Supporting information for:

Solid-phase helicase dependent amplification and electrochemical detection of *Salmonella* on highly stable oligonucleotide-modified ITO electrodes.

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SUPPORTING METHODS

Materials and instrumentation

1-Naphthyl phosphate (α-NPP), 3,3´,5,5´-tetramethylbenzidine (TMB) containing hydrogen peroxide as substrates of peroxidase (POD), bovine serum albumin (BSA), phosphate buffered saline (10× PBS; 0.1 M phosphate, 1.54 M NaCl pH 7.4), 20× saline sodium phosphate (20× SSPE; 200 mM sodium phosphate, 3 M NaCl, 20 mM EDTA pH 7.4), (3-aminopropyl)triethoxysilane (APTES), 6-(ferrocenyl)hexanethiol, hexaammineruthenium (III) chloride, D-(+) Glucose, and indium tin oxide coated glass slide (square, surface resistivity 8-12 Ω/sq) were purchased from Sigma-Aldrich (Spain). Blocking casein (1% casein in PBS), sulfo-succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carbonate (Sulfo-SMCC) and PicoGreen® Assay for dsDNA were obtained from Thermo Scientific (Spain) and the enzyme conjugates anti-fluorescein-alkaline phosphatase (antiFITC-AP) and anti-fluorescein-POD Fab Fragments from Roche diagnostics (Spain).

Four different buffers were used: crosslinking buffer (CB; 50 mM sodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4), 0.15 M sodium chloride and 10 mM EDTA kept at pH 7.2), hybridization buffer (2× SSPE, pH 7.4; prepared by tenfold dilution of 20× SSPE), casein blocking buffer (0.5% casein in 1× PBS), and AP buffer (0.5 M Tris-HCl, pH 9.8, 1 mM $MgCl_2$).

Synthetic oligonucleotide sequences were obtained from Laboratorios Conda (Spain) purified by HPLC; and their sequences are listed in Table S1. All oligonucleotides stock

solutions were prepared in Milli-Q water and stored at -20° C. The thiolated oligos were commercially supplied as the respective disulfides. Prior to use, these products were treated with dithiothreitol (DTT, Sigma-Aldrich, Spain) and then purified by elution through a Sephadex G25 column (NAP-10, Amersham Biosciences) with Milli-Q water[1].

Table S1: Synthetic oligonucleotides used in this work.

	Function	**Name**	**Sequence (5'→3')**
Hybridization assay	Capture Probe	HS-CP	HS-C_6 CCGTTCTGACGCTGGCCCACTTCAC
	Signaling Probe	SP-6FAM	CCGGACGAATATCGTCGTAATG GCTGAAGGTGGAGTACA-6FAM
86bp Target Generation	Forward Primer	FP	GGTCTGCTGTACTCCACCTTCAG
	Reverse Primer	RP	TTGGAGATCAGTACGCCGTTCT
Solid Phase Amplification	Forward Primer (*in solution*)	6-FAM-FP_2	6FAM-GGTCTGCTGTACTCCACCTTCAGC
	Reverse Primer (*immobilized*)	HS-T_{10}-RP_2	HS-C_6-$(T)_{10}$TTGGAGATCAGTACGCCGTTCTGACGCT
	Reverse Primer (*in solution*)	RP	TTGGAGATCAGTACGCCGTTCT

All other reagents were of analytical grade. Unless otherwise indicated, double-deionized water (Milli-Q Millipore Corporation) was employed to prepare all aqueous solutions.

Electrochemical measurements of the hybridization assay were carried out with a conventional three-electrode electrochemical cell driven by a computer-controlled μ-Autolab type II potentiostat with GPES 4.9 software (Ecochemie, The Netherlands). A homemade platinum wire electrode acted as auxiliary electrode. All the potentials are referred to Ag | AgCl | KCl saturated reference electrode. The working electrode was kept in a micro-cell designed by our group and immersed in a regular cell maintaining the electrical contact through a salt bridge at the bottom. The measurements that required temperature control were carried out in a thermostatized cell by a HAAKE DC1 circulation thermostat.

For the integrated electrochemical solid phase HDA, measurements were carried out on ITO wells, each one connected through a copper strip and using external auxiliary and reference electrodes placed into a syringe (Figure 3 main text).

Optical measurements were performed with an Eon microplate spectrophotometer (BioTek Instruments, Inc.).

Sensing Surface Construction

For the hybridization assay, indium tin oxide coated glass slides were cut into pieces of about 8 × 5 mm and modified as follows. Each ITO surface was cleaned with acetone, ethanol and water and then treated with 1 M HCl for 10 min. Afterwards an oxidizing step was carried out by immersing the pieces into a solution of 1:1:5 (v/v) $H_2O_2/NH_4OH/H_2O$ during 1 h followed by rinsing with water and drying under a stream of nitrogen. These treated ITO was silanized with 1 % (3-aminopropyl)triethoxysilane (APTES) in absolute ethanol at room temperature overnight. After this reaction, the modified substrates were rinsed with ethanol and water to remove the physically adsorbed silanes and dried under a stream of nitrogen. To provide the electrical contact, a copper strip was coupled to each ITO fragment. Then, a 2 mm in diameter electrode surface was defined with an adhesive tape. Finally, lateral sides were protected from the solution with Teflon[2]. Sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carbonate (Sulfo-SMCC) was used as a heterobifunctional reagent with an amine-reactive group at one end and a thiol-reactive group at the other end that connects the silanized platform with the thiolated capture probe to form the sensing phase. Thus, amine modified ITO surfaces were incubated in 2 mg/mL sulfo-SMCC prepared in CB for an hour at room temperature and protected from light. The resulting maleimide-modified surface was rinsed with CB and dried with nitrogen. Finally, oligonucleotides were linked to the surface by incubation with 10 μM thiol-capture probe (HS-CP) in CB for 2 hours. The resulting surface was then washed with CB followed by water.

For the solid-phase HDA, ITO coated glass slides were scratched with a diamond to short-circuited the different cells. After silanization, different wells, each 8 mm in diameter, were defined by sticking a polycarbonate piece onto the modified ITO slide. The thiolated-reverse primer, $HS\text{-}T_{10}\text{-}RP_2$, was anchored to the surface as previously described for the thiolated capture probe, HS-CP.

Measurement of active groups for the DNA anchoring

Accessible on-surface maleimide groups, serving as attachment sites for thiolated DNA, were estimated as follows. ITO surfaces, functionalized with sulfo-SMCC and transformed into individual electrodes, were incubated with 1 mM of 6-(ferrocenyl)hexanethiol in CB for 2 h. Afterwards, the electrodes were thoroughly washed with CB and water, and the potential was scanned in 0.1 M $HClO_4$ by cyclic voltammetry (scan rate 100 mV/s). The anodic peak area (i.e: the quantity of charge consumed during the oxidation of the surface-confined ferrocene) was used to calculate the surface coverage of ferrocene, Γ_{Fc}.

Generation of Salmonella amplicons used as a target in the hybridization only assay

Genomic DNA of *Salmonella enterica* subsp. *enterica* was purchased from Spanish Type Culture Collection, Valencia, Spain (CECT 878) in lyophilized form. It was dissolved in Milli-Q water, aliquoted (5×10^6 genomic units per microliter, GU/µL), and stored at -20ºC.

For the electrochemical hybridization assay, an 86 bp DNA sequence specific of pathogenic *Salmonella* was used as a target and obtained by PCR amplification of *Salmonella* genome, using the IMMOLASE™ DNA Polymerase kit (Bioline, Spain), as well as a set of primers, FP and RP (see Table S1), previously described[3]. The reaction mixtures contained 1× PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 µM each primer, 1 U Immolase™ DNA polymerase, 2.5 µL of 10^6 GU of *Salmonella* genome and water to a total reaction volume of 25 µL. The amplification reactions were performed in a thermal cycler (GeneAmp® PCR System 2700 thermocycler (Applied Biosystems, Spain)). PCR amplification conditions were 10 min at 95 °C, then 40 cycles composed by 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. A final step was carried out at 72 °C for 7 min to extend any incomplete product. The 86 bp PCR product was purified with MinElute PCR purification kit (Qiagen) and quantified spectrophotometrically at 260 nm (GENESIS™ 10S UV-vis Spectrophotometer, ThermoScientific).

Hybridization assay

Hybridization experiments were carried out in a sandwich-like format. First, the homogenous hybridization between the target (86 bp *Salmonella* amplicon) and the signaling probe (SP-6FAM) takes place. 25 µL of solution containing 2 µM SP-6FAM and varying concentrations of 86 bp *Salmonella* amplicons in the hybridization buffer (2× SSPE; pH 7.4) were heated at 98 °C for 5 min and cooled down in ice-water bath for 5 min. After bringing the mixture to room temperature (30 min), 25 µL of 5 % BSA solution in 2× SSPE were added. 6 µL of this mixture was placed onto the sensing phase for 2 h (RT, in darkness). Then, the surface was washed with 1× PBS solution and 6 µL 0.5 U/mL of the antiFITC-AP conjugate in the casein blocking buffer was added to the electrode for 30 min (RT, in darkness). After a washing step with AP buffer, the electrode was placed in a micro-cell with 400 µL of AP buffer containing 4 mM of α-NPP and, after 10 min the current due to the oxidation of α-naphthol was measured by DPV from 0 to 0.8 V (modulation amplitude 50 mV, scan rate 10 mV s^{-1}).

Hybridization efficiency

Hybridization efficiency was estimated by measuring the fluorescence of a dsDNA-binding dye, PicoGreen®, with a Bio-tek FL600 fluorescence reader. With this aim, upon performing the sandwich hybridization assay onto sensing surface-modified ITO wells, these were filled with 50 µL of 0.5×PicoGreen® in 1×TBE buffer (10-times diluted form 10×TBE buffer (Tris-borate-EDTA), ThermoFisher Scientific) and the fluorescence emission intensity was recorded at 525 nm (excitation wavelength: 490 nm, optical path length was defined by the total volume in the well). The recorded signal was translated into dsDNA by means of a calibration curve carried out in solution with 0.5× PicoGreen® in 1× TBE buffer and dsDNA concentrations ranging from 0 to 500 ng/mL, using ITO wells.

Electrochemical/optical solid-phase HDA

The set of primers for isothermal solid-phase HDA amplification was designed starting from that described for PCR (FP and RP, Table S1), with minor modifications to bring them closer to the specific guidelines for HDA, i.e. primer size within the interval 24-33 nt, melting temperature (Tm) ranging from 60 to 74 °C, and GC% 35-60 %; considering specificity (Primer Blast[4]) and lack of complementary as well. As a result, FP_2 (24mer, Tm=68.5 °C, GC% of 58) and RP_2 (28mer, Tm=71.8 °C, GC% of 54) were selected, the latter being attached to the solid support through its 5' thiolated terminus with 10 thymines as spacer. Likewise, a shorter reverse primer, RP (22mer, Tm=62.1 °C, GC% of 50), was added to the solution.

Salmonella genome was amplified using the IsoAmp® II kit (BioHelix, Beverly, USA). The reactions were carried out in 50 µL total volume. Two separate reaction mixtures, A and B, were prepared. Mix A (25 µL) contained 11.25 µL of nuclease-free water, 2.5 µL of 10× annealing buffer, 3.75 µL 1 µM of forward primer, 2 µL 125 nM of reverse primer and 5 µL of *Salmonella* genome at several levels to obtain a final concentration between 10 and 10^6 GU. Mix B (25 µL), contained 6 µL of nuclease-free water, 2.5 µL of 10× annealing buffer, 1.5 µL of 100 mM $MgSO_4$, 3 µL of 500 mM NaCl, 3.5 µL of IsoAmp dNTP solution, 3.5 µL of IsoAmp enzymes mixture and 5 µL of 2.5% BSA in water. Mix A was incubated at 95 ºC for 2 min and at 65 ºC for another 5 min. Mix B was then added and the new mixture was incorporated to each well. A transparent adhesive film, devised for real-time PCR measurements, was used to seal the wells, reducing the possibility of cross-contamination between thereof and the evaporation of samples. This amplification reaction was conducted in an oven at 65 ºC for 90 min.

After two washing steps with 1× PBS buffer and additional one with casein blocking buffer, the enzymatic labeling was performed in 50 µL of the blocking solution containing 2 U/mL of anti-fluorescein-AP conjugate for the electrochemical detection or 0.5 U/mL of anti-fluorescein-POD conjugate for the optical detection. After 30 min of incubation time, the surface was washed three times with 1× PBS buffer. For the electrochemical detection, the cell was covered

with 100 µL of 4 mM α-NPP in measuring buffer. After 10 min of enzymatic reaction, a DPV voltammogram from 0 to 0.8 V was recorded (modulation amplitude 50 mV, scan rate 10 mV/s). For the optical detection, the enzymatic reaction was started by adding 50 µL of TMB + H_2O_2 solution and stopped, after 5 minutes, with 50 µL 1 M of sulfuric acid to measure the absorbance at 450 nm.

SUPPORTING FIGURES

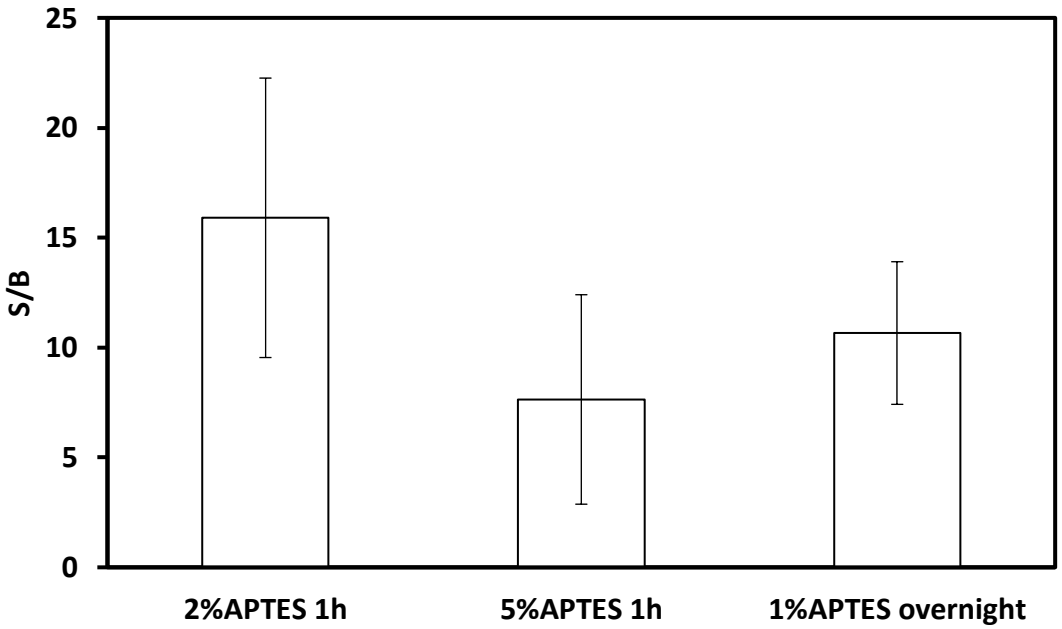

Figure S1: Silanization step optimization. Three different conditions for the silanization step with the amino silane compound were evaluated, modifying the concentration of silane and the reaction time. The surfaces were then modified with 10 µM capture probe and tested with and without 100 nM of 86 bp target. Though the highest signal/blank ratio was obtained with 2% APTES for 1 h, improved reproducibility was achieved with the lowest APTES concentration overnight.

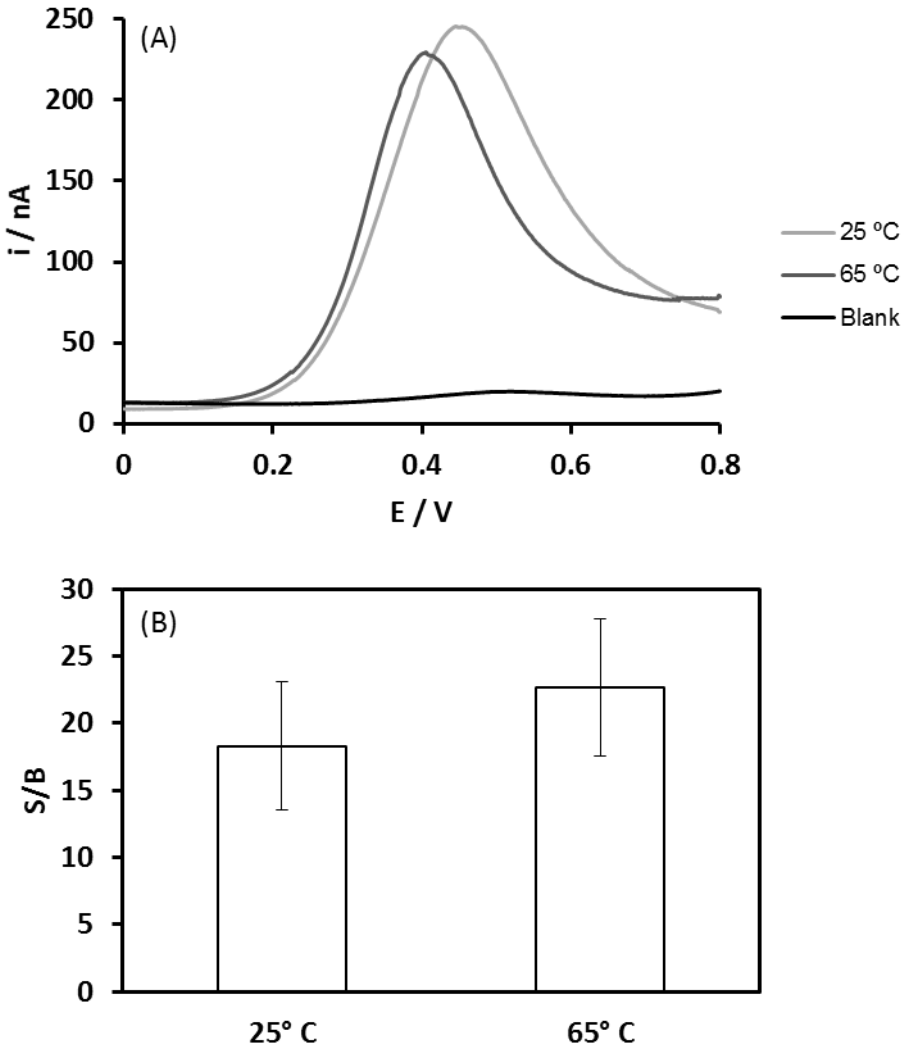

Figure S2: Thermal stability. (A) Differential pulse voltammograms and (B) signal to blank ratio obtained with the electrochemical sandwich assay in the presence of 100 nM 86 bp dsDNA target. Sensing surfaces built onto ITO slides were subjected to room temperature (25 ºC) or HDA temperature conditions (65 ºC) for 1 h.

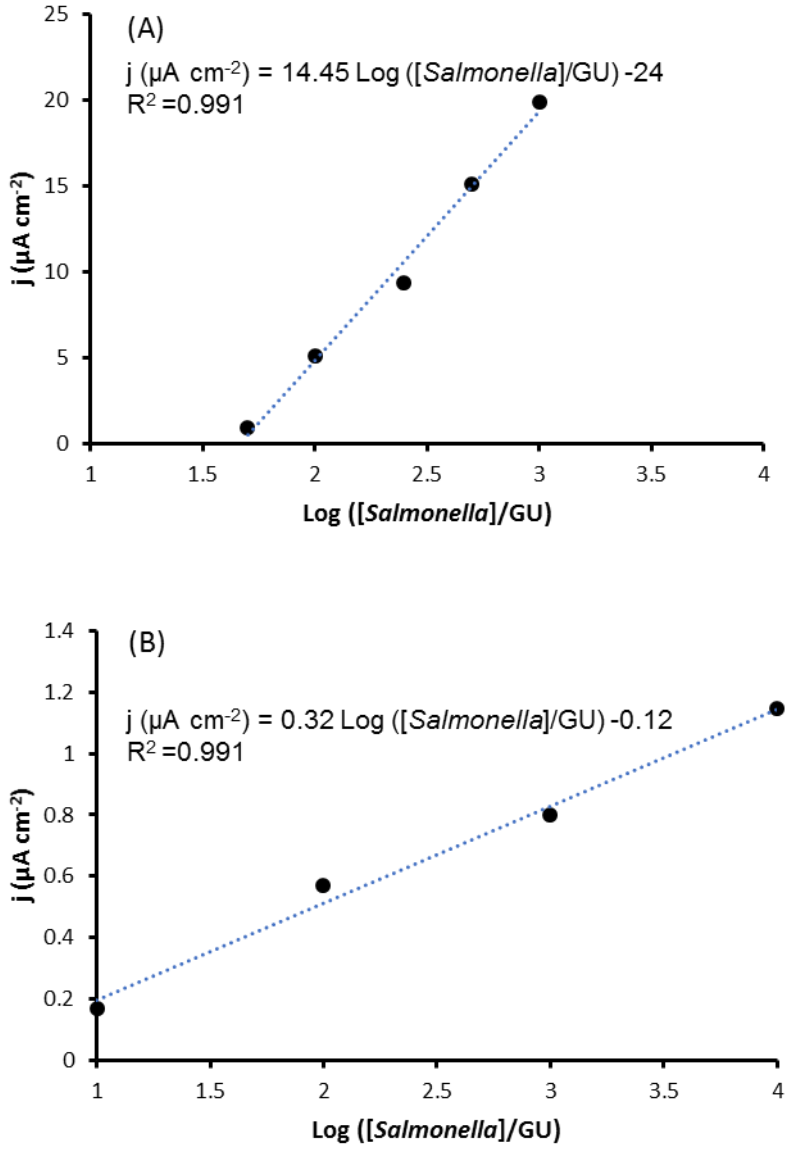

Figure S3: Electrochemical monitoring of HDA amplification for increasing amounts of *Salmonella* genome (A) liquid phase amplification with 75 nM of both reverse and forward primer, followed by the detection of amplicons by a sandwich assay on ITO electrodes (B) on-surface amplification with anchored RP_2 primer and a RP to FP_2 ratio 1:15 in solution.

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