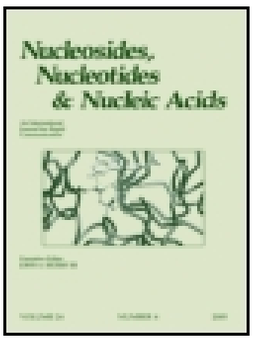

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ANIONIC MANGANESE meso-TETRAPHENYL PORPHINE/OLIGONUCLEOTIDE CONJUGATES - SYNTHESIS AND UTILIZATION IN CHEMILUMINESCENT DNA-PROBE DETECTION

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Abstract: The synthesis and application of oligodeoxynucleotides derivatized by the water-soluble anionic manganese-(III)-5-(4-carboxyphenyl)-10,15,20-tris-(4-sulphonato-phenyl)-porphine complex are described. These conjugates were found to be potent catalysts for the chemiluminescent oxidation of luminol, which in turn favour their application as non-radioactive DNA probes.

During the past few years there has been a growing interest in the use of modified oligonucleotides as research tools in molecular biology. The potential for analytical and therapeutical applications can be strongly increased by covalent linking of reporter groups at one of the ends of the oligonucleotides. Significant progress in chemical methods has recently resulted in the ability to synthesize modified oligonucleotides which have many attractive advantages over other DNA fragments[1]. Among these, redoxactive metal complexes covalently bound to oligonucleotides have exhibited some very interesting features. Metal chelates such as EDTA-Fe(II)[2] or Phenanthrolin-Cu(II)[3] covalently linked to oligonucleotides have been used for site-directed chemical modification, stimulating strand scission reactions on complementary polynucleotides.

Oligonucleotides bearing covalently attached cationic porphyrin derivatives have been utilized as artificial endonuclease systems[4-8].

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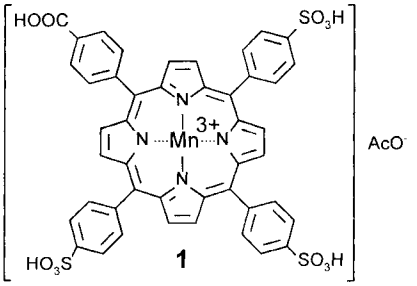
†Professor Dr. Dieter Cech deceased on November 19th, 1996.

Metalloporphyrin-linker-oligonucleotide-conjugates have been studied for *in vitro* cleavage of double-stranded DNA targets present in the HIV-1 genome[9]. Furthermore, strand cleavage was achieved by using oligonucleotide probes with natural occurring cobalt-corrin complex conjugates in the presence of ascorbic acid[10].

As a part of our comprehensive studies on non-radioactive detection systems for different DNA probes based on pure chemical reactions, we recently published[11] the use of methylene-blue as photosensitizer in oligonucleotide-conjugates. These results encouraged us to study the applicability of redoxactive metalloporphyrins as artificial peroxidase mimics in DNA analysis. Additionally, this concept was stimulated by recently published results of Meunier et al.[12], who studied different substituted metalloporphyrins as structural analogues of prosthetic groups of peroxidases and found that substituted metalloporphyrins show even a higher activity in the luminol system as the holoenzyme itself. Motsenbocker et al.[13] developed a new detection system of metalloporphyrin-protein-conjugates in immunoassays based on the catalytic influence of metalloporphyrins on the luminol oxidation. However, the described system presents a few shortcomings related to efficiency and widespread application for biomolecules. Among the various synthetic metal meso-tetraphenyl porphine derivatives so far examined, the manganese-(III) derivative of tetrasodium meso-tetrakis-(4-sulphonato-phenyl)-porphine (MnTPPS4) has proved to be the most active system, but any attempt at covalent coupling to proteins has as yet failed. To overcome this difficulty, the less active Mn-(III)-5,10,15,20-tetrakis-(4-carboxyphenyl)-porphine complex has been used, which was coupled via an active ester approach to antibodies. We have been interested in suppressing these drawbacks through the development of a method for covalent linking of sulphonated manganese meso-tetraphenyl porphine complexes to oligonucleotides or biomolecules. We describe in the present paper the chemical synthesis of new manganese-(III)-complexes suitable for covalent linking, their introduction into synthetic DNA-fragments, and the first analytical application of these conjugates.

An inspection of the literature has confirmed, that a method for coupling synthetic anionic metal meso-tetraphenyl porphines with sulphonic acid functionalities to



1

oligonucleotides or DNA is as yet unknown.

Stable activated derivatives of sulphonic acid functions in meso-tetraphenyl porphine capable of forming amide bonds with free amino groups of biomolecules are also unknown. That is very remarkable, as other dyes (e.g. Texas Red[14]) thoroughly react in the form of their corresponding sulphochlorides with amino groups of proteins or aminoalkylated oligonucleotides. Both Motsenbocker[13] and later Seliger[6], who studied porphyrin-functionalized oligonucleotides as antisense structures have accommodated the fact that all attempts of the synthesis of covalently attached derivatives of sulphonated porphyrins of biomolecules failed and have consistently used the tetracarboxylated derivatives. This convenience has its price. The compromise comes clearly at the expense of sensitivity in the analytical application of the complexes, which is for the sulphonated derivatives almost twice as high as for the carboxylated derivatives. Furthermore, the utilization of the tetracarboxylated derivatives gives rise to additional difficulties with respect to isolation and purification of the monoactivated conjugates. To overcome these obstacles, a compromise between the desired sensitivity and the chemical availability of the attachment to DNA-fragments seems to be necessary. Thus, from a chemical point of view, the desired metalloporphyrin should contain at least one activated carboxylic group for the covalent attachment to nucleic acids, which is realized in a structure of type **1**.

We found the complex **1** to meet all the requirements discussed above. The complex combines both the high catalytic activity of the sulphonated complex in chemiluminescent oxidation of luminol and the possibility of a selective activation of the porphyrin structure by formation of an active N-hydroxysuccinimido ester of the carboxylic group and its subsequent covalent attachment to 5′-alkylated oligonucleotides or other biomolecules with primary amino groups.

Using the conditions of the luminol system of Motsenbocker[13] the sensitivity of detection of **1** was 0.4 fmol compared to 0.2 fmol of the tetrasulphonated manganese-

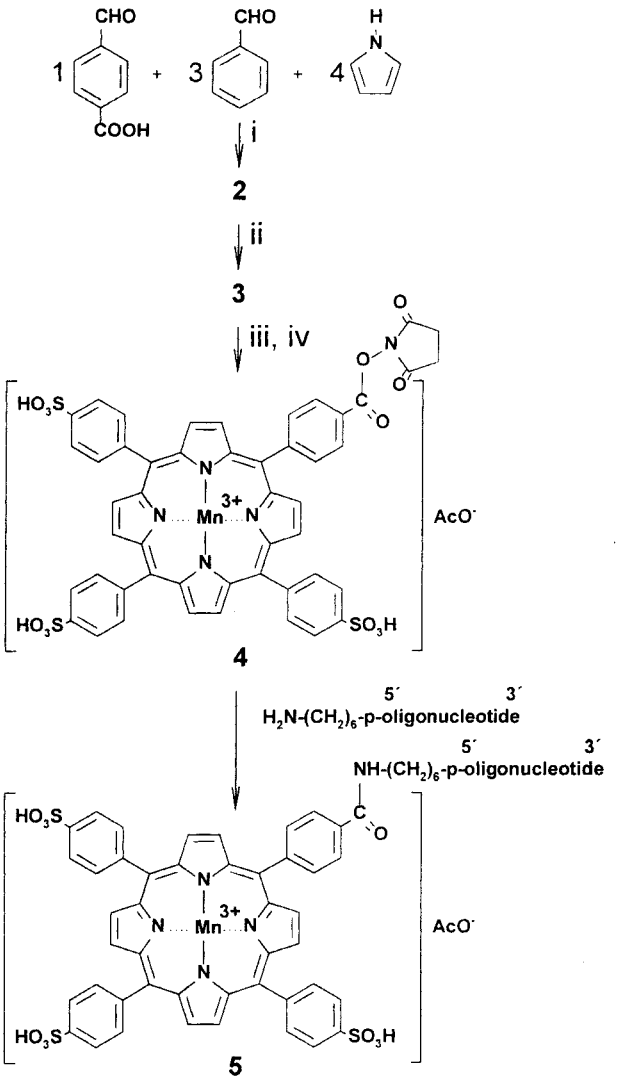


FIGURE 1

Synthetic pathway to **1** and oligonucleotide labelling with complex **1**
(i: propionic acid/acetic anhydride/reflux; ii:sulfuric acid; iii: manganese(II)acetate/O_2/H_2O; iv: TSTU/DMF)

(III)-meso-tetraphenyl porphine (MnTPPS4) and 1 fmol of the tetracarboxylated manganese-(III)-meso-tetraphenyl porphine (MnTPPC4).

The synthesis of **1** is outlined in Fig.1. The monocarboxylated tetraphenyl porphine **2**, which was synthesized via a statistical Rothemund reaction[15], served as starting

material for the synthesis of **1**. Recrystallization of the reaction product and chromatographical separation on silica gel led to **2** in unexpected high yields (see experimental part). Next, **2** was treated with sulphuric acid giving 5-(4-carboxyphenyl)-10,15,20-tris-(p-sulphonatophenyl)-porphine **3** as the key intermediate for the following reactions in a moderate yield. The obtained porphyrin derivative **3** was then metalized in water with manganese-(II)-acetate in the presence of atmospheric oxygen yielding **1**. Consequently, **1** was activated through formation of the N-hydroxysuccinimido ester using O-(N-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU) in absolute DMF giving **4** according to reference[16].

The 21mer oligonucleotide 5'H_2N-$(CH_2)_6$-p-d(AGC TGT ATC GTC AAG GCA CTC)-3' complementary to a particular region of K-ras gene was synthesized using standard phosphoramidite chemistry with commercially available phosphoramidite synthons. After deprotection, reaction of the terminal amino group of this oligonucleotide with **4** yielded the conjugate **5**.

However, the purification of the reaction mixtures either by gel electrophoresis or size exclusion chromatography (NAP-10) was troublesome and led to considerable losses of products. Otherwise, unreacted metalloporphyrin can be removed by BIORAD-P2 gel filtration without cleavage of the conjugates. The crude products of the reactions were additionally purified by HPLC. Baseline separation of starting material and the products were possible both in analytical and preparative scale (see Fig. 2). Detection at 260 nm and 467 nm allowed the differentiation between peaks of unreacted starting material (A) and the product (B). It should be mentioned, that the HPLC detection at 467 nm was done with a 4 fold higher sensitivity of the multi wavelength detector compared with the UV (260 nm) detector, hence the peak heights do not correspond to the ratio of absorption coefficients. Impurities from the oligonucleotide synthesis and some 5'-unmodified 21mer might be responsible for the two small peaks and the shoulder at the

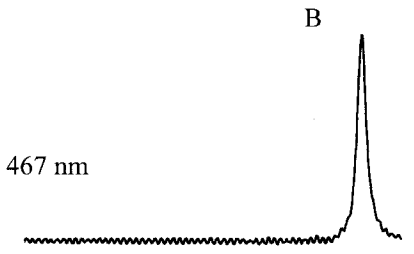


FIGURE 2

HPLC elution profile of the reaction products of **4** with a 21mer aminoalkylated oligonucleotide, conditions see experimental part

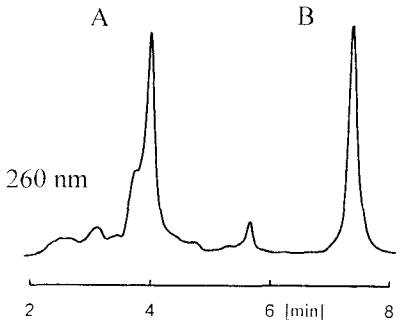


FIGURE 2 Continued

peak of the starting material, respectively. The yield of the coupling reaction ranged from 50-70% estimated by integration of the HPLC peaks.

Finally, the purified product was characterized by Matrix Assisted Laser Desorption Ionisation Mass Spectrometry (MALDI-MS). The expected molecular peak was found at m/z = 7521 [M^+] for **5** (succinic acid was used as matrix). The UV/Vis spectra of purified and isolated oligonucleotide conjugate displayed the expected absorption maximum at 260 and 467 nm, respectively. The ratio of the corresponding maximum E_{260}/E_{467} was appr. 4.

The determination of the Tm values of the complexes formed by hybridization of the oligonucleotide-porphyrin conjugate **5** or the corresponding unmodified oligodeoxynucleotide with their complementary sequence, 67 °C and 69 °C respectively, has shown that the label does not disturb any hybridization.

The analytical potential of the oligonucleotide-metalloporphyrin-conjugates was investigated in a novel, microtiterplate formated DNA hybridization assay developed for point mutation analysis in K-ras genes[17-20] and compared with currently used 5'-digoxigenin labelled oligonucleotides. In this assay, amplified and labelled fragments of K-ras genes in which point mutations cause malignant transformation of cells, are hybridized sequence-specifically against oligonucleotides, which are complementary to known oncogenic mutated sequences (so called capture probes) immobilized at microtiterplate wells. This approach allows early detection of malignant transformation in the genotype of cells.

A dilution series of 10 ng to 1 pg of conjugate **5** per well were hybridized against complementary probes covalently immobilized at the microtiterplate surface. Each well contained between 1 and 2 **micrograms** of the immobilized capture probe[20], i.e. a large excess. Amounts of up to 5 pg (0.61 fmol) of the hybridized conjugate **5** could be detected using a chemiluminescent microtiterplate reader with an integrated dispenser for delivering of the luminol reagent described by Motsenbocker[13]. The dependence of the

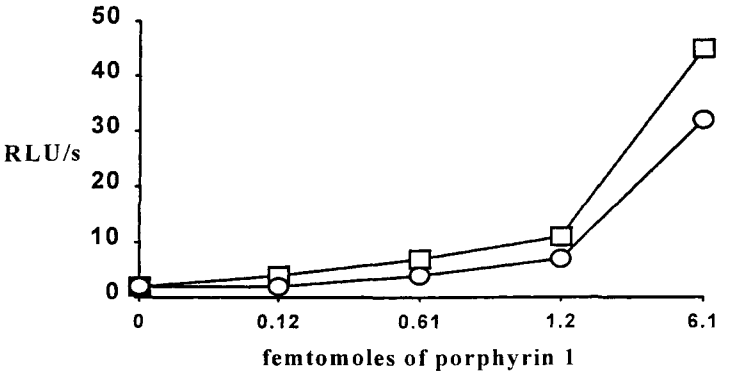


FIGURE 3

Detection of compound **1** and **5** in mikrotiterplate wells (details see experimental part). Signal intensity in relative light units per second (RLU/s) versus the concentration per well of the free complex **1** (squares) and the hybridisized conjugate **5** (circles)

signal intensitiy versus the concentration of the hybridisized probe **5** as well as unbound complex **1** is depicted in Figure 3. Unlabelled oligonucleotides or labelled sequences not complementary to the immobilized capture probe gave after the hybridization protocol signals, which were not higher than the average background level.

Comparing the sensitivity of this new detection system with 5` digoxigenated probes and detection of this probes with anti-DIG/alkaline phosphatase conjugates using the chemiluminescent decomposition of CSPD™, the detection limit was approximately one order of magnitude lower and should be comparable with the corresponding colorimetric mode of detection.

However, the detection does not require any additional reaction step to attach signal producing components as is usually necessary in indirect systems. That means detection is possible immediately after the last washing step of the hybridization protocol. This fact dramatically reduces the time of the total assay and simplifies the whole detection procedure substantially. Furthermore, this sensitivity should be sufficient to analyse PCR fragments both in qualitative as well as in quantitative assays. The simplicity of performance, high speed and the low cost of all reagents make the described procedure

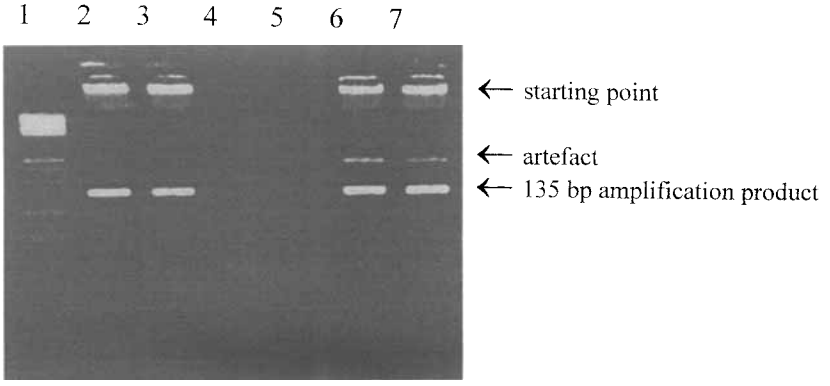


FIGURE 4

Electrophoresis of PCR products in 1% agarose gel containing ethidium bromide: lane 1 = Molecular weight standard; lane 2 and 3= phorphyrin modified primer (**5**) as one part of the primer pair; lane 4 and 5= PCR product without template DNA (negative control); lane 6 and 7 = PCR product from digoxigenated primer

well suited for automation and high throughput routine analysis, for example in clinical practice.

Oligonucleotide primer bearing the complex **1** were used in the PCR. The reaction was carried out using primers from ref. 18 yielding amplicons of 135 base pairs of the K-ras oncogene (for details see experimental). The results are displayed in Fig.4. Amplification reactions were done using primer pairs in which one primer bore the described porphyrin modification (lane 2 and 3, Figure 4) or the corresponding digoxiginated counterpart (lane 6 and 7). Lane 4 and 5 correspond to amplification reactions without template (negative control); lane 1 represents the molecular weight marker. Neither any interference with the activity of the polymerase nor the primer specificity during PCR were observed. Furthermore, no loss of catalytic activity of the label in the detection reaction was realized.

Taking together the results of our work, it was shown that anionic metal porphyrin complexes are suitable for introduction into oligonucleotides. The procedure can be generalized to any sequence of choice. These conjugates are a possible alternative of oligonucleotide enzyme conjugates in chemiluminescent detection systems, e.g. for PCR

products labelled with metal porphyrin in a quantitative hybridization assay of DNA on microtiterplates. The application of the presented system in clinical practice will be published in detail in a proceeding paper.

Experimental

^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AMX 300 spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane. UV/Vis spectra and melting profiles were recorded with a CARY-3 UV/Vis spectrometer connected to a thermocontroller (Varian, Australia). Chemiluminescent measurements were performed with the microtiterplate reader Luminoskan® (Labsystem, Finland) in black 96-well microtiterplates from the same company. HPLC analysis was carried out with a ICI-System (GAT, Germany) using a linear gradient of 80% buffer B (0.1 M triethylammonium acetate, pH 7.5/acetonitrile, 98/2, (v/v)) to 50% in buffer A (0.1 M triethylammonium acetate, pH 7.5/acetonitrile, 50/50, (v/v)) during 31 minutes on a reversed phase column (LiChrosorb RP 18, 4x125 mm).

Oligonucleotides were synthesized using commercial available phosphoramidites (Pharmacia) on a Gene Assembler® oligonucleotide synthesizer (Pharmacia). Aminoalkylation was done according to the instruction of the supplier with Aminolink® (Pharmacia). The synthesized oligonucleotides were deprotected and desalted by standard procedures.

Polymerase chain reaction was carried out with a TRIO-Thermocycler (BIOMETRA, Germany). All Reagents of the polymerase chain reaction were purchased from Perkin Elmer, Germany. The molecular weight marker Nr.5 (8-587 bp) was obtained from Boehringer Mannheim, Germany.

O-(N-succinimidyl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TSTU) was purchased from Novabiochem (USA). All other reagents and solvents were from Aldrich (Germany). Sodium hydroxide was 99.99 % semiconductor grade and deionized water (Mili-Q^{50}, Milipore) was used for the measurements of chemiluminescence.

5-(4-carboxyphenyl)-10,15,20-triphenylporphine (2). 4-carboxybenzaldehyde (5.25 g, 37 mmol), benzaldehyde (11.73 g, 110 mmol), pyrrole (9.8 ml, 140 mmol), propionic acid (270 ml) and acetic anhydride (14 ml) were heated under reflux and stirred

for 90 min. Propionic acid was removed under vacuum and the final layer mixed with 200 ml of glacetic acid, shortly refluxed again and slowly cooled to room temperature, than the precipitate was separated by filtration at room temperature. Final purification by silica gel column chromatography with chloroform/methanol (98/2) yielded **2** which appears as single spot in TLC. Yield: 630 mg (2,6%); mass spectra m/z= 656 [M^+]; ^{1}H-NMR: 300 MHz (d_8-THF) δ(ppm) = 8.8 (s 8H (pyrrole), 8.4 (dd 4H, J= 7.91 Hz), 8.2 (m 15H), -2.9 (s 2H(NH); ^{13}C-NMR: 300 MHz (d_8-THF) δ(ppm) = 127.5, 128.6, 128.9, 135.3, 143.1, 147.5, 167.8, 177.1; UV/Vis spectra (THF) λ_{max} = 417 nm.

5-(4-carboxyphenyl)-10,15,20-tris-(4-sulphonatophenyl)-porphine (3). (210 mg, 0.310 mol) **2** was stirred with concentrated sulphuric acid (5 ml) at 100 °C over 5 h. The reaction mixture was poured on ice, neutralized with Na_2CO_3 and the volume was reduced under vacuum. After filtration, the residue was extracted with ethanol until the filtrate layer appeared colourless. The filtrate was evaporated to dryness to yield **3** which appears as a single spot in TLC. Yield of **3** 180 mg, 64 %; mass spectra m/z= 898 [M^-]; ^{1}H-NMR : 300 MHz (D_2O) δ(ppm) = 8.8 (s 8H (pyrrole), 8.36 (d 2H J_{AB}= 6.62 Hz), 8.19 (d 8H $J_{A'B'}$= 7.36 Hz), 8.05 (d 6H $J_{A'B'}$= 7.36 Hz)-2.9 (s 2H(NH); ^{13}C-NMR : 300 MHz (D_2O) showed the complete set of signals between 120-180 ppm; UV spectra (H_2O) λ_{max} = 415 nm.

Metalation of 3 (1). 99 mg (0.11 mmol) **3** and manganese-(II)-acetate (27 mg, 0.11 mmol) were resolved in 3 ml water and heated under reflux. TLC control on silica gel plates with methanol/chloroform (1/1) exhibited a quantitative reaction after 3 h. After evaporation to dryness 103 mg of **1** were isolated (98 % yield), mass spectra m/z= 951 [M]$^+$; UV spectra (H_2O) λ_{max} = 467 nm.

N-Hydroxysuccinimido ester of 1 (4). A solution of **1** (18 mg, 0.017 mmol) in 2 ml DMF (dry) was mixed with (12 mg, 0.034 mmol) TSTU, diisopropylethylamine (DIPEA) (4.5 mg, 0.034 mmol) and the mixture was allowed to remain overnight at room temperature. The reaction mixture was dropped into an excess of chloroform after which the product precipitated. The raw product was filtered off and washed with chloroform. Purification by column chromatography on silica gel with chloroform/methanol (5/4) as eluent yielded 12 mg of **4** (57 %); mass spectra: m/z= 1048 [M^+].

Synthesis of conjugates (5). Labelling reactions of aminoalkylated oligonucleotides (4 O.D., 17 nmol) were performed using **4** (1.2 mg, 1 μmol) in 200 μl 1 M potassium carbonate in water (pH 9.0) overnight in the dark. Separation by size exclusion chromatography on BIORAD-P2 gel with ammonium acetate buffer (20 mM, pH 8) gave a mixture of labelled and unlabelled oligonucleotides without unreacted **4**. The product **5** was separated by HPLC. Yield around 50% (between 1.5 and 2.5 $O.D._{260}$ of the labelled product).

Tm measurement. Melting temperatures were recorded in melting buffer containing 0.25M Na_2HPO_4, 1mM EDTA (disodiumsalt), pH 7.2.

Purification of the luminol. Ultra pure luminol was prepared by repeated recrystallization (4 times) from 1 M NaOH according to reference[21].

Chemiluminescent measurement without hybridization. Samples of complex **1** or oligonucleotide **5** were placed in 10 μl of water in the wells. Than, 100 μl of the detection solution (luminol 1mM, linolenic acid 0.1% in 0.1N NaOH in water, pH 13.0) were automatically injected under mixing modus and the light output was measured subsequently in intervals of 5 seconds.

Hybridization of 5 in microtiterplate wells. Complementary capture oligonucleotide was bound to the surface as described in literature[20]. Hybridization of **5** followed over 30 min at 42 °C in 50 μl 1x SERVA buffer. The wells were washed three times with 1x SSC buffer for 5 min and straightforward detected as described above.

Detection limits. All analysis were done triplicate and the signals were regarded as positive at a signal-to-noise ratio of 2.

Preparation of samples for PCR. DNA was prepared from peripheral human blood cells (Invisorb DNA Kit, Invitek, Germany) according to the manufactures recommendations.

Polymerase chain reaction. 100 μl reaction volume were prepared by mixing of the DNA sample (62 μl bidest. H_2O/2 μg DNA), 10 μl 10x reaction buffer (ammonium-sulphate 167 mM, TRIS 167 mM; pH 8.8) 10 μl 10x gelatine (2mg/ml), 2 μl 50x magnesium chloride (100 mM), 0.5 μl Taq (5 U/μl), 8 μl dNTP (1 mM) and primer 1 and 2 (each 50 pmol), respectively. After 5 min denaturation at 94 °C, 30 cycles of the

program with 30 sec 94 °C, 30 sec at 52 °C, 1 min 70 °C and finally 5 min at 70 °C were performed.

Primer sequences were: primer 1 { 5′-d(ACT GAA TAT AAA CTT GTG GT)-3′} and primer 2 {5′-d(AGC TGT ATC GTC AAG GCA CTC)-3'}.

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