTTTGCCCGCCAGTTA

The following probe oligonucleotides were used for mass spectrometric analysis:

Probes for positive ion mode detection:

Probe 1:

5'-(C6-Aminolink containing a phosphorothioate linkage instead of the usual phosphate bridge) TptGptCGTTGTGCCACGCGGTTGGGAATGTA

Probe 2:

5'-(C6-Aminolink containing a phosphorothioate linkage instead of the usual phosphate bridge) TptGptCATTGTGCCACGCGGTTGGGAATGTA

Probes for negative ion mode detection:

Probe 1: 5’-CmpTmpCmpCATTGTGCCACGCGGTTGGGAATGTA

Probe 2: 5’-CmpTmpCmpCGTTGTGCCACGCGGTTGGGAATGTA

FEN reaction on a PCR product

Oligonucleotide set for the Invader assay at SNP 390 in the β-2-adrenergic receptor gene:

Probe 1: 5'-(C6-Aminolink containing a phosphate)

GptGptGGAAGCCATGCGCCGGACCACGACGT

Probe 2: 5'-(C6-Aminolink containing a phosphate)

CptCptGAAAGCCATGCGCCGGACCACGACGT

Invader: 5'-CCCGCTGAGGCGCCCCCAGCCAGTGCGCTTA

Charge-tagging of oligonucleotides was done as described for the GOOD assay for positive ion mode detection.

10 µl of PCR reactions developed for the β-2-adrenergic receptor gene or SNP R670G in the PECAM gene described for the simplified GOOD assay were mixed with two probe oligonucleotides (each 20 pmol) and 1 pmol invader oligonucleotide. 1 µl 10 X buffer ((400 mM Trisbase (pH 8.8), 320 mM $(NH_4)_2SO_4$, 500 mM KCl), 1.5 µl $MgCl_2$ (20 mM) and 200 ng FEN were filled up with water to a 20 µl volume. The mixture was incubated at for 2 h 60 °C.

The alkylation and preparation of products containing phosphorothioates was done according to GOOD assays. Probe oligonucleotides containing methylphosphonate bridges instead of phosphorothioates and a phosphate linkage as charge-tag were also alkylated but only to perform efficient phase separation. Dilution as is done for the simplified GOOD assay was not feasible.

MALDI target preparation and mass spectrometric analysis were performed as described above but 100-300 laser shots were accumulated to obtain good spectra.