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Article

QCM Genosensor for Detection of Golden Mosaic-Resistant Transgenic Common Beans in Non-Amplified Samples

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Abstract

A quartz crystal microbalance-based biosensor for the specific detection of the first transgenic common bean (*Phaseolus vulgaris* L.) cultivar (BRS FC401 RMD) with resistance to *bean golden mosaic virus* (BGMV) was developed. The immobilization chemistry relies on the strong bond between the thiolated probe and the gold electrode surface. The probe sequence is internal to a region of the BGMV *rep* gene that was introduced into the common bean genome. The sensor's analytical performance was determined using synthetic oligonucleotides. Real samples of transgenic and wild-type bean seeds were also tested. Sample pretreatment consisted only of enzymatic fragmentation, followed by a thermal denaturation step combined with blocking oligonucleotides. Different biosensor regeneration approaches were studied. Immobilization showed good reproducibility (CV% of 5.8%). The biosensor proved specific for both synthetic oligonucleotides and non-amplified genomic DNA. A linear detection range of 0–1.4 ng/μL was observed, with a detection limit of 0.18 ng/μL. Three sequential detections were performed without loss of surface activity. The results demonstrate the biosensor's potential for direct, real-time, label-free detection of DNA samples for field screening of transgenic common bean cultivars.

Keywords: *bean golden mosaic virus*; biosensor; common bean; genetically modified organism; immobilization; quartz crystal microbalance; regeneration

1. Introduction

Brazil is among the world's leading common bean producers. BRS FC401 RMD is the first genetically modified common bean (*Phaseolus vulgaris* L.) cultivar developed in the world and the only one registered and protected in Brazil as effectively resistant (immune) to golden mosaic [1]. Caused by *Bean golden mosaic virus* (BGMV), symptoms of golden mosaic include leaf yellowing, plant stunting, pod and grain deformation, and flower abortion. That is the main bean viral disease in Brazil, responsible for production losses ranging from 40% to 100% [2].

The transgenic cultivar possesses an intron-hairpin gene construct containing 411 base pairs of the BGMV *rep* gene (GenBank Accession NC 004042, position 1836–2247), positioned in sense and antisense, aiming to express an RNA that will form a hairpin structure, with double-stranded regions. The cellular machinery converts this double-stranded RNA (dsRNA) into small interfering RNAs (siRNA). These small RNAs trigger the degradation of the viral messenger RNA (mRNA) specific to the *rep* gene, a process known as RNA interference. Since the *rep* gene is essential for virus replication, its silencing leads to plants that are immune to golden mosaic virus [2,3].

BRS FC401 RMD is phenotypically identical to non-transgenic cultivars, being morphologically indistinguishable. Therefore, several safety concerns have been erroneously raised about this cultivar, including suspicions of loss of resistance, contamination with non-transgenic cultivars, or the emergence of other viruses transmitted by the same vector [4]. Furthermore, to comply with biosafety and food safety regulations in countries that adopt mandatory regulations (European Union (EU), Japan, Australia, New Zealand, Thailand, China and Brazil), transgenic cultivars must be traceable at all stages of the production chain, from seed and grain production to packaging and marketing [5].

Currently, the most popular DNA-based methods for screening genetically modified organisms (GMO) use polymerase chain reaction (PCR), with real-time PCR as the gold standard for GMO quantification. These procedures are laborious, expensive, and time-consuming, making them unsuitable for routine analyses [5]. Therefore, the development of fast, low-cost, easy-to-use, and field-applicable tools to detect the presence of GMOs is crucial [6].

An alternative to traditional methods is the use of DNA biosensors, known as genosensors. Among the various transduction methods [7–9], piezoelectric genosensors based on quartz crystal microbalances (QCMs) have recently gained prominence as the most attractive due to their potential for real-time, label-free detection, high sensitivity, simplicity, and low instrumentation cost [10,11]. In these devices, a quartz crystal wafer is sandwiched between two gold electrodes. The electrodes provide the connection between the reaction surface and the external oscillator circuit, which drives the quartz crystal at its resonance frequency [12]. DNA detection in QCM is performed by measuring the mass change at the sensor surface due to DNA hybridization. The linear relationship between the frequency change and the mass deposited on the quartz crystal surface is described by the Sauerbrey equation [13]:

$$\Delta f = \frac{-2.3 \times 10^{-6} f_0^2 \Delta m}{A}, \quad (1)$$

where $\Delta f(\text{Hz})$ is the frequency shift of the crystal resonance, $f_0(\text{Hz})$ is the fundamental frequency of the crystal, $A(\text{cm}^2)$ is the surface area and $\Delta m(\text{g})$ is the deposited mass. Thus, the immobilization, hybridization, and hydrolytic cleavage of DNA can be monitored in situ by measuring the quartz crystal's resonance frequency. The mass-sensitive QCM technique has been successfully used to detect GMOs in several previous studies [14–17].

Therefore, the purpose of this study was to develop a genosensor capable of detecting the BRS FC401 RMD event and differentiating transgenic from conventional cultivars in non-amplified samples using quartz crystal microbalance technology. Biosensors were prepared by immobilizing a probe complementary to the characteristic DNA sequence present in the transgenic common bean. The probe sequence was internal to the BGMV *rep* gene sequence that was inserted into the common bean genome. The reproducibility of the immobilization procedures – thiol-gold interaction with a thiol-blocking solution – was evaluated. The analytical performance of the genosensor was evaluated for sensitivity, repeatability, reproducibility, and specificity. Furthermore, different bioreceptor regeneration approaches were investigated.

2. Materials and Methods

2.1. Chemicals

Hydrochloric acid (HCl), sulfuric acid (H₂SO₄), hydrogen peroxide (H₂O₂), 6-mercapto-1-hexanol (MCH), sodium hydroxide (NaOH), saline-sodium phosphate-EDTA buffer (SSPE 20X), ethanol, and tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) were purchased from Merk (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), potassium phosphate monobasic (KH₂PO₄), ethidium bromide, and agarose were purchased from Sigma-Aldrich® (Madrid, Spain). Ethylene diamine tetraacetic acid (EDTA), tris-borate-EDTA (TBE 10X) buffer, and saline-sodium citrate (SSC 20X) buffer were obtained from Thermo Fisher Scientific (Waltham, MA, USA). All buffers and solutions used in the experiments were prepared with ultrapure water obtained from a Millipore Milli-Q® (Madrid, Spain) purification system (18.2 MΩ·cm at 25 °C).

2.2. Synthetic Oligonucleotides Samples

Synthetic oligonucleotides were designed based on studies of the BGMV *rep* gene sequence. The base sequences of the 5'-functionalised probe (25-mer), complementary target (25-mer) and non-complementary target (25-mer) oligonucleotides and blocking oligonucleotides (10-mer) are reported in Table 1.

Table 1. Synthetic oligonucleotide sequences used in this study.

Elements	Sequence
Probe	5' HS-(CH2)6 - ATGCGGCATCCGAAGCATTGAATGC 3'
Complementary target	5' GCATTCAATGCTTCGGATGCCGCAT 3'
Non-complementary 1	5' TTGATGACATCGCACCGCACTATCT 3'
Non-complementary 2	5' AGCGGCACCTCTCCCGAAATAATCA 3'
Blocking oligo 1	5' ATATTCACAA 3'
Blocking oligo 2	5' TGCATGGCTT 3'

All the oligonucleotides were purchased from GenOne Biotechnologies (Rio de Janeiro, Brazil). Lyophilized samples were dissolved in ultrapure water to a final concentration of 100 µM (pmol/µL) and stored at -20°C. Both the target and non-complementary sequences were used to evaluate the analytical performance of the biosensor. Blocking oligonucleotides were applied in the genomic DNA (gDNA) denaturation procedure.

2.3. Genomic DNA Samples

2.3.1. Genomic DNA Extraction

Initially, the bean grains (both wild type and transgenic) were ground for 20s (IKA A11 basic) to ensure greater exposure of the genetic material during cell lysis. Genomic DNA was then extracted using three commercial kits: ZymoBIOMICS (Zymo Research, United States), DNeasy Plant Pro (Qiagen Co. Ltd., Germany), and DNeasy *mericon* Food (Qiagen Co. Ltd., Germany), following recommended protocols. The DNA extract was quantified and its purity evaluated with a spectrophotometer (Thermo Scientific NanoDrop One) by determining the A_{260/280} and A_{260/230} ratios. Sample integrity was verified next by electrophoresis on a 1.5% agarose gel in TBE 1X buffer containing ethidium bromide, and samples were photodocumented with the Locus Biotechnologia L.PIX Transilluminator (manual profile; 696 x 520 resolution; 16-bit format). Finally, the DNA was stored at -20 °C until use.

2.3.2. Enzymatic Digestion

After extraction, gDNA was enzymatically digested using the restriction enzyme *FastDigest AluI* (Thermo Scientific™). The choice of enzyme was based on the presence of restriction sites located in the flanking regions of the target sequence. The reaction was allowed to proceed at 37 °C for 15 min in a dry bath/thermoblock (Nova Ética/model 330 - 3D). Enzyme inactivation was not performed. The fragmented DNA was immediately used in the denaturation step.

2.3.3. Thermal Denaturation with Blocking Oligonucleotides

DNA fragments obtained by enzymatic digestion have a double helix structure and the two strands should be separated (denatured) to allow the hybridization with the probe immobilized on the sensor. To obtain a single strand, the digested DNA was submitted to thermal denaturation with a blocking oligonucleotide procedure [18], as shown in the Figure 1. This method is based on combining thermal dissociation effect with the steric hindrance caused by the binding of two short oligonucleotides to the thermally separated strands. One oligonucleotide binds to the strand containing the target while the other binds to the strand containing the probe. In this way, re-

association of the strands is prevented, leaving the target strand available for hybridization. After adding 50 μL of each oligonucleotide (10 $\text{ng}/\mu\text{L}$), the sample was incubated at 95 °C for 5 minutes and then for 1 minute at 40 °C. This second temperature is the appropriate temperature for the annealing of the oligonucleotides to the complementary DNA sequences. The sample was injected into the cell flow immediately after completion of the procedure. The sample containing wild-type DNA was used as negative control.

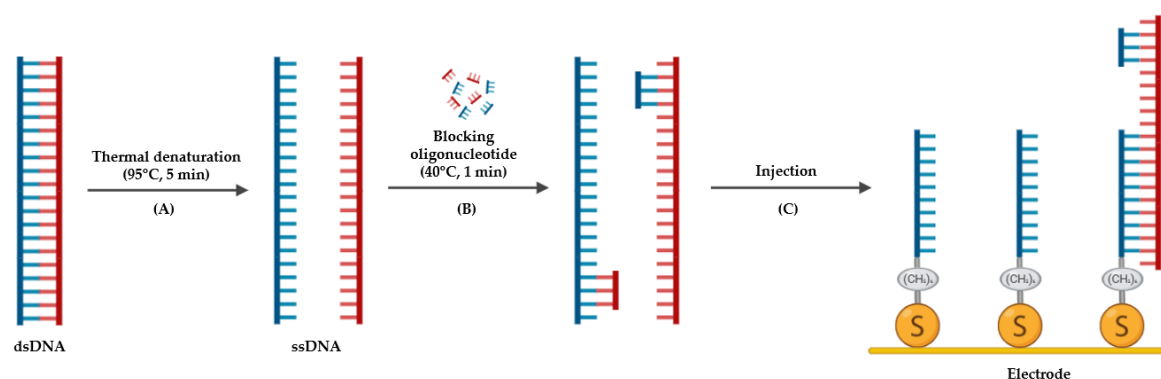


Figure 1. Thermal denaturation followed by blocking to dissociate the dsDNA. (A) high temperature denaturation (95°C for 5min), (B) blocking with oligonucleotides (1 min incubation with oligonucleotides at 40 °C), (C) injection into the flow cell to hybridization with the probe immobilized on the electrode.

2.4. QCM Crystals and the Experimental Set-Up

A quartz crystal resonator with an AT-cut and a nominal frequency of 10 MHz, oscillating in the fundamental harmonic (Novaetech S.r.l., Italy), was used for piezoelectrical measurements of hybridizations of synthetic oligonucleotides and genomic DNA. The device has an approximate thickness of 160 μm and a total diameter of 13.9 mm, positioned between two circular gold electrodes with a diameter of 6 mm and a thickness of approximately 200 nm. The adhesion of the gold to the quartz surface was enhanced by the deposition of an intermediate layer of titanium (Ti). The crystal exhibits a nominal sensitivity of $4.42 \times 10^{-9} \text{ g Hz}^{-1} \text{ cm}^{-2}$ (C_f), with thermal stability characterized by a variation of $\pm 20 \text{ kHz}$ at 23 °C.

The quartz crystal was previously cleaned with a Piranha solution composed of H_2O_2 (30%) and H_2SO_4 (98%) in a 1:3 ratio. Approximately three drops of the solution were deposited on the electrode surface and held for 10 minutes. Then, it was rinsed with ultrapure water. The crystal was used immediately or kept in absolute ethanol until use.

The quartz crystal was housed inside a flow-cell and the resonance frequency was continuously recorded using an open-source quartz crystal frequency counter (openQCM proximity electronic, 1–25 MHz) developed by Novaetech S.r.l (Pompei, Italy). Samples and solutions were passed through the QCM flow-cell setup at a specific flow rate (100–500 $\mu\text{L}/\text{min}$) using an open-source, precise peristaltic pump [19]. The frequency shifts (ΔF) reported in the paper are the difference between two stable values ($\pm 1 \text{ Hz}$). All measurements were conducted in a controlled laboratory environment to minimize external interferences. Additionally, the ambient temperature was monitored using the OpenQCM software's real-time temperature monitoring feature to prevent signal drift.

2.5. Immobilization of the Oligonucleotide Probe on Gold

Quartz crystal functionalization was carried out as described in previous works [14,20], with some modifications. The immobilization solution (1 M KH_2PO_4 , pH 3.8) was injected into the flow-cell until a stable baseline frequency was achieved. Then, 50 μL of the thiolated probe (10 $\text{ng}/\mu\text{L}$) prepared in the same solution was injected and recirculation was maintained for 2 hours. The surface modification is based on the direct chemisorption of DNA probes onto transducer surfaces due to the

formation of gold–thiol bonds. Next, the crystal was washed with immobilization solution and water to remove non-linked probes and prepare it for the blocking step. To block the surface and prevent non-specific adsorption, the crystal was treated with 1 mL of a 1 mM aqueous solution of blocking thiol (6-mercapto-1-hexanol, MCH) for 1 hour. Both probe immobilization and blocking surface procedure were conducted with recirculation of each solution at a continuous flow rate (100 $\mu\text{L}/\text{min}$). Frequency value shifts were recorded when a stable frequency signal was obtained. Sauerbrey equation was used to determine the amount of mass deposited on the crystal surface by immobilized thiolated probe and blocking thiol.

2.6. Hybridization with Synthetic Oligonucleotides

Hybridizations using synthetic oligonucleotides were conducted to characterize the analytical capabilities of the genosensor in terms of stability, specificity, and reproducibility. The flow cell was filled with hybridization buffer (SSPE 1x) and left in this condition until baseline stabilization. Hybridization was conducted using a solution containing 10 ng/ μL of the complementary synthetic target, prepared in the same buffer, for 30 minutes under continuous recirculation (100 $\mu\text{L}/\text{min}$). Then, the crystal was washed with the hybridization buffer to remove unbound target molecules. The single-stranded probe regeneration was performed by treatment with a 1 mM HCl solution for 1 minute at a continuous flow rate (500 $\mu\text{L}/\text{min}$). Finally, the hybridization buffer was injected, re-establishing the experimental conditions necessary for subsequent detection cycles.

2.7. Hybridization with Real Samples

Genomic DNA was first enzymatically digested to obtain fragments that could be easily denatured and recognised by the immobilised probe. The procedure for hybridising digested genomic DNA is the same as that described for oligonucleotides, with a few exceptions due to the greater complexity of the sample. The samples were diluted in hybridization buffer to ~ 1.2 ng/ μL . The hybridization reaction was allowed to proceed for 30 min. In addition to the treatment with 1 mM HCl for 1 minute, NaOH (50 mM and 100 mM) and 200 mM Tris-HCl (pH 7, 0.1X SSC, and 0.1% p/v SDS) were used to optimize the regenerations, varying the temperature and exposure time (detailed in supplementary material). The regeneration solutions were injected into the flow cell and discarded at a continuous flow rate of 500 $\mu\text{L}/\text{min}$.

3. Results and Discussion

3.1. Electrode Functionalization

Immobilization was achieved by directly interacting the gold surface of the quartz crystal with the thiolated probe solution. The driving force for coating thiolated probes on gold surfaces is due to the formation of strong thiol–Au bonds, which have a chemisorption energy of ~ 50 kcal/mol [21]. The application of this technique is easy and straightforward and is possibly the most widely employed surface chemistry approach for immobilizing bioreceptors on Au-based molecular detection platforms [22]. The QCM gold electrode serves as a transduction interface, and the immobilization process can be observed in real time, eliminating the need for a labeling system. In this study, a frequency variation of -162 ± 9 Hz ($n=3$) was observed upon 2h immobilization of 10 ng/ μL of the thiolated probe, corresponding to a coefficient of variation (CV%) of 5.8%. This value corroborates the literature results [14] and demonstrates good reproducibility, also indicating that the procedures adopted for crystal cleaning, solution preparation, and flow condition control are robust. According to the Sauerbrey equation ($\Delta f = -C_f \times \Delta m$) for a 10 MHz quartz crystal with a nominal sensitivity of 4.42×10^{-9} g Hz $^{-1}$ cm $^{-2}$, a frequency decrease of 1 Hz corresponds to a mass increase of 1.25 ng. Therefore, the estimated chemisorbed mass was ~ 203 ng, equivalent to a probe density of 5.6×10^{13} molecules.cm $^{-2}$ (MW of thiolated-probe; 7691 g/mole).

After the immobilization with the thiolated probe, the crystal was treated with 1 mM of MCH for 1 hour at a continuous flow rate (100 $\mu\text{L}/\text{min}$) to control surface density and remove non-specific interactions [23]. The frequency variation associated with MCH treatment was $+46 \pm 4$ Hz, indicating partial desorption of poorly anchored probes and monolayer reorganization.

Surface activity was assessed by hybridization with 10 $\text{ng}/\mu\text{L}$ of complementary DNA in 1x SSPE buffer. The hybridization signal was -35 ± 4 Hz ($n = 3$), corresponding to a hybridization ratio of $\sim 30\%$ (calculated as $(\Delta F_{\text{hybridization}}/\Delta F_{\text{immobilization}}) \times 100$ adjusted after MCH passivation). The hybridization ratio typically observed in QCM biosensors ranges from 30% to 80%, depending on the probe design, surface chemistry, and experimental conditions[24,25]. Specifically for hybridization with a thiol-modified probe, a ratio of 40-42% is reported[25].

This results highlight opportunities for optimization by fine-tuning probe density to balance monolayer stability, reproducibility, and accessibility for hybridization. Among the possible optimizations, reducing the concentration of probes in the immobilization fluid or decreasing the exposure time stands out, resulting in a less dense monolayer and more favorable interactions with the target. Another possibility focuses on the concentration of the blocking agent: in excess, it can displace probes from the QCM surface and reduce subsequent hybridization. At low concentrations, the probes may not assume an upright orientation, thereby reducing their availability for binding to the target [26]. Alternatively, decreasing the flow rate can reduce mass transport, resulting in less compact monolayers [27]. All of this, combined with surface studies (Atomic Force Microscopy (AFM), X-ray photoelectron spectroscopy (XPS), etc.), allows for a more detailed investigation of the influence of each variable on electrode functionalization.

3.2. Analytical Performances of the Biosensor

Analytical performance of the developed biosensor was evaluated for sensitivity, repeatability, reproducibility and specificity using standard solutions of synthetic oligonucleotides. As shown in Figure 2, a calibration curve was obtained using the complementary target in a concentration range of 0–10 $\text{ng}/\mu\text{L}$. The sensor demonstrated linear behavior over the 0–1.4 $\text{ng}/\mu\text{L}$ concentration range, described by the regression equation $\Delta F \text{ (Hz)} = (11.909) C \text{ (ng}/\mu\text{L}) + (0.8971)$, with a high correlation coefficient of $R^2 = 0.9858$. The experimental limit of detection (LOD) calculated following the standard approach ($\overline{X}_B + 3\sigma B$) for the complementary target sequence was 0.18 $\text{ng}/\mu\text{L}$.

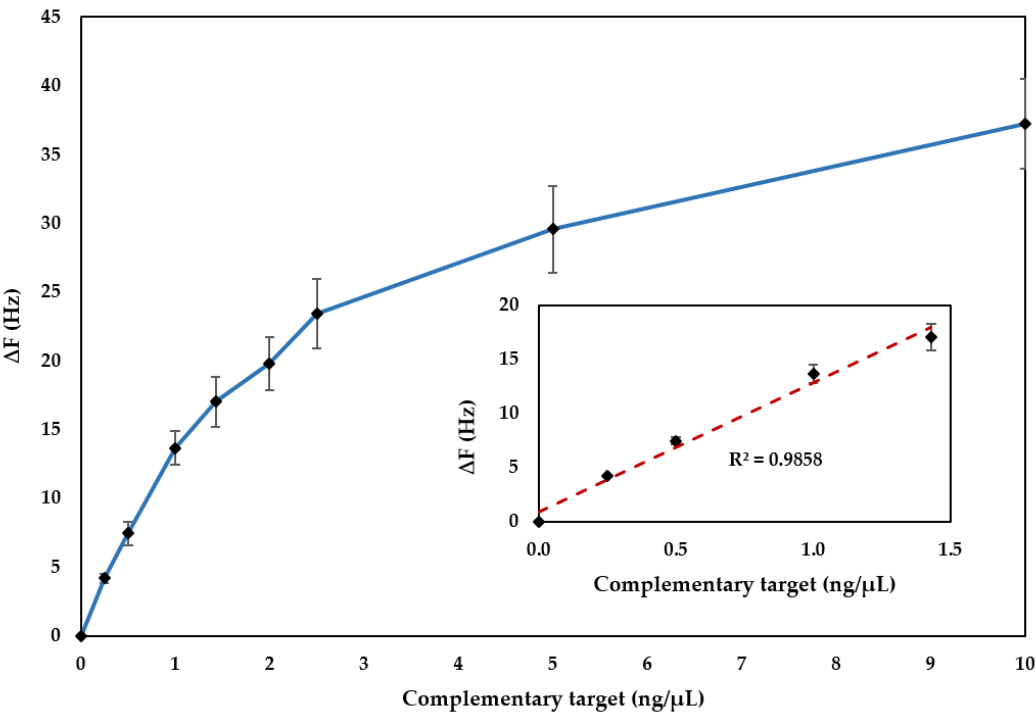


Figure 2. Calibration curve for complementary target detection obtained from thiolated probe/ blocking thiol immobilization procedure.

Repeatability of the biosensor was evaluated by performing five consecutive measurements on the same electrode, using the same complementary target concentration (10 ng/μL). Electrode regeneration was performed by injecting 1 mM HCl at a flow rate of 500 μL/min. The average frequency variation was -30 Hz, with a standard deviation of ±2 Hz, yielding a coefficient of variation of 6%. Similar studies reported CVs of 8% (n=4)[17] and 11% (n=3)[20] for assays using the same regeneration solution. Thus, the low variability observed indicates good repeatability of the system and confirms the stability of the detection signal resulting from hybridization of ssDNA on the quartz crystal surface.

Reproducibility was investigated by performing three detection tests on three independent electrodes under the same experimental conditions. A frequency variation of -35 Hz was observed, with a standard deviation of ±4 Hz. The CV of 11% indicates good inter-device reproducibility. This value, although slightly higher than that observed for repeatability, remains within the range reported in the literature for piezoelectric DNA biosensors[16,28], indicating robustness of the manufacturing and immobilization process. The observed variation may be associated with slight differences in probe density, heterogeneity in the self-assembled monolayer, and differences in hybridization efficiency across electrodes.

Specificity was demonstrated by comparing the biosensor's response to 10 ng/μL complementary target DNA with that of two non-complementary sequences. Negligible signals (< 1Hz) from non-complementary sequences confirm the sensor's ability to recognize the intended DNA target specifically. This result is crucial for the device's applicability, ensuring that only genetically modified targets are detected, minimizing false positives.

3.3. Genomic DNA Extraction

For each method of DNA detection and/or quantification in biomolecular analyses, at least one extraction protocol applicable to the sample matrix must be available, tested, and validated. Table 2 presents the results of genomic DNA extraction from transgenic bean grains using three commercial kits. The essence of extraction is to obtain a sufficient quantity of DNA with adequate quality for subsequent analyses. Quality refers to the length, structural integrity and physicochemical purity of the extracted DNA[29].

Table 2. Results of gDNA analysis using the NanoDrop spectrophotometer.

N	gDNA Extraction Kit	ng/μL	A _{260/280}	A _{260/230}
I	ZymoBIOMICS DNA Miniprep	59 ± 9	1.95 ± 0.00	1.49 ± 0.07
II	DNeasy Plant Pro	52 ± 20	1.86 ± 0.03	0.13 ± 0.06
III	DNeasy mericon Food	127 ± 16	1.85 ± 0.04	1.28 ± 0.00

Kits I and II showed similar average extraction yields, indicating comparable efficiency in the lysis and recovery of genomic DNA from the plant matrix. However, kit II showed lower reproducibility between replicates. Kit III, on the other hand, showed a yield twice that of the previous ones. The A_{260/280} ratio was greater than 1.7 for all extracts, indicating that all three kits were efficient in removing proteins, since values between 1.7 and 2.0 are accepted as indicative of pure DNA. The evaluation of DNA purity by measuring the absorbance ratio at 260 and 280 nm (A_{260/280}) is a widely used technique for indicating, for example, the presence of residual peptides and phenolic compounds. These compounds exhibit strong absorption around 280 nm, resulting in low A_{260/280} ratios when they coexist with DNA, which absorbs at 260 nm [15]. The A_{260/A230} ratio showed distinct behavior: all samples exhibited values below the ideal range (2.0–2.2), indicating residual presence of contaminants that preferentially absorb at 230 nm, such as lipids, salts and phenols[29].

The integrity of the extracts obtained from the bean seeds was evaluated by agarose gel electrophoresis (Figure 3). Eight samples were analyzed.

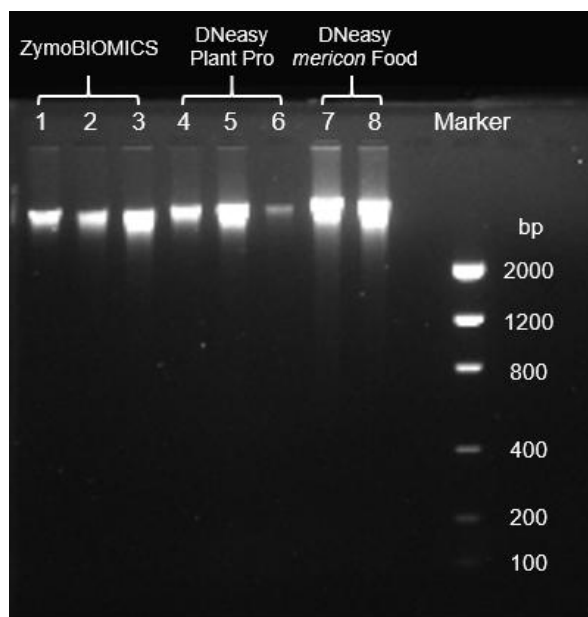


Figure 3. Agarose gel electrophoresis of genomic DNA extracted from transgenic carioca bean seeds using the commercial kits ZymoBIOMICS, DNeasy Plant Pro and DNeasy mericon Food.

All samples exhibited intense bands located near the top of the gel, a region of high molecular weight, indicating the presence of genomic DNA with good structural integrity [16]. The exception was sample 6, extracted with kit II, whose band showed reduced intensity, suggesting a lower amount of DNA extracted. The extraction method and sample characteristics influence the yield and quality of the extracted DNA, and both are crucial to the results of GMO detection and quantification. Overall, kit III showed better yields and purity. However, although purity ratios are important indicators of sample quality, the best indicator of nucleic acid quality is its functionality in subsequent applications of interest [16]. Therefore, all samples were subjected to enzymatic digestion steps and detection assays to assess the quality of the signal obtained.

3.4. Sample Pretreatment: Enzymatic Fragmentation and Thermal Denaturation Combined with Oligonucleotides

DNA fragmentation and denaturation are necessary pretreatment steps for samples that require short fragments for rapid hybridization and high-sensitivity target detection [30]. Plant genomic DNA is a structurally complex, long, and difficult-to-dissociate molecule, which sterically hinders the approximation of target sequences to probes bound to the crystal surface [31]. Therefore, after extraction, the gDNA was enzymatically digested using the restriction enzyme *FastDigest AluI*. The enzyme was chosen based on the presence of restriction sites located in the flanking regions of the target sequence, allowing the generation of a fragment of 138 nitrogenous base pairs. The length of the fragment obtained is smaller than that reported in similar studies (approximately 400-900 bp) [20,31]. It is worth noting that smaller fragments tend to exhibit greater diffusional mobility and less steric hindrance during association with the probe.

The high-temperature thermal denaturation procedure (95 °C for 5 min, followed by 1 min in ice) is widely described in literature for hybridization assays [14]. However, this approach may be unfavorable for experiments performed on instrumentation that employs flow systems for sample injection [18], such as the one developed in this work. This limitation is due to the rapid reannealing of denatured DNA strands before they come into contact with the sensor surface. Therefore, the

digested sample was subjected to a thermal denaturation protocol associated with the presence of oligonucleotides. In this strategy, after denaturation, the DNA is incubated for 1 minute at the oligonucleotide annealing temperature (40 °C). The binding of these oligonucleotides to single-stranded DNA (ssDNA) slows reannealing, increasing the time that denatured sequences remain available for hybridization. The effectiveness of sample pretreatment was inferred from the analytical hybridization signal.

3.5. Hybridization with Non-Amplified gDNA Samples

In the digested DNA sample, the target sequence is present at reduced copy number and embedded within a highly complex matrix of fragmented genomic DNA. Even so, the biosensor detected the complementary sequence in all samples analyzed. This result demonstrates that the sample pretreatment strategy—involving extraction, enzymatic digestion, and thermal denaturation combined with blocking oligonucleotides—was effective in making the target sequence available in a form accessible to molecular recognition.

The reproducibility assay in three distinct crystals revealed a hybridization frequency variation of -40 ± 16 Hz with a CV% of 40%. It should be noted that it is not possible to directly compare the coefficient of variation of the signals obtained with real samples and with samples of synthetic oligonucleotides, since these matrices present distinct levels of complexity. While synthetic oligonucleotides constitute well-defined and homogeneous systems, genomic DNA extracted from plant matrices is a complex mixture of fragments, possible residual contaminants from the extraction process, and variability inherent in the pretreatment steps, all of which tend to increase the dispersion of analytical signals.

The comparison of the different extraction kits revealed no direct correlation between hybridization signal intensity and the extraction method. Although differences in DNA yield and purity were observed, particularly with the DNeasy *mericon* Food kit, these variations were not significantly reflected in the final biosensor response.

The biosensor's specificity was confirmed using a negative control, consisting of wild-type DNA subjected to the same extraction, digestion, and heat treatment procedures. Under these conditions, a negligible frequency shift (≤ 4 Hz) was observed ($n=3$). The small frequency shift can be attributed either to direct DNA adsorption on the gold electrode without hybridization with the probe or to weak, non-specific interactions between the probe and partially complementary sequences in the complex sample.

3.6. Surface Regeneration

To regenerate the bioreceptor after hybridization with gDNA, different approaches were evaluated using 1 mM HCl, NaOH (50 mM and 100 mM), and 200 mM Tris-HCl (pH 7), with 0.1X SSC and 0.1% w/v SDS. The efficiency of each treatment was evaluated by the percentage of baseline recovery before hybridization. No regeneration occurred with 1mM HCl and 50mM NaOH. Only partial regeneration occurred with 100 mM NaOH (25°C), accompanied by incomplete removal of hybridized target DNA, resulting in lower surface activity. On the other hand, regeneration with I: (100 mM NaOH (25°C) for 1 min + 200 mM Tris-HCl(...)(25°C) for 2 min) and II: (200 mM Tris-HCl(...)(95°C) for 10 min + H₂O (95°C) for 15 s) resulted in a regeneration percentage greater than 100%, indicating that in addition to the removal of the hybridized target DNA, there was also partial removal of the probes immobilized on the sensor surface. The maintenance of the surface's analytical capacity was evaluated by the percentage retention of the analytical signal after two regeneration/hybridization cycles (Figure 4).

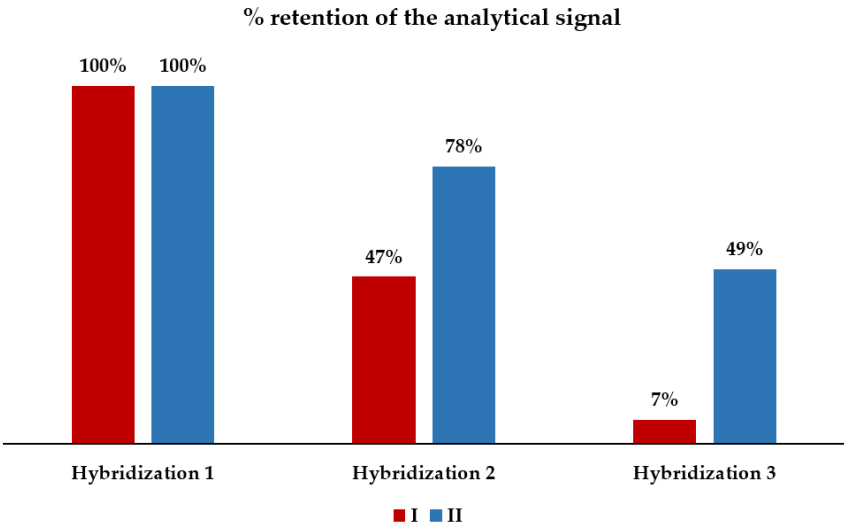


Figure 4. Percentage retention of the analytical signal after two regeneration/hybridization cycles.

Regeneration with treatment I showed a cumulative loss of approximately 93% of the analytical signal compared to the initial value after the third cycle. In comparison, regeneration with II resulted in a 51% loss. This difference is attributed to the milder chemical conditions employed in treatment II, in which only the thermal effect was used to promote the separation of the DNA strands, combined with the Tris-HCl buffer solution (pH 7, 0.1X SSC, and 0.1% w/v SDS). The use of temperature to induce DNA denaturation is a well-established technique and, when adequately controlled, can be applied without causing irreversible damage to the integrity of the nucleic acid [30]. Despite this progressive decrease, the signal remained clearly distinguishable from noise and the negative control after treatment II, allowing for the unequivocal identification of the analyte in all cycles evaluated. This behavior indicates that although the functional layer shows partial degradation, a significant fraction of the probes remains active and accessible for molecular recognition. At least three hybridization reactions can proceed without the need for a new immobilization procedure.

5. Conclusions

In this work, a QCM-based genosensor for the detection of common bean resistant to golden mosaic was developed. By immobilizing a sequence internal to a region of the BGMV *rep* gene on the QCM sensor surface, the system achieved selective, sensitive detection of the transgenic event directly from non-amplified samples. Although some challenges remain, the developed biosensor introduces an assay format that allows for real-time detection of the transgenic event without markers, without the need for extensive sample preparation or complex analytical procedures. The assay is compatible with miniaturized instrumentation, making it particularly suitable for field applications. In Brazilian agriculture, this method can transform the monitoring of modified common beans, speeding up inspections, reducing costs, and expanding analytical reach to regions with less laboratory infrastructure.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Approaches studied for bioreceptor regeneration after hybridization with genomic DNA.

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Abbreviations

The following abbreviations are used in this manuscript:

BGMV	bean golden mosaic virus
dsRNA	double-stranded RNA
siRNA	small interfering RNAs
GMO	genetically modified organisms
PCR	polymerase chain reaction
gDNA	genomic DNA
ssDNA	single-stranded DNA
AFM	atomic force microscopy
XPS	x-ray photoelectron spectroscopy

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