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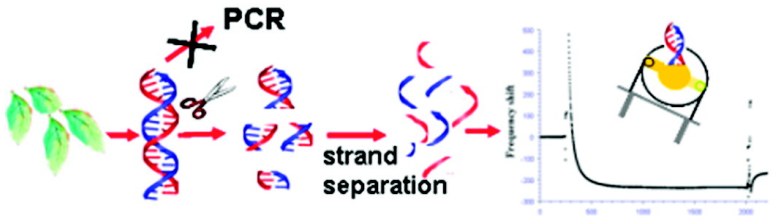

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Detection of Fragmented Genomic DNA by PCR-Free Piezoelectric Sensing Using a Denaturation Approach

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A great effort has been recently devoted to the development of new devices for the detection of specific sequences of DNA, due to the increasing need of fast, cheap, and miniaturized analytical systems able to detect target sequences for screening purposes. In particular, polymerase chain reaction (PCR) is widely used to amplify target DNA fragments, but it represents an additional step in sample pretreatment and is still time consuming and requires skilled personnel to be performed; PCR also requires expensive reagents and is prone to false positive/negative results. Development of well-automated and miniaturized gene analysis methods is the objective at which research is currently aiming. Recent works report examples of miniaturized PCR devices[1] pointing at high-speed PCR. However, a great improvement in DNA sequence analysis would come by direct detection in nonamplified genomic DNA. Biosensors represent an interesting candidate for DNA detection. In particular, several DNA-based sensors have been reported.[2] Most of the work was applied to PCR-amplified samples, and only few works, operating directly with genomic DNA, appeared in the literature with different detection principles.[3−6]

In this paper, a piezoelectric sensor for direct detection of sequences in nonamplified genomic DNA is reported. Recently, we have demonstrated the ability of a piezoelectric sensor to detect highly repeated DNA sequences (satellite 13) in nonamplified bovine DNA.[6] The successful application of this transduction principle to the PCR-free detection of highly repeated sequences was a starting point to go further toward the detection of target DNA present in single copy per genome. The system relies on real-time and label-free detection of the hybridization reaction between an immobilized probe (25-mer) and the complementary sequence in solution. The DNA probe is immobilized on the sensing surface (gold electrodes of 10 MHz quartz crystals) following the procedure reported in Mannelli et al.[2] The hybridization with the target (10 min for oligonucleotides and 20 min for PCR products and genomic DNA) was performed by adding 100 μL of the sample solution to the cell well. The difference between the value of the frequency when the crystal is in contact with buffer after and before the hybridization is the analytical datum. In all of the experiments, the single-stranded probe was regenerated by two consecutive treatments of 30 s with 1 mM HCl, allowing further use of the crystal. All of the experiments were performed at room temperature.

Genomic DNA was extracted from the plant, *Nicotiana glauca*, used as a model system. The target sequence was a portion of the promoter region (35S), a part of the gene cassette introduced as foreign DNA into the genetically modified plant. This sequence is present in various genetically modified organisms (GMOs) and can be used as a marker for GMO screening.[7] The ability of biosensors to detect 35S in PCR-amplified DNA samples using different transducers has already been reported in the literature.[2,8−10] To achieve sequence detection of 35S directly in nonamplified DNA, different denaturation approaches were studied here, first applied to oligonucleotides and PCR-amplified DNA, and then transferred to genomic DNA.

PCR was conducted according to the EU protocol for 35S amplification (243 bp),[11] while genomic DNA was extracted from the leaves of *Nicotiana glauca*. The wild type (WT) and the genetically modified (GR4) plants were both used; they have the same genome except for the introduced gene cassette carrying the target sequence 35S. The 35S sequence of interest is present in single copy in the whole GR4 plant genome, while it is absent in the WT, which was used as the negative control. Genomic DNA consists of long chains of double-stranded DNA (dsDNA), but to achieve surface hybridization, it should be available for hybridization as single-stranded DNA (ssDNA). When dealing with PCR-amplified samples, a simple thermal treatment is sufficient to allow a significant analytical signal. On the contrary, when nonamplified genomic DNA is analyzed, the thermal treatment is not enough and alternative strategies are required.[12] For this reason, different denaturation methods have been studied.

Three approaches were tested: thermal (95 °C for 5 min, cooling in ice for 1 min), chemical[9] (20% formamide, 0.3 M NaOH at 42 °C for 30 min), and thermal combined with blocking oligonucleotides.[6] This denaturation procedure combines two effects. At first, the DNA is dissociated by keeping it at a very high temperature, then its re-association is prevented by creating steric hindrance. The steric hindrance is accomplished by the bond of two oligonucleotides, one to the strand containing the target sequence and the other to the other strand. The blocking sequences stick close to the target/probe sequences but do not overlap them so that the target is free to hybridize to the immobilized probe (short), but it cannot bind the digested fragment (long). The denaturation treatments were also applied to ssDNA (oligonucleotides) to study any possible interference with the sensor and were then applied to PCR-amplified samples.

The complementary 35S oligonucleotide (25-mer, 1 ppm), a noncomplementary oligonucleotide (25-mer, 8 ppm), the 35S PCR fragment (243 bp), a PCR negative control (180 bp), and PCR blanks were analyzed after the three denaturation procedures (Figure 1). The effect of the chemical denaturation on oligonucleotides resulted in an increase (46%) of the signal if compared with the one obtained with the thermal treatment. This increase could be due to a nonspecific effect since it is also observed with the noncomplementary oligonucleotide. On the contrary, the thermal and blocking oligonucleotides' treatment did not affect the hybridization signal. The same findings were obtained with the same treatments applied to PCR fragments and PCR negative controls. The same hybridization signal was obtained after treating the 35S

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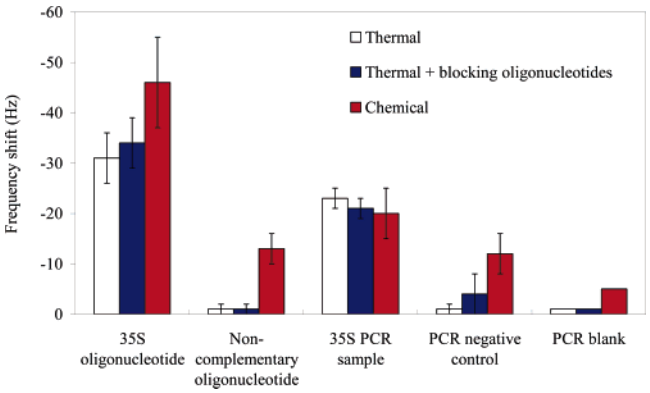

Figure 1. Effect of the denaturation treatment on oligonucleotides and PCR-amplified samples. The samples analyzed were the complementary oligonucleotide, 35S, the noncomplementary oligonucleotide, the 35S PCR fragment (54 ppm), the PCR negative control (35 ppm), and the PCR blank ($n = 3$).

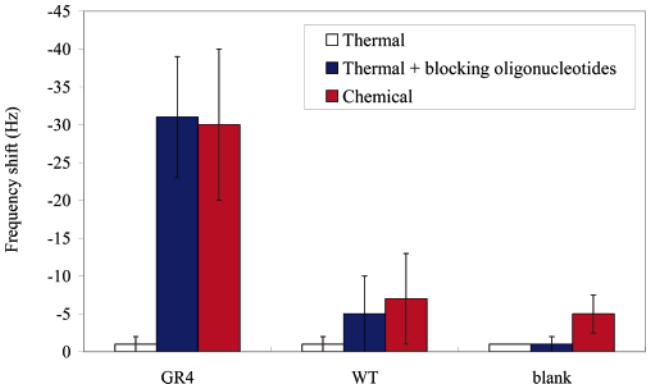

Figure 2. Experiments with digested genomic DNA (10 ppm), transgenic GR4 DNA ($n = 6$), nontransgenic WT DNA ($n = 6$), and blanks ($n = 3$).

samples with the three denaturation methods, but the reproducibility (expressed as coefficient of variation, CV%) ($n = 3$) was quite different (9% for the thermal one, 12% for the thermal and blocking oligonucleotides one, and 25% for the chemical one). The same denaturation treatments applied to the negative controls gave less homogeneous results since a high signal was observed in the case of the chemical treatment, while negligible results were found in the cases of thermal plus blocking and thermal treatments.

After the initial evaluation of the sample pretreatments on the sensor behavior, these were finally applied to more complex, nonamplified genomic DNA. This experiment was conducted to reach the final aim of achieving PCR-free target sequence detection.

Nonamplified genomic DNA consists of long chains of DNA difficult to dissociate and to hybridize with a complementary probe immobilized on the sensor.[12] To achieve this hybridization, the above-mentioned denaturation methods were finally applied to genomic DNA, previously fragmented by enzymatic digestion by restriction enzymes (BaMH 1) to facilitate their dissociation. To ensure that the fragmentation does not affect the ability of the target sequence to hybridize to the immobilized probe, it was verified that the consensus sequence recognized by the enzyme was not present inside the target DNA sequence. The length of the fragments containing the target sequence, complementary to the immobilized probe, was 872 bp. All of the denaturation procedures were applied to 10 ppm of sample. The results are reported in Figure 2. A significant signal was obtained by samples treated with thermal plus blocking oligonucleotides and chemical denaturation. The thermal treatment alone did not result in a measurable signal. A better reproducibility was achieved with the thermal plus oligonucleotide procedure if compared to the chemical one. It should be noted that the lifetime of the sensor was dependent on the sample denaturation treatment. With chemical denaturation, the surface could be regenerated just five times, while with the thermal plus blocking oligonucleotides procedure, the sensor surface could be regenerated up to 13 times before losing sensitivity and specificity (data not shown). The system was able to distinguish the positive sample (GR4) from the negative control (WT), except when the thermal treatment alone was applied. Moreover, the best results were found when the thermal plus blocking oligonucleotides' treatment was applied, as demonstrated by an evaluation of the reproducibility and the lower nonspecific effect when testing the negative controls, as confirmed by the findings observed with ssDNA and PCR-amplified DNA.

This work demonstrated that it is possible to detect the target sequence directly in nonamplified genomic DNA, even considering the low concentration of the target in the sample (4×10^5 copies in 10 ppm of sample). PCR-amplified DNA (243 bp) represents an enriched sample where the target sequence is present in a very high number of copies (4×10^{11} copies). To explain the detection of such a low number of copies of target DNA, additional contributions (i.e., viscoelastic effects) to the biosensor signal other than mass loading may be taken into account. Moreover, it must be considered that the signals due to oligonucleotides, to the PCR samples, and to the genomic digested DNA on the surface cannot be compared due to the very different matrix complexity of all these samples and to the different secondary structure once the DNA is hybridized to the probe.

The real-time and label-free DNA sequence detection in nonamplified DNA, as reported here, represents an important improvement in DNA analysis. Since the specificity of the system relies on the probe immobilized on the surface, the applicability of direct genomic sensing is wide, from environmental to food and clinical analysis.

Supporting Information Available: Details on instrumentation and procedures. This material is available free of charge via Internet at http://pubs.acs.org.

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