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Electrical microarrays for highly sensitive detection of multiplex PCR products from biological agents

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ABSTRACT

For the sensitive detection of amplicons derived from diagnostic PCR, a novel electrical low-density microarray is applied and compared to state-of-the-art quantitative real-time PCR. The principle of the electrochemical method and the effective use for analysis are described. Interdigitated array gold electrodes (IDA-E) embedded into a silicon chip are the core technology of the fully automated compact biosensor system, basing on enzyme coupled electrochemical detection. The biointerface is built up with thiol-modified capture oligonucleotides on gold and mediates the specific recognition of hybridised target DNA amplified with uniplex or multiplex PCR. In here we show the potential of the designed electrical microarray to function as an advanced screening method for the parallel detection of a panel of the four pathogens *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis* and ortho pox viruses (genus), which are among the most relevant biowarfare agents. PCR products, generated from 10 to 50 gene equivalents, have been detected reproducibly. The experiments with varying pathogen amounts showed the good reliability and the high sensitivity of the method, equivalent to optical real-time PCR detection systems. Without PCR the total assay time amounts to 27 min. The advantage of the combination of multiplex-PCR with electrical microarray detection avoiding intensive PCR probe labelling strategies is illustrated.

1. Introduction

Considering the global political situation of today and with the general availability of technical means and know-how to culture microorganisms in large quantities there is worldwide intensive discussion about the possible danger of bioterroristic attacks with a number of different pathogens with high risk to public health. Thus, in this field there is a special interest to develop analytical screening tools with the advantage of portability and fast and simple detection for first responder teams as well as specialised laboratories (Higgins et al., 1999). In case of terroristic biowarfare attacks in a civil environment it is quite realistic that victims are not immediately recognised. In such an event, symptomatic patients would show up delayed but in large numbers at medical practices and hospitals (Franz et al., 1997).

Molecular diagnostics widely uses PCR based techniques, most often real-time PCR with fluorescently labelled hybridisation probes in various formats (Mackay, 2004). PCR is known to be highly specific for one target of choice, but can also be multiplexed for the amplification of various targets in one reaction (Wittwer et al., 2001). Additionally, screening methods for simultaneous detection of a higher number of pathogens can use nucleic acid microarrays, typically with optical data read-out (Palacios et al., 2007). Several microarrays for pathogen detection, using either direct nucleic acid hybridisation (Cleven et al., 2006) or highly sensitive target amplification by PCR (Tamioka et al., 2005), have been described. Typically the use of these microarrays involves many manual handling steps, long hybridisation times and no direct combination with an automated biosensor system.

We developed electrical microarrays and a compact, fully automated and easy-to-use biosensor system (Albers et al., 2003). The microarray chips can be manufactured at low costs and easily be customised for different applications (Los et al., 2005; Liu et al., 2007). The method also offers speed, flexibility and mobility for on-site detection and point-of-care diagnostics as an advanced screening tool for a broad range of target sets. Most important key features are the interdigitated array gold electrodes (IDA-E) arranged in 16 positions on a microarray, the independent data read-out by a unique multiplexing 16-channel potentiostat, the

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Table 1
Oligonucleotide sequences of PCR primer and 5′ nuclease PCR probes.

Name	Target/Sequence	Function
Orthopoxvirus rpo18 gene, 205 bp		
OPV rpo18 F	CTGTAgTTATAAACgTTCCgTgTg	Forward primer
OPV rpo18-R bio	Bio-TTATCATACgCATTACCATTTCgA	Reverse primer
OPV rpo18	F-ATCgCTAAATgATACAgTACCCgAATCTCTACT-q	5′ Nuclease probe
Bacillus anthracis rpoB gene, 175 bp		
BA rpoB F bio	Bio-CCACCAACAgTAgAAAATgCC	Forward primer
BArpoB R	AAATTTCACCAgTTTCTggATCT	Reverse primer
BA rpoB	F-ACTTgTgTCTCgTTTCTTCgATCCAAAgCg-q	5′ Nuclease probe
Yersinia pestis IS gene, 178 bp		
YP inv/IS F bio	Bio-TTATTTACCTgCACTCCCACAA	Forward primer
YP inv/IS R	CCAgACCgCAAACTgTTTTA	Reverse primer
YP inv/IS	F-TTCTgggCACAATAAAgCAACACgCT-q	5′ Nuclease probe
Francisella tularensis FopA gene, 101 bp		
FT 821F bio	Bio-TTgggCAAATCTAgCAggTCA	Forward primer
FT 921R	ATCTgTAgTCAACACTTgCTTgAACA	Reverse primer
FT FopA	F-AAgACCACCACCAACATCCCAAgCAT-q	5′ Nuclease probe

Bio, 5′-biotin modifier; F, 6-carboxyfluorescein attached to 5′-terminus (FAM); q, tetramethylrhodamine (TAMRA); bp, base pair.

versatile biointerface and the enzyme mediated electrical signal generation in a process called redox cycling (Niwa et al., 1990; Paeschke et al., 1995).

Herein the electrical microarray and the biosensor system are adapted for the recognition of PCR amplified gene segments of *Bacillus anthracis* (BA), *Yersinia pestis* (YP), *Francisella tularensis* (FT) and ortho pox viruses (OPV). These four pathogens are among the biowarfare agents of the highest threat potential (Klietmann and Ruoff, 2001). The PCR assays are modifications of original real-time PCRs and used as such for the identification of the microorganisms at the Robert Koch Institute, Berlin, Germany (Nitsche et al., 2004; Ellerbrok et al., 2002). Electrical microarray detection was carried out with uniplex PCR as well as with multiplex PCR and compared to results from real-time PCR. The C_T-values derived from real-time PCR assays are used for the determination of detection limits and to get semi-quantitative results from the described experiments.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA) was purchased from Carl Roth GmbH & Co. (Karlsruhe, Germany), ExtrAvidin® alkaline phosphatase conjugate (EX-AP), Polyoxyethylensorbitan monolaurate (Tween 20) and Tris–(hydroxymethyl)aminomethane (TRIZMA Base, Tris) were obtained from Sigma (Taufkirchen, Germany), 4-aminophenyl phosphate (*p*-APP) from Universal Sensor Inc. (Kinsale-Sandycove, Ireland). Ethylenediaminetetraacetic acid disodium (EDTA-Na_2), hydrochloric acid and all salts for buffers were purchased from VWR International (Darmstadt, Germany). PCR reagents, including buffers, magnesium chloride solution, dNTPs and Hotstart Taq Polymerase were from Invitrogen (Karlsruhe, Germany).

2.2. Oligonucleotides

Oligonucleotides have been selected to be specific for the sequences of the four different pathogens, respectively. The sequences of the different targets can be obtained from the National Center for Biotechnology Information (NCBI) database and were aligned with the program BioEdit V5.0.9. PCR primer and 5′ nuclease probes were obtained from TIB Molbiol (Berlin, Germany). The oligonucleotides used for real-time PCR and asymmetric PCR are given in Table 1. Electrical DNA microarrays, type M-II-Array, for the detection of the four PCR amplicons were obtained and can be ordered from AJ eBiochip GmbH (Itzehoe, Germany). For manufacturing, 5′-thiol-C6-modified oligonucleotides synthesised by Thermo Electron Corporation (Ulm, Germany) are used.

2.3. Electrical biochip

The chips were manufactured in an industrial semi-conductor production line on 6 inch silicon wafers as described earlier (Paeschke et al., 1995). The interdigitated gold array electrode structures were embedded in a silicon oxide layer, employing photolithographic techniques. They consist of two interlocking, comb-like structures each with 204 electrode fingers, 800 nm wide and with a 400 nm gap between anode and cathode fingers. The chip dimensions were 9 mm × 10 mm, carrying 16 electrode positions with a diameter of 500 μm each. Additionally, a counter and an internal iridium oxide reference electrode are integrated on the chip (Elsholz et al., 2006).

2.4. Instrumentation

For electrochemical detection the ePaTOX Array Analyser (AJ eBiochip GmbH, Itzehoe, Germany) was used in a fully automated mode. The core components of the instrument are the multichannel potentiostat, the electrical microarray and the fluidic system. Its components and the functional parameters are described in more detail in Elsholz et al., 2006. In principle the multichannel potentiostat consists of an analogue circuit, an interface between analogue and digital components and an element for processing of the digital data. The circuit consists of two measuring channels, which can be switched with the help of a special multiplexer component on up to 16-test electrodes. Thus the current measurement is realized during simultaneous potential supply at the electrodes. The use of a high resolution A/D transducer makes a resolution of the signal current of 5 pA as well as a measuring range of ±204.8 nA possible. The collection of the current signals during a measuring period generally takes place each 640 ms.

2.5. Microarray design and preparation

Four different capture probes designed for the specific detection of BA, OPV, YP and FT were printed on 12 electrodes for the four types of pathogens, each in triplicate, orthogonal to the flow direction.

Additional three positions were coated with a mix of biotin labelled and unlabelled oligonucleotide (PC = 5′-(Thiol-C6)-TTGTGGTGTTGGTGGTGTTGTTG-(Biotin)-3′ and 5′-(Thiol-C6)-TTGTGGTGTTGGTGGTGTTGTTG, 1:1000, v/v) as positive control (PC) and internal standard for normalising signals. The last free position is a negative control, consisting of the above non-biotinylated control oligonucleotide. For manufacturing 32 nL (80 drops, 400 pL ea.) of a solutions of 5′-thiol-modified oligonucleotides (100 μM, 25 mM Na_2HPO_4, pH 7.0) were dispensed on the gold electrodes using a piezoelectric nanoplotter NP 2.0 (GeSiM, Grosserkmannsdorf, Germany) and evaporated to dryness. The chip batch was brought into a humidity chamber (130 mM sodium chloride, pH 7.0) for uniform rehydrating, incubated for 3 h at ambient temperature and washed six times with 500 μL purified water. Chips were stored dry at ambient temperature under nitrogen and exclusion of light.

2.6. DNA preparation

DNA of the four pathogens BA, YP, FT and vaccinia virus (VACV) as a model for orthopoxviruses was prepared with a Qiagen kit according to standard procedures. The concentration was determined with a spectrophotometer. For quantitative comparison of real-time PCR and microarray detection, plasmids containing the target region of each PCR were cloned and quantified as described earlier (Nitsche et al., 1999). For plasmid construction, the corresponding sequence was amplified using the specific primers and inserted into a TA-Cloning Vector (Invitrogen), according to the manufacturer's instructions. After plasmid preparation, the DNA concentration was determined with a spectrophotometer at 260 nm, and the corresponding copy number was calculated. Serial dilutions of 10^1 to 10^7 plasmids were prepared in H_2O.

2.7. Quantitative real-time PCR

PCR was performed in an ABI Prism 7900 (Applied Biosystems Foster City, USA) in 96-well microtiter plates using a final reaction volume of 25 μL. Optimum reaction conditions were obtained with 2.5 μL 10× PCR buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), 4.0 mM $MgCl_2$, 1.0 mM dNTP, 0.5 U Platinum Taq DNA polymerase (Invitrogen, Karlsruhe, Germany), 200 nM specific sense primer (s), 200 nM specific antisense primer (as), 120 nM specific probe (TM) (Table 1) and 1 μM ROX (6-carboxy-X-rhodamine). Finally, 5 μL of template DNA were added to the reaction mixture. Amplifications were performed starting with a denaturation step (180 s) at 94 °C, followed by 45 cycles of denaturation at 94 °C for 15 s and combined primer annealing/extension at 60 °C for 30 s. Fluorescence increase of FAM was automatically measured during PCR. All samples were amplified in duplicate.

2.8. Uniplex and multiplex PCR for microarray detection

In general, the same PCR reactions were performed as for quantitative real-time PCR. Introduced modifications are the use of one biotinylated primer, used in fivefold excess compared to the non-biotinylated primer and a reaction volume of 50 μL. Briefly, a reaction contained 5.0 μL 10× PCR buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), 4.0 mM $MgCl_2$, 1.0 mM dNTP, 0.5 U Platinum Taq DNA polymerase (Invitrogen, Karlsruhe, Germany), 40 nM non-biotinylated primer, 200 nM biotinylated primer (Table 1) and 5 μL of template DNA and was amplified as described above. The multiplex PCR combined all primers of the uniplex PCR reactions (for 50 μL: 5.0 μL 10× PCR buffer, 4.0 mM $MgCl_2$, 1.5 mM dNTP, 1.5 U Platinum Taq DNA polymerase, 40 nM of each non-biotinylated primer and 200 nM of each biotinylated primer and 5 μL of template DNA). All samples were diluted with hybridisation buffer (4× SSPE: 0.72 M NaCl, 40 mM NaH_2PO_4 and 4 mM EDTA sodium salt, pH 7.7) to a volume of 300 μL.

2.9. Detection principle

The principle of detection is based on labelling of the nucleic acid target sequence during asymmetric PCR with one 5′-biotinylated primer, followed by hybridisation to the capture sequence. After binding of the ExAP conjugate to biotin (1:500 in TTBS (v/v), 30 mM Tris, 100 mM NaCl, 0.05% Tween 20, 1% BSA, pH 7.0), the enzyme initializes the hydrolysis of *p*-aminophenyl phosphate (1:1000 *p*-APP in TBS (w/v), 30 mM Tris, 100 mM NaCl, pH 8.0) to liberate the electroactive substrate *p*-aminophenol (*p*-AP) near the electrode surface for the electrochemical amplification process redox cycling. Further details were described earlier (Elsholz et al., 2006). For quantitative results, the current slope on each electrode is determined by linear regression during 10 s in the stop flow mode with an offset of 2 s. Total assay time was 27 min, including 15 min hybridisation, all other reactions and washing steps.

3. Results and discussion

3.1. Design and preparation of the microarray biointerface

High quality microarrays were achieved by noncontact dispensing 5′-thiol-modified capture oligonucleotide solution onto the 16 electrode positions using a piezoelectric nanoplotter with customised integrated image control.

Increasing the salt concentration during immobilisation enhanced the density of the capture oligonucleotide layer (Herne and Tarlov, 1997), but did not result in significant higher binding of target DNA (data not shown). The hybridisation efficiency was somewhat reduced (Southern et al., 1999). The use of additional alkan thiols can increase the accessibility to the immobilised capture probes and thus the hybridisation efficiency in a well organised mixed monolayer (Steel et al., 1998). However, during the development of our method mixed monolayers lead to reduced signal intensities, most likely due to blocking of effective conversion of *p*-AP on the electrode surface. Beside the density, the structure of the biointerface is also highly important for the sensitivity of the electrochemical detection. The 5′-end of the capture oligonucleotides was modified with thiol-hexyl-linker. To increase the hybridisation efficiency an additional spacer sequence of four nucleotides, non-complementary to the target sequences was introduced between alkylspacer and capture sequence. The binding site of the capture sequence and the biotinylated part of the target region were chosen to be as close together as possible. The later was an essential criterion for the underlying electrical detection principle, where the biotin mediated linkage of an enzyme complex generates an electroactive substrate most favourable in closest proximity to the electrode surface for effective redox cycling.

Introduction of the described parameter for the design of our microarray lead to higher sensitivity and to lower chip-to-chip-variations. The formed biointerface is schematically illustrated in Fig. 1.

The positive control (PC) on the electrical microarray generated signals with low standard deviations and was independent from hybridisation processes. As such, the PC guaranteed the functionality of each biochip in each analysis. A ratio of 1:1000 between the biotinylated oligonucleotide versus the non-biotinylated oligonucleotide of the same sequence with an overall concentration of 100 μM was found to give best results. For other arrays the height of the PC is well adjustable by varying the concentration in the mix of

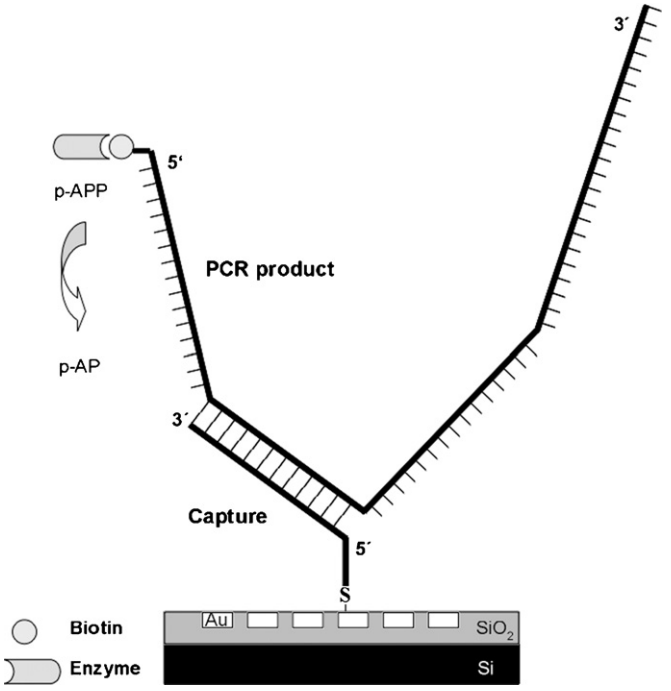

Fig. 1. Biointerface scheme, showing hybridisation of a target PCR product with immobilised capture oligonucleotide on the microarray and the detection principle with enzyme coupling via biotin and the catalysed substrate reaction of *p*-aminophenylphosphate (*p*-APP) into *p*-aminophenol (*p*-AP).

biotinylated and non-biotinylated species. The chip-to-chip variation in assay measurements was determined from the averaged electrical response from the PC positions with $n = 35$ chips (105-electrode signals) to a coefficient of variation of 15.4%. The average signal from the PC positions was used as internal standard and for normalisation of the absolute signals. The negative control positions resulted in very low electrical background signals of about 2 nA/min on average.

3.2. Detection of PCR products and data analysis

PCR protocols for electrical detection were slightly modified, compared to real-time assays. One PCR primer for each assay was 5′-biotinylated, the 5′ nuclease probe was left out. During PCR this allowed for a controlled way of label-introduction into the target sequence, required for position specific binding of the enzyme conjugate. An excess of single stranded target PCR product was generated by asymmetric PCR conditions, leading to highly improved hybridisation efficiency on the microarray surface. The data readout of the array analyser from the electrical microarray was carried out by online transfer of the measurement data points to the Origin® software for data analysis and visualisation. An original measurement is illustrated in detail in Fig. 2A and described below. Fig. 2A shows the amperometric response (current vs. time), monitored during the different phases of the substrate reaction.

Phase 1 in Fig. 2A is the start of the substrate reaction. Here, the substrate solution enters the flow cell and the enzyme mediated substrate conversion starts. In phase 2 the reaction during constant flow reaches an equilibrium, characterised by a final constant current corresponding to the surface enzyme concentration on each electrode. In the given example, due to high concentration of hybridised biotinylated PCR product (FT assay), the current is highest at approximately 20 nA. Via direct enzyme binding on PC positions an average current of 11 nA is produced. The current on negative positions varies in the range of 1–6 nA and is mainly determined by the individual density of the layers. These differences become much more significant in phase 3, where the substrate flow is stopped. Here, the position specific production of *p*-AP is kinetically determined without further influence by flow induced removal of formed *p*-AP and *p*-quinoneimine. The positive positions show a strong rise in current, whereas negative positions typically share a constant or a decrease in current within the first seconds.

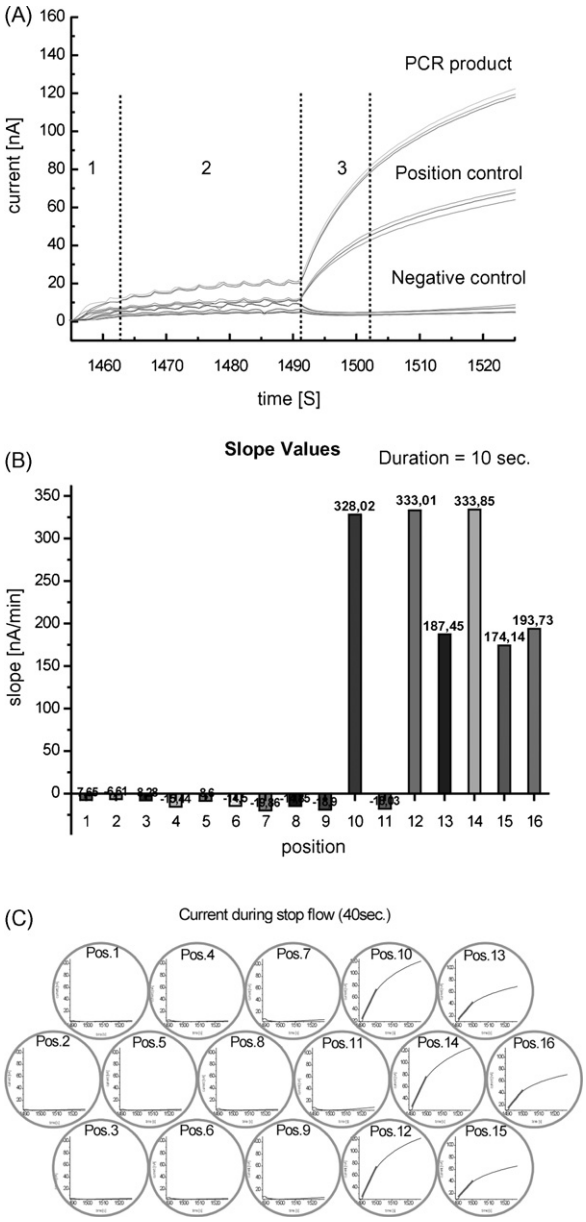

Fig. 2. Data presentation for automated measurements on electrical microarrays: (A) amperometric curves during enzymatic substrate conversion with begin of the substrate reaction (phase 1), equilibration at constant flow (phase 2) and substrate conversion in stop flow mode (phase 3), (B) diagram with current curves for the 16 individual electrode positions during stop flow mode and with linearised slopes and (C) bar chart of current slopes from (B) for each of the electrodes 1–16 (in nA/min).

Another visualisation of the results is given by the automatically generated graph shown in Fig. 2B. This column plot diagram is the standard evaluation of the data. The calculated current slope values (nA/min) are obtained from linear regression from all 16-current curves after an offset of 2 s and over a period of 10 s. The FT PCR product (position 10, 12 and 14) gave an average of 331.6 ± 2.6 nA/min (C_T-value of 26.8, approx. 1100 genome equivalents, ge), the PC

(position 13, 15 and 16) was 185.11 ± 7.31 nA/min. The quality of the coated microarrays is obvious through the very low coefficient of variation for intra-chip variation. In this case the intra-chip variation for the FT PCR product was determined to 0.7% and to 3.9% for the PC. The chip-to-chip variation for the FT PCR product was 13.1%.

The diagram in Fig. 2C gives a direct overview of all 16 positions in the microarray format as arranged on the biochip. It shows all single extracted current curves with bold lines, indicating the range used for linear regression. It simplifies the comparison of different positions and allows visual inspection to determine whether linear regression parameters are set correctly.

Typical results for the analysis of the four different uniplex PCR products, covering a broad range of target amounts are shown in Fig. 3.

The column plots represent relative signal intensities as the average slope of equal target positions from three different chips (9 electrode signals), normalised to the slope average of the PC positions on each microarray. PCR-experiments were made in parallel with identical template dilution series for the microarray and for corresponding real-time PCR assays. All four assays with electrical microarray detection gave results, very consistent with those from real-time PCR over a broad range of template concentrations or C_T-values (threshold cycle). For uniplex reactions, typically C_T-values of 37 (approx. 10–50 ge, OPV assay in Fig. 3D) related to normalised values of 15.2 ± 2.1 rel. slope [%] and positive current curves, well distinguishable from negative positions with a hybridisation time of 15 min and a PCR volume of 50 μL diluted in 250 μL hybridisation buffer. All four PCR assays showed similar signal intensities on the microarray at a comparable template concentration with the exception of the PCR for FT detection, which gave consistently higher signals (Fig. 3). At a C_T-value of 33 (142.5 ± 13.7 rel. slope [%], 20 ge, Fig. 3A) the average signal is significantly higher than in all other assays, although the comparison to C_T-value for the YP with C_T = 31 (48.4 ± 5.3 rel. slope [%], 200 ge, Fig. 3C) and for BA with C_T = 21 (72.2 ± 6.8 rel. slope [%], 10^6 ge, Fig. 3B), indicated higher template concentrations. The reasons for the high sensitivity for FT can be found in the exceptional well performing PCR assay and the small amplicon size (101 bp).

3.3. Sensitivity and linear range

The sensitivity of the microarray was investigated starting with DNA plasmid samples over a broad range of template concentrations or C_T-values. Exemplary, results for the BA PCR are further described in the following and shown in Fig. 4A with the characteristic diagram profile. The electrical measurements were carried out as end-point detection, with PCR products applied to the microarray after completion of thermocycling (40 cycles, 45 min).

Generally, high template concentrations or low C_T-values in real-time PCR lead to high slope values on the microarray and with decreasing template concentration or increasing C_T-values the signal decreased as well. The decrease in absolute signal (nA/min) with the decrease in template concentration (C_T-values) was found to be not linear. Between C_T-values of 21 and 31 the average signal is only lowered to approx. 80% of the maximum value. Down to a C_T-value of 37 the average signal is still strong but lowered to 30% of the signal at a C_T-value of 21. The non-linear relation is in conformance with typical yields from PCRs carried out with template dilution series, which reach a product plateau phase towards the end-point of the PCR reaction. The electrical microarray showed to be sensitive enough to analyse very low PCR product yields or reactions that amplified as low as 10–50 ge per reaction (BA, C_T = 37, 74.32+/+9.6 nA/min). The other datapoints in the sensitivity range were determined to 1050 ge (C_T = 31.4, 183.91 ± 25.2 nA/min);

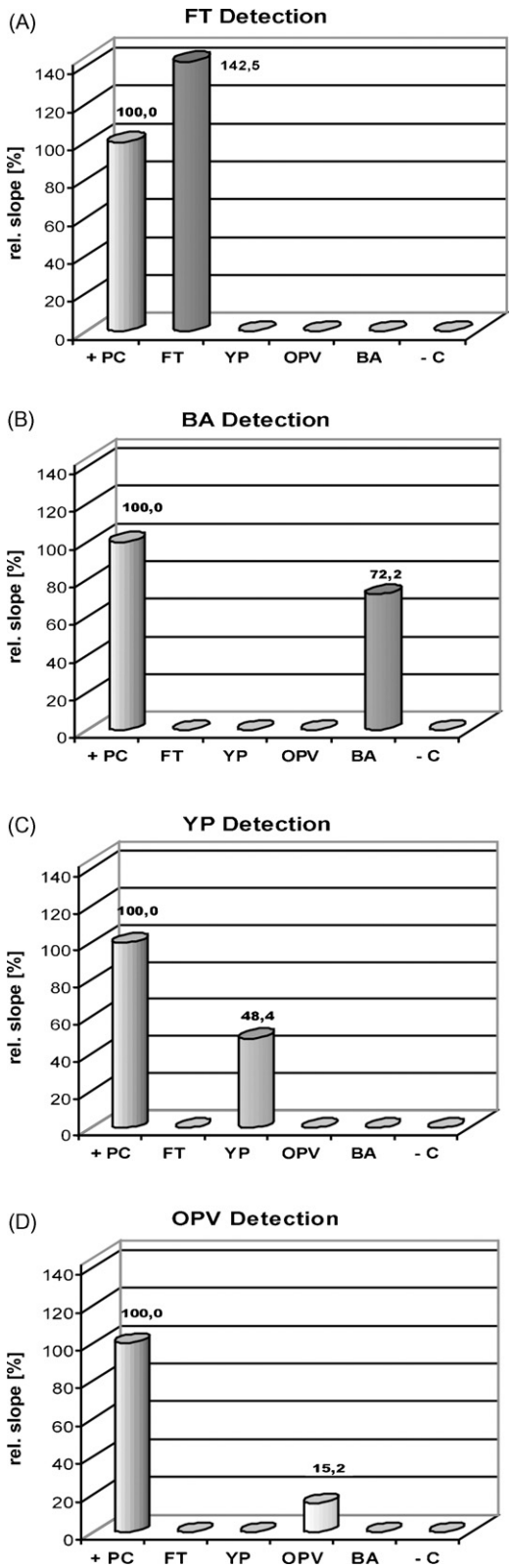

Fig. 3. Normalised, average current responses in electrical microarray detection with uniplex PCR assays. Template amounts correspond to C_T-values of parallel real-time PCR. (A) *Francisella tularensis* (FT): C_T = 33; electrical detection: 142.5 nA/min, (B) *Bacillus anthracis* (BA): C_T = 21; electrical detection: 72.2 nA/min, (C) YP (*Yersinia pestis*): C_T = 31; electrical detection: 48.4 nA/min and (D) OPV (orthopoxviruses, *Variola* spp.): C_T = 37; electrical detection: 15.2 nA/min.

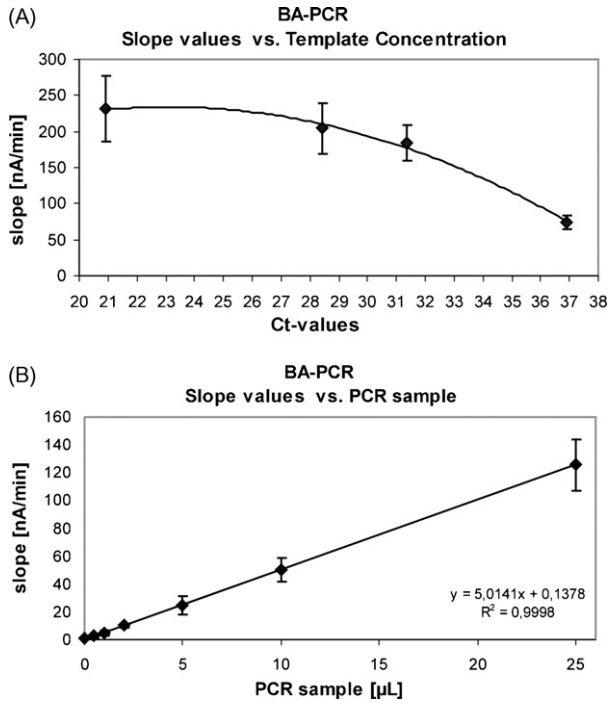

Fig. 4. Sensitivity of the electrical detection method for PCR products with template DNA from *B. anthracis* (BA). (A) Plot of average current response (slope values in nA/min) with decreasing template concentration vs. C_T-values from real-time PCR and (B) plot of slope values (in nA/min) vs. amount of PCR sample volume (in μL).

9000 ge (C_T = 28.5, 204.28 ± 35.0 nA/min) and for the highest concentration 10^6 ge (C_T = 21, 231.1 ± 45.4 nA/min).

We also clearly detected OPV-PCR products from reactions started with approximately 10–50 gene equivalents corresponding to C_T-values of 37 as shown in Fig. 3D and YP-PCR products were analysed down to a C_T-value of 35, FT-PCR products down to a C_T-value of 37 (data not shown). With the investigated high and low concentrated PCR products for all four assays the enzyme mediated electrical detection method is highly selective and virtually background free. The method is in this aspect superior to typical optical detection on microarrays. Fluorescent microarray images often require complex analysis tools for background corrections (Tran et al., 2002) and background signals are highly dependent on immobilisation chemistries and blocking strategies (Lindroos et al., 2001; Taylor et al., 2003).

Fig. 4B shows the linear response of the electrical biosensor for the measurement of the current slopes corresponding to different PCR product concentrations. The concentration of the sample was varied by mixing different volumes from a pooled BA PCR product with a corresponding C_T-value of 24 (approx. 2×10^5 ge) with hybridisation buffer to give an end volume of 300 μL for each measurement. The PCR volume, added to the sample for microarray detection, was varied between 0.5 and 25 μL. The error bars indicate the standard deviation. The data points are obtained from n = 3 chips with 9 electrode positions. The calculated linear coefficient of regression is R^2 = 0.9998.

3.4. Analysis of multiplex PCR products

The full advantage of the electrical low-density microarray can be achieved when its use is combined with multiplex PCR. In contrast to the electrical microarray, the detection of the four pathogens from unknown samples by one multiplexed real-time PCR would require four differently labelled detection probes or in general a complex labelling strategy with dye labels, showing fluorescence at four different wavelengths. Naturally, this problem increases with the number of targets which need to be detected. The design of multiplex real-time PCR, especially aiming for higher degrees of multiplexing, will usually be complex, less efficient or even hindered due to interaction between detector probes and PCR primers. In contrast to that, the electrical microarray allowed the direct target identification in unknown samples. In this regard, using detection at the end-point of PCR after 45 cycles is advantageous because the capture probes are separated and cannot influence the performance of the PCR. The susceptibility to errors and to variability from sample preparation and PCR samples is substantially lower, when multiplex instead of parallel uniplex PCR is used.

For this purpose a mix of all 4 PCR primer pairs was used and tested with each of the different target DNAs. As one additional adjustment the amount of Taq-DNA polymerase was increased, due to the higher amount of primer in the reaction mix. First experiments were made using real-time PCR assays with the same FAM/TAMRA detector probes (Table 1) used before. For comparison, the multiplex reactions were carried out in parallel with uniplex PCRs. No substantial differences in sensitivity were observed when C_T values were compared. For analysis on the electrical microarrays, multiplex PCR reactions were carried out as real-time PCR assays but leaving out the detection probes. The resulting PCR products allowed for clear specific identification of the four different pathogens without background on other positions of the microarray. The samples included each pathogen in two different concentrations as well as two negative control samples (Table 2).

4. Conclusion

In this study we developed a multiplex PCR based electrical oligonucleotide microarray for the simultaneous identification of

Table 2
Analysis of 10 samples with slope values (nA/min) for electrochemical microarray detection with multiplex PCR in comparison with C_T-values and genome equivalents from uniplex real-time PCR.

Sample	1	2	3	4	5	6	7	8	9	10
Electrical microarray detection (nA/min), n = 3										
OPV	–	–	–	128.0 ± 21.6	–	–	–	6.7 ± 0.4	–	–
BA	–	570.6 ± 44.8	–	–	–	–	–	–	–	520.1 ± 8.4
YP	–	–	–	–	283.1 ± 42.5	–	233.8 ± 9.9	–	–	–
FT	–	–	470.9 ± 10.1	–	–	–	–	–	331.6 ± 3.2	–
Real-time PCR detection (C_T-values and genome equivalents)										
C_T-OPV	–	–	–	29.1–3300	–	–	–	34.8–80	–	
C_T-BA	–	23.3–2×10^5	–	–	–	–	–	–	–	29.6–4000
C_T-YP	–	–	–	–	25.8–1×10^4	–	29.6–500	–	–	
C_T-FT	–	–	24.4–2×10^5	–	–	–	–	–	26.8–1×10^4	–
C_T-nc	45	–	–	–	–	45	–	–	–	–

the four pathogenic microorganisms *Bacillus anthracis* (BA), *Yersinia pestis* (YP), *Francisella tularensis* (FT) and ortho pox viruses (OPV) with uniplex and multiplex PCR samples. The fully automated analysis could be carried out in 27 min. Latest results in our group make total analysis times of less than 8 min without compromising the sensitivity possible. Multiplex PCR detection has been established without differences in signal intensity in comparison to uniplex formats. The measurement data was directly correlated to the C_T-values of real-time PCR. The sensitivity of the electrical detection method was comparable to real-time PCR. PCR products, generated from 10 to 50 gene equivalents, have been detected reproducible. The experiments showed that it is very efficient to transfer and combine real-time PCR-assays to multiplex format for simultaneous analysis on the electrical microarray. The device and method for electrical microarray detection demonstrated to be a robust and simple to use technique with clear data interpretation for on-site or point-of-care detection in molecular diagnostics.

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