Supplementary Information (SI) for Sensors & Diagnostics.
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SUPPLEMENTARY INFORMATION

Rapid and portable molecular test for *Mycobacterium tuberculosis* based on double-tagged amplification and electrochemical readout

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S1. Buffer and solution composition

The composition of the buffers used in this work are described below:

For the electrochemical-magneto genosensing

- For the anti-DIG-HRP dilution: Phosphate buffer solution (PBS): 100 mM Na_2HPO_4, 137 mM of NaCl, pH=7.4
- For washing and resuspension of streptavidin magnetic particles: Binding and washing buffer (B&W): 10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, pH=7.5
- For the dilution of amplicons sample: Ultrapure water (Millipore System, resistivity 18.2 MΩ cm)

For electrochemical readout

- ePBS: 100 mmol L^{-1} Na_2HPO_4, 100 mmol L^{-1} KCl, pH 7.0,
- ePBS MIX: ePBS plus 2 mmol L^{-1} Hydroquinone, 4 mmol L^{-1} H_2O_2 (30% (v/v))

S.2. Optimization of the DNA extraction and double-tagging polymerase chain reaction

The extraction protocol was adapted from the GenoType MTBDRplus and MGIT culture (Hain Lifescience GmbH, Germany) with the aim of performing a simple, fast and efficient extraction minimizing the total number of steps. However, different conditions for DNA extraction were tested and these samples were amplified with the two sets of primers to check their influence on the final extraction protocol. Table S1 summarized the conditions tested to obtain four DNA samples following the extended protocol, omitting or including steps 6 and 7.

The extensive DNA extraction protocol was as follows:

1. Transfer 1mL of sample to an eppendorf tube; 2. Centrifuge at 10,000 *g* for 15 min; 3. Discard supernatant; 4. Resuspend pellet with 300 µl of water and mix with vortex; 5. Incubate at 95 °C for 20 min; 6. (*Optional)* Incubate in an ultrasonic bath for 15 min; 7. (*Optional)* Add 30 µL of sodium acetate and mix*;* 8. Centrifuge at maximum speed for 5 min*;* 9. Transfer supernatant to a new tube and store DNA at -20 °C.

Table S1. Summary of the conditions tested for the optimization of DNA extraction protocol.

	DNA extraction protocol (Nº)			
	1	**2**	**3**	**4**
Step 1 to 5 (mandatory) transfer, centrifugation, supernatant removal, pellet resuspension, and heat lysis	YES	YES	YES	YES
Step 6 (optional) Ultrasonic pretreatment	NO	NO	YES	YES
Step 7 (optional) Sodium acetate treatment	NO	YES	NO	YES
Step 8 and 9 (mandatory) entrifugation, supernatant transfer, and DNA storage	NO	YES	NO	YES

The resulting amplicons were analyzed with conventional agarose gel electrophoresis on 0.8 % agarose gels containing TBE 0.5 % buffer and Sybr Safe DNA gel stain and using a 250 bp ladder as a molecular weight marker. Figure S1 shows the electrophoresis gels obtained with the different protocols. As observed, the amplification obtained with protocol 2 and 4, in which step 7 was included in the DNA extraction, were the ones with the lower signal intensity, resulting in a lower amplification efficiency. However, where protocol 1 and protocol 3 were used, in which step 7 was not included, the signals were more intense leading to a higher PCR yield product. According to the results, further experiments were performed without including step 6 and 7, since the PCR product yield was clearly lower when sodium acetate was added in the DNA extraction protocol and no improvement was observed when the ultrasonication treatment, step 6, was included, thus leading to a simplification in the analytical procedure.

In addition, the primers selected for the amplification of *IS6110* and *gyrB* genes were previously tested with no tags (BIO/DIG), using the sequences described in Table 1, to verify their efficiency in DNA amplification. Since the sequences contain a high guanine and cytosine (GC) content, characteristic of mycobacteria, the required annealing temperature is typically somewhat higher. For this reason, an optimization of the annealing temperature was carried out ranging from 60 to 65 °C with no tagged primers. Figure S1 shows successful amplification of the previously extracted DNA was observed using the two set of primers even if the annealing temperature was modified between 60-65 °C.

Finally, for the double-tagging PCR for the detection of tuberculosis, 62 °C was selected as the annealing temperature, ensuring high efficiency in the PCR, and with the objective of minimizing the lack of specificity or the production of undesired secondary structures. The final double-tagging PCR used in this work, consisted of an initial step at 94 °C for 5 min followed by 30 cycles of 94 °C at 30 s, 62 °C for 30 s, 72 °C for 45 s, and a last step of 5 min at 72 °C.

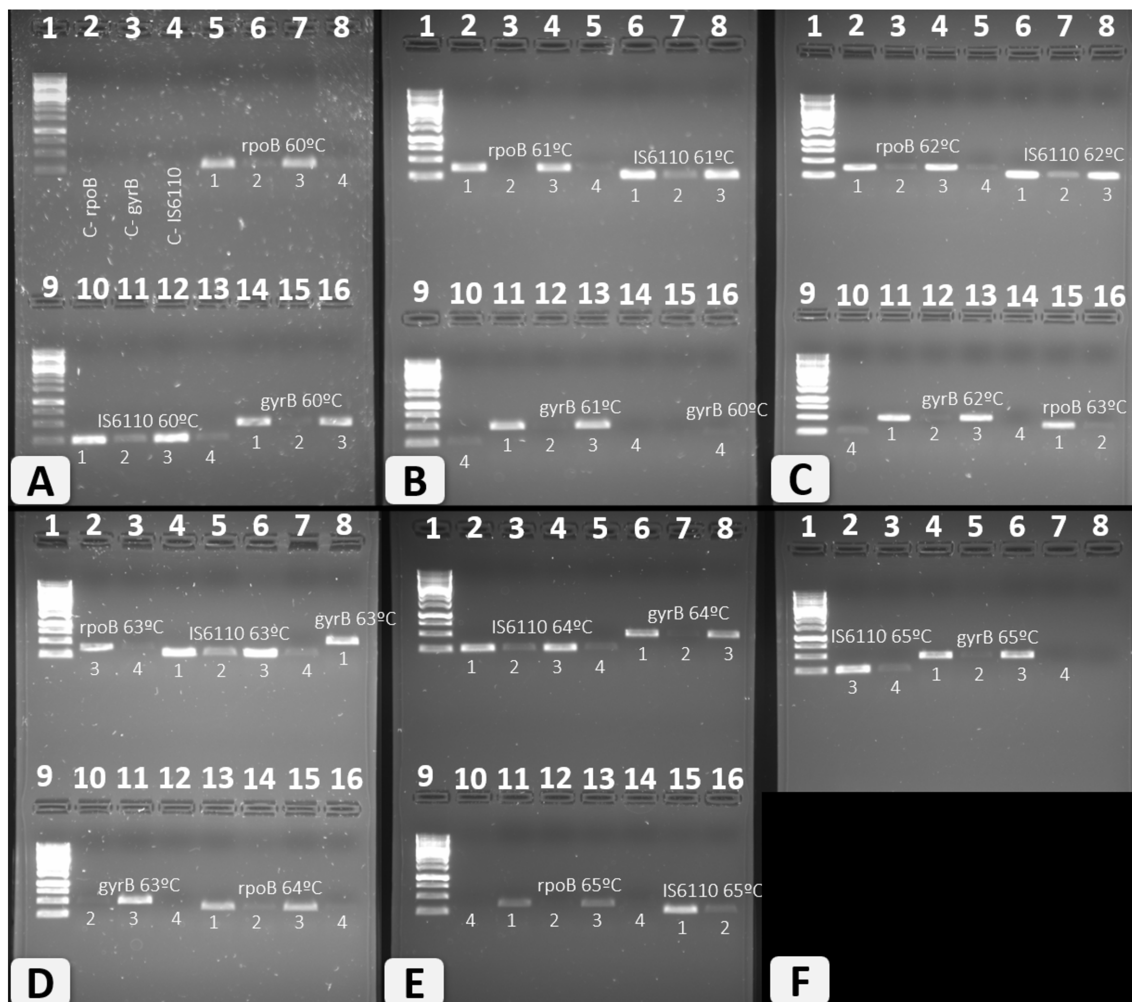

Figure S1. Results of the agarose gel electrophoresis of the amplicons obtained by the PCR from a sample of *M. bovis BCG-Pasteur* culture using the non-labeled primers coding for *IS6110, gyrB and rpoB.* In the panels were analyzed the samples of amplicons providing from DNA extraction protocols 1 to 4, represented as 1, 2, 3 and 4 according to the procedure followed for extract the bacterial DNA. All these samples were tested with each set of primers with different annealing temperature ranging from 60 to 65 °C. **Panel A.** Negative controls setting 60 °C of annealing temperature, lane 2, 3 and 4 for *rpoB, gyrB* and *IS6110* primers, respectively. Positive controls for 60 °C using *rpoB* in lane 5, 6, 7, 8, *IS6110* in lane 10, 11, 12, 13 and *gyrB* in lane 14, 15, 16. **Panel B.** Positive controls for 61 °C using rpoB in lane 2, 3, 4, 5, *IS6110* in lane 6, 7, 8, 10 and *gyrB* in lane 11, 12, 13, 14. Positive control for 60 °C using *gyrB* for protocol 4 in lane 16. **Panel C.** Positive controls for 62 °C using *rpoB* in lane 2, 3, 4, 5, *IS6110* in lane 6, 7, 8, 10 and *gyrB* in lane 11, 12, 13 14. Positive controls for 63 °C using *rpoB* in lane 15 and 16. **Panel D.** Positive controls for 63 °C using *rpoB* in lane 2, 3, *IS6110* in lane 4, 5, 6, 7 and *gyrB* in lane 8, 10, 11, 12. Positive controls for 64 °C using *rpoB* in lane 13, 14, 15, 16. **Panel E.** Positive controls for 64 °C using *IS6110* in lane 2, 3, 4, 5, *gyrB* in lane 6, 7, 8, 10. Positive controls for 65 °C using *rpoB* in lane 11, 12, 13, 14 and *IS6110* in lane 15 and 16. **Panel F**. Positive controls for 65 °C using *IS6110* in lane 2, 3 and *gyrB* in lane 4, 5, 6, 7.

S3. Optimization of the electrochemical magneto-genosensing procedures

The reagent concentrations for the electrochemical magneto-genosensor were previously optimized in order to obtain the maximum signal to noise ratio. Firstly, the concentration of the streptavidin magnetic particles (strep-MPs) was optimized. A volume of 10 μL of strep-MPs at different dilutions from the stock of ½, 1/10, 1/20 (approx. 3.25×10^8, 6.5×10^7, 3.25×10^7 MP mL^{-1}) were tested in combination with 100 μL of anti-digoxigenin-HRP (anti-DIG-HRP) antibodies at 1000 mU mL^{-1}. Then 10 μL of positive controls containing double-tagged amplicons (BIO/DIG) and negative controls were incubated for 15 min at RT, performing a total volume of 120 μL of sample.

After the incubation step, the sample was transferred into the disposable cartridge (BioEclosion SL, Spain) for electrochemical readout. The cartridge acts as a self-contained microchamber that enables magnetic actuation, washing, and amperometric measurement in a controlled environment. In the open position, residual supernatant was removed and one washing step was performed by adding three drops of ePBS MIX buffer to eliminate unbound reagents. The washing solution was then discarded, and three fresh drops of ePBS MIX were added in the closed position to establish electrical contact between the electrodes and the sample for amperometric measurement. The electrochemical signal was recorded under enzyme-saturation conditions at –0.12 V for 30 seconds.

Secondly, the concentration of the anti-DIG-HRP antibody was optimized. A concentration of 250, 500, 1000, 2000 mU mL^{-1} of anti-DIG-HRP was tested in combination with 10 μL of strep-MPs at ½ dilution from the stock and 10 μL of sample, including positive and negative controls.

Then, the electrochemical magneto-genosesing and the measurements were done as described before. In all cases, amplicons obtained from the *IS6110* tagged primers were used as a model for the optimizations and were tested by triplicate (n=3). Finally, the ½ dilution from the stock for the strep-MPs and 500 mU mL^{-1} for the anti-DIG-HRP antibody were selected for further experiments according to the results described in Figure S2, panel A and B.

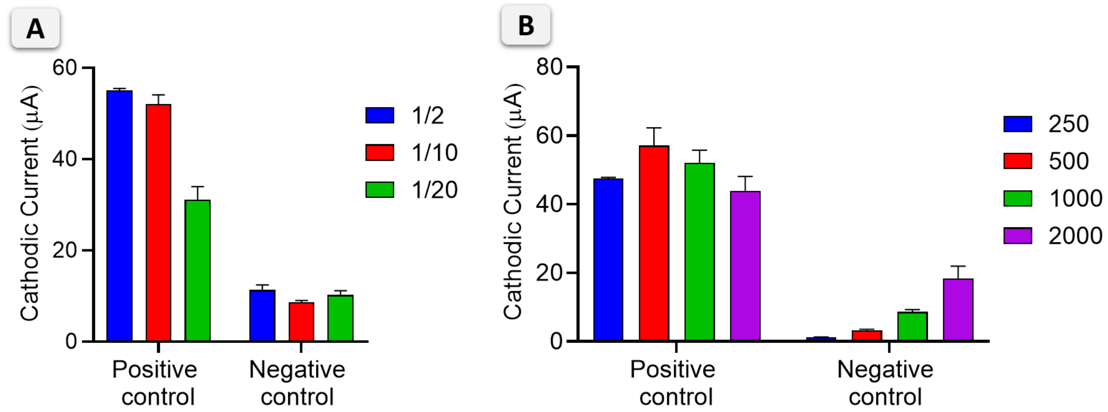

Figure S2. Plot for the optimization of the reagent concentrations for the electrochemical magneto-genosensor. Panel A shows the optimization of the strep-MPs concentration, while Panel B, the optimization of the anti-DIG-HRP concentration. Positive and negative controls (from o to 2.6 x 10^7 CFU mL^{-1} of *M. bovis BCG-Pasteur*, respectively) (n=3).

S4. Comparison of mycobacteria detection using the handheld device and commercial electrochemical systems

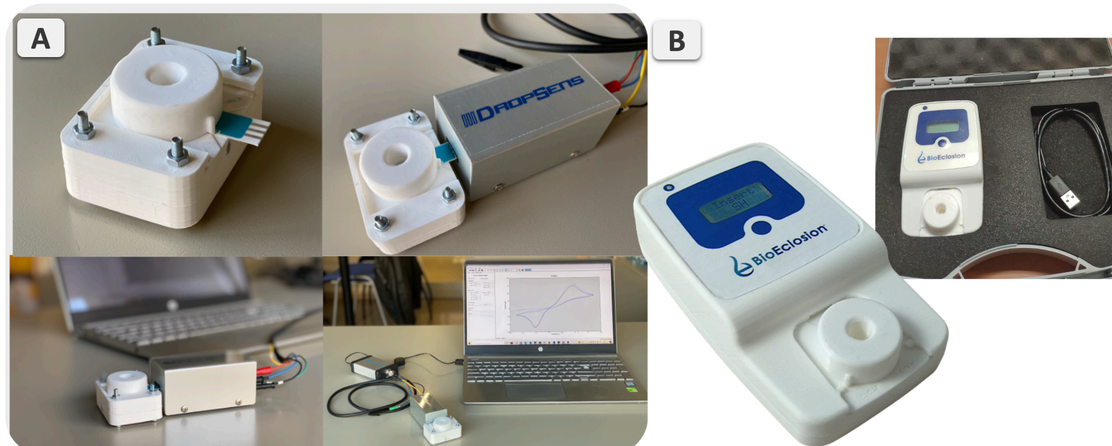

Figure S3. Electrochemical platforms for tuberculosis electrochemical genosensing. (A) Laboratory proof-of-concept setup used as a Reference device consisting of a disposable cartridge for magnetic actuation connected to a portable commercial potentiostat (DropSens, Spain). The electrochemical readout was performed using a laptop equipped with DropView 2.2 software via USB connection. (B) Handheld integrated platform, comprising a disposable cartridge and a digital reader operated by batteries. The reader integrates an electronic circuitry for quantitative electrochemical measurements, displaying the results directly on screen or transmitting data wirelessly via Bluetooth to a mobile application.