Bioimpedance Spectroscopy as a potential technique to detect label-free PCR products

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Abstract. PCR is a molecular technique that multiplies DNA fragments in a logarithmic way. qPCR uses fluoroscopic dyes or probes to quantify amplicons but it is a complex and expensive technique that should be performed by highly trained personnel. PCR has been used in a wide variety of disciplines such as in food sciences, organ transplant, odontology, oncology and lately, as the standard diagnostic technique for COVID-19. Even when qPCR is a reliable and robust technique, it is hardly accessible for developing countries for its complex labelling procedures and expensive instrumentation, for that, it is of big relevance to search for simpler and cheaper alternative technologies for the detection and analysis of DNA. In this work, we explore the feasibility of using multifrequency bioimpedance measurements to detect label-free PCR products as a proof of principle for the future development of a gene biosensor on the basis of PCR and bioimpedance measurements.

1. Introduction
Polymerase Chain Reaction (PCR) is a molecular technique that multiplies Deoxyribonucleic Acid (DNA) fragments in a logarithmic way, resulting in billions of copies of a specific DNA fragment [1]. PCR became an important method of DNA analysis and detection and it has been widely used in medicine in the last decades [2]. PCR uses the enzyme DNA Polymerase to replicate a specific DNA fragment delimited by the forward and reverse primers. Polymerase uses single-base nucleotides as building blocks for the DNA replication in a temperature-controlled manner consisting of three stages: denaturation, annealing and extension. These three stages make up for one cycle that theoretically doubles the quantity of the target DNA fragments; a PCR assay is completed when around 30 thermocycles have been performed. The amplified PCR products are conventionally detected by a technique called agarose gel electrophoresis, in which amplicons are separated by molecular weight and then observed by ultraviolet illumination. This technique is relatively simple, but it lacks sensitivity and should be performed by trained personnel [3]. Conventional PCR has been replaced by real-time quantitative PCR (qPCR), a type of PCR that introduces fluoroscopic dyes or probes to indirectly...
measure DNA amplification; this technique even allows the estimation of the initial amount of template DNA fragments [2]. Even when this technique is robust and efficient, this type of PCR relies on sophisticated, complex and expensive labeling reagents, procedures and technology [3, 4], which hinders the introduction of this technique in developing countries.

Medicine has benefited from PCR since its discovery in 1985 in a variety of applications. PCR has been used for the identification of compatibility in organ transplants [5] replacing the blood type method; in odontology for the study of periodontic diseases, dental caries, and even oral cancer [6]; in oncology for the diagnosis of different cancer types [7], cancer staging [8], the prognosis of remission and disease progression [9, 10] and even for the individualization of the treatment based on the molecular profile of patients [5, 9]; and lately, PCR has become the gold standard diagnostic technique for the recent COVID-19 global pandemic [11, 12].

Driven by the fact that qPCR is fast, efficient, accurate, and a relatively simple technique, we believe that the disadvantages that it faces rely directly on the functional principle of the detection of genetic amplification, which adds to the reaction fluoroscopic reagents thus expensive and complex fluoroscopic instrumentation is needed. So, instead of seeking for a completely different technique, we think it is pertinent to seek a simpler, but not weaker, way of detecting the amplicon products of PCR. Electrochemical detection of PCR products can be a great choice for its intrinsic characteristics such as possible miniaturization, ease of use, inexpensive instrumentation [13] and its capability to detect DNA’s differences in concentration [14, 15] and fragment length [16] in a label-free manner. Bioimpedance capabilities to distinguish PCR products have been already stated in microfluidic devices using an electroactive intercalator [17]. In this study, we explore the feasibility of using bioimpedance measurements to detect label-free PCR products in a macroscopic scale as a proof of principle for the future development of a gene biosensor based on PCR and bioimpedance measurements.

2. Methods

2.1. Experimental design

In order to evaluate the bioimpedance’s capacity to differentiate between a PCR product sample with amplified DNA from one without amplified DNA, we developed a simple test using two samples: a negative control and a positive control. Both samples consisted of the same mixture of reagents with one crucial difference: the negative control was not added with template DNA, instead, milli-Q water was added, while in the positive control template DNA was added. Both samples went through a PCR thermocycling assay of 30 cycles. Bioimpedance measurements were made prior to and after the thermocycling to study the changes in bioimpedance caused by DNA amplification by DNA Polymerase.

2.2. PCR protocol

Two PCR samples were prepared for conventional end-point PCR. For the positive control, the PCR sample was prepared using 12.5µl of PCR master mix (ThermoFisher Scientific, United States) containing 0.05 U/µl of the enzyme Taq Polymerase, 0.4 mM of each dNTP, 4 mM of MgCl2, and the reaction buffer; 2.5 µl of each primer mix, reverse and forward, designed for human beta globin gene; 5 µl of template DNA and 2.5 µl of Milli-Q water to sum 25 µl of total sample volume. For the negative control, the PCR mix was exactly the same but with one crucial difference: instead of adding 5 µl of template DNA, we added 5 µl of milli-Q water to avoid unspecific amplification, having a final total volume of 25 µl as well. The temperature profile of the PCR assay is described in Table 1.

Agarose gel electrophoresis was performed to the PCR products to validate DNA amplification in the positive case and the lack of it in the negative case.

2.3 Bioimpedance Measurements.

Typical 0.2 ml PCR tubes (Applied Biosystems, United States) were used as sample containers, the lid was adapted with two silver cylinders as electrodes. Electrical Bioimpedance Spectroscopy (EBiS) measurements were made using the ScioSpec ISX-3 system (Sciospec Scientific Instruments Inc, Germany) in bipolar configuration at a frequency range from 100 Hz to 10MHz in 128 logarithmically spaced steps and an amplitude of 250 mV.
6 µl of sample were extracted, placed in a new PCR tube with the adapted lid and centrifugated at 10000 RPM for 30 seconds, then, EBiS measurements were acquired. The same process was carried out for an initial condition, prior to the thermocycling, as well as in a final condition, after the thermocycling process.

| Thermocycling stage | Temperature (°C) | Time (seconds) |
|---------------------|------------------|----------------|
| Initial denaturation | 95               | 300            |
| Denaturation         | 95               | 30             |
| Annealing           | 62               | 30             |
| Elongation          | 72               | 30             |
| Final elongation    | 72               | 120            |

3. Results

3.1. Bioimpedance measurements

Figure 1 and 2 shows the magnitude and phase spectrum of the negative and positive case, respectively. Two curves are shown in each graph corresponding to the initial (prior to thermocycling) and final (after thermocycling) condition. The final condition shows lower impedance magnitude and a higher phase than the initial condition for the negative case; while in the positive case, the magnitude of the final condition is higher than the initial condition and the phase is lower than the initial condition which appears to be due to DNA amplification. In both cases, the bandwidth where the differences are more evident are from 1kHz to 1MHz for the magnitude spectrums and from 100 Hz to 10 kHz in the phase spectrum for both cases. In the frequencies greater than 4.5 MHz the measurements show unexpected behaviour (positive phase) so those results were not analyzed nor shown.

Additionally, to compare the negative and the positive case, the difference between the final and the initial conditions of bioimpedance magnitudes were calculated. Figure 3 shows the negative and positive case differences in magnitude between the final and initial conditions.

![Figure 1](image1.png)

**Figure 1.** Magnitude and Phase of the negative case. Two curves are shown corresponding to the initial (blue) and final (orange) condition.

3.2. Agarose gel electrophoresis

Figure 4 shows the agarose gel electrophoresis results. Band 1 corresponds to the molecular weight marker and the bands 2 and 3 are the negative and positive cases, respectively. The electrophoresis assay
shows that there was no amplification for the negative case and for the positive case amplification of a 300-base pair DNA fragment appears, consequent with the β human globin gene length.

Figure 2. Magnitude and Phase of the positive case. Two curves are shown corresponding to the initial (blue) and final (orange) condition.

Figure 3. Magnitude differences between the final and initial condition of the positive and negative case.

Figure 4. Agarose gel electrophoresis of both cases negative and positive. Band 1 is the molecular weight reference and bands 2 and 3 corresponds to the negative and positive cases, respectively.

4. Discussion
Results from the agarose gel electrophoresis show expected behaviour: in the negative case (band number 2), no amplification is detected in the range of the molecular weight reference which goes from 100 to 1000 base pairs, while in the positive case (band number 3) the amplification of a DNA fragment consequent to the target gene length is observed. In band 1, corresponding to the negative case, a small background signal was observed below the 100 base-pair reference, but not visible in the digital image, suggesting that there was little unspecific amplification caused potentially by the formation of primer-dimer complex, a phenomenon observed in the initial cycles of a PCR and in PCR mixes without template DNA, as described in [18]. This could explain that the final impedance of the negative case was lower than the initial measurement because primer-dimer complexes are of a small fragment length, and since the charge/mass of the DNA double helix is uniform [19], the DNA negatively-charged molecules’ movement caused by the electric field could be similar in these greater charges of the primer-
dimer complex to that of the free smaller charges, yielding in a better conductive sample. On the other hand, a greater phase in the final condition than in the initial condition could be also explained by the same phenomenon, but the electric effects that this causes in a solution are not fully understood, in that sense, more experiments with PCR sample lacking template DNA are needed to fully understand the effect of primer-dimer complex formation in the electrical properties of the solution. Also, in this study no proper detection of the primer-dimer formation was carried out; one technique to detect this phenomenon could be High-Performance Liquid Chromatography. Either way, free molecules, such as dNTPs and MgCl₂, in the solution are more susceptible to polarization since they are free to respond to the force caused by the electric field, so the charges are free to migrate to its complementary pole of the electric field applied.

For the positive case, the electrophoresis confirmed that there was the amplification that the assay was intended for, we can observe an evident amplification of products of approximately 300 base pairs of length in band number 3, which is consequent to the target gene length of the β human globin. The expected amplification could explain that the final magnitude spectrum is lower than the initial measurement because, in a PCR mix, MgCl₂ is added to the solution as Mg²⁺ serves as a polymerase enzyme cofactor. When the amplification is effective, the negative charges of the DNA’s phosphate backbone attract the positive charges of the Mg²⁺ dissolved in the sample, neutralizing the charges and leaving less free ions in the solution, so the sample is less conductive. This phenomenon occurs in the low (kHz range) and intermediate (MHz range) frequencies [20], consequent to the frequency range used in this study. In the phase spectrum, it was observed that the phase shift is lower in the final condition than in the initial measurement. This could be explained by the fact that one of dNTP’s most important physical properties is its dipole moment [21], a property that changes when the dNTP is paired in a DNA double helix by hydrogen bonds, possibly increasing the energy needed to polarize the molecule. Also, the counterion condensation on the DNA backbone could be weakening the dipole moment of the DNA double helix, diminishing the molecule’s capacity to polarize. Both effects could be causing less capacitive effects in the macroscopic electrical properties of the sample.

Figure 3 shows a clear difference between the electrical behaviour of the positive and negative cases: in the positive case, the difference between the final and initial condition resulted positive in the whole spectrum, while in the negative case, such difference is negative. We believe that this distinction could serve as the classification criteria in blind samples: if the difference between the final and initial magnitude is positive, the sample contains the target gene for which the assay was designed; on the other hand, if the difference is negative, the sample does not contain the target gene. This observation, even when supported with literature, should be confirmed in a study with a greater sample size and with negative controls with actual template DNA but designed to avoid amplification, since the negative control used in this study lacked template DNA. As a first approach, we believe our results suggest a clear distinction between a sample with amplified DNA than one without amplified DNA, even when the lack of amplification was originated from a lack of template DNA.

5. Conclusions
For developing countries, it is of big importance to find more accessible technologies for DNA analysis because of its big impact in science and healthcare. Multifrequency bioimpedance measurements showed potential value as an emerging technique to detect label-free PCR product. Bioimpedance spectra show different signatures to differentiate PCR products with amplified DNA from those without amplified DNA. Further studies to confirm the observations with greater sample sizes are needed to fully understand the electrical behaviour of different PCR scenarios and to assert a reliable descriptive explanation of the electrical changes caused by genetic amplification.

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