Comparing nucleic acid lateral flow and electrochemical genosensing for the simultaneous detection of foodborne pathogens

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Abstract

Due to the increasing need of rapid tests for application in low resource settings, WHO summarized their ideal features under the acronym ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid & Robust, Equipment-free, Delivered to those who need it). In this work, two different platforms for the rapid and simultaneous testing of the foodborne pathogens *E.coli O157:H7* and *Salmonella enterica*, in detail a nucleic acid lateral flow and an electrochemical magneto genosensor are presented and compared in terms of their analytical performance. The DNA of the bacteria were amplified by polymerase chain reaction using a quadruple-tagging set of primers specific for *E. coli eaeA gen* (151 bp) and *Salmonella enterica yfiR gen* (375 bp). During the amplification, the amplicons were labelled at the same time with biotin/digoxigenin or biotin/fluorescein tags, respectively. The nucleic acid lateral flow assay was based on the use of streptavidin gold nanoparticles for the labelling of the tagged amplicon from *E. coli* and *Salmonella*. The visual readout was achieved when the gold-modified amplicons were captured by the specific antibodies. The features of this approach are discussed and compared with an electrochemical magneto genosensor. Although nucleic acid lateral flow showed higher limit of detection, this strategy was able to clearly distinguish positive and negative samples of both bacteria being considered as a rapid and promising detection tool for bacteria screening.

Keywords: Nucleic acid lateral flow, electrochemical magneto genosensing, foodborne bacteria, simultaneous detection, magnetic particles

1. Introduction

The polymerase chain reaction (PCR) (Mullis and Faloona, 1987; Saiki et al., 1988) has found widespread application in many areas including the diagnostic of infectious diseases. As main advantages, the PCR can improve test sensitivity up to 100-fold over immunoassays and with much more rapid turnaround times compared with classical culturing. However, PCR requires thermocycling platforms, trainee personnel, and infrastructure including reliable power supply, which can be a barrier for its application in some low-resource settings (Urdea et al., 2006). To overcome this issue, recent work has focused on PCR platforms that are cheap, portable and operated with batteries (Marx, 2015) that are now commercialized, including Palm PCR™ (Ahram Biosystems Inc.), Freedom4 (Ubiquitome), miniPCR (Amplyus), among others. The detection of PCR products can be easily achieved by electrochemical genosensing

(Pividori et al., 2002). Since this early report, novel routes based on tagging-PCR procedures to increase the sensitivity of the electrochemical detection and, at the same time to achieve the immobilization of the amplicon on different platforms were explored (Lermo et al., 2007; Brasil de Oliveira Marques et al., 2009). Among this platforms, the magnetic particles (MPs) greatly enhance the performance of the biological reaction by increasing the surface area, improving the washing steps and, importantly, minimizing the matrix effect (Lermo et al., 2008). MPs also allow reduction of reaction times and reagent volumes. In addition, MPs can be easily magneto-actuated using permanent magnets (Brandão et al., 2015b). Recently, a triple-tagging multiplex PCR amplification strategy for the simultaneous electrochemical genosensing of foodborne pathogens was reported (Brandão et al., 2015a). In this work, a set of tagging primers were selected for the specific multiplex amplification of the bacteria, being one of the primers for each set (the forward primers) labelled with fluorescein (FLU), biotin (BIO) and digoxigenin (DIG) coding for *Salmonella*, *Listeria* and *E. coli*, respectively, while the reverse primers are not labelled. Afterwards, silica magnetic particles were used as a platform for the immobilisation by physical adsorption of the amplicons which were further labelled with three different specific antibodies (conjugated with horseradish peroxidase, HRP), in three separated reaction chambers for each pathogen: antiFluorescein-HRP (antiFLU-HRP) coding for *Salmonella*, streptavidin-HRP (strepAv-HRP) coding for *Listeria,* and antiDigoxigenin-HRP (antiDIG-HRP) coding for *E. coli,* respectively. Magnetic actuation in three differentiated magneto-electrodes for each pathogen was then performed. As a main advantage of this approach, the use of the same electrochemical reporter (HRP) allowed the simultaneous electrochemical detection in an array of electrodes to be performed in the same electrochemical cell, by using the same substrate and mediator for the enzyme. Recently, there have been significant developments in the detection of amplicons directly without the need of an instrument or gel electrophoresis (Tomita et al., 2008, Niemz et al., 2010) using, for instance, lateral-flow assay (LFA) (Darren et al., 2007; Singh et al., 2015). This technology introduced in 1988 by Unipath is the most common commercially available point of care (PoC) diagnostic format. The LFA incorporates porous membranes, antibodies, and a visible signal-generating system (commonly colloidal gold or dyed polystyrene or latex spheres). It depends upon fluid migration or flow technology (Chun, P., 2009; O'Farrell, B., 2009). LFAs are currently used for qualitative –and to some extent quantitative– monitoring in non-laboratory environments. Although there are many commercial available examples for biomedical diagnosis including the pregnancy test, other applications are still under development (Gubala et al., 2012). Lately, many methodological improvements have been done (Posthuma-Trumpie et al.,

2009), although in general the sensitivity observed for this technology should be improved (Seo et al., 2003; Moongkarndi et al., 2011).

This work addresses, for first time, the simultaneous detection of two of the most important foodborne pathogens, *Salmonella enterica* and *E. coli O157:H7* (Altekruse, S. F. et al.,1997; WHO, 2015) based on the quadruple-tagging PCR amplification of DNA and by comparing two different approaches for the readout: electrochemical magneto-genosensing and NALF. A set of tagged primers for the quadruple-tagging PCR were selected for the amplification of yfiR (375 bp) and eaeA (151 bp) genes specific for *Salmonella* and *E. coli,* respectively. During PCR, the DNA of each pathogen is amplified and double-labelled at the same time by BIO/FLU and BIO/DIG tags. In the electrochemical magneto-genosensor strategy, the BIO tags, carried by the reverse primers, is common for both pathogens and used for the immobilization of the amplicons on streptavidin-magnetic particles (streptAv-MPs), while the FLU and DIG-tags, carried by the forward primers, are used for the labelling with the specific antibodies, antiFLU-HRP and antiDIG-HRP, coding for *Salmonella* and *E. coli,* respectively, and performed in two separated reaction chambers. The simultaneous electrochemical readout of the two pathogens is based on HRP as electrochemical reporter and performed in the same electrochemical cell, as previously reported (Brandão et al., 2015a). On the contrary, in the NALF strategy, the common BIO-tag is used for the labelling and the visual readout based on streptavidin-gold nanoparticles (streptAv-AuNPs), while FLU and DIG tags, for capturing the amplicon in separated location on the strip by the specific antibodies antiFLU, coding for *Salmonella*, and antiDIG, coding for *E. coli*. The analytical performance of the NALF test and the electrochemical magneto-genosensor are discussed and compared. The NALF test showed promising features, including outstanding limit of detection (LOD) for bacteria screening of the most relevant pathogens in food.

2. Experimental section

2.1. Instrumentation and materials

Glass fiber conjugate pad (GFCP083000) and cellulose fiber sample pad strips (CFSP203000) were purchased from Millipore. Adhesive Backing Cards were obtained from Kenosha C.V. (Netherlands) and nitrocellulose membranes (FP120HP) as well as the absorbent pads (CF7) were purchased from GE Healthcare Europe. Lateral Flow Reagent Dispenser from Claremont Bio (Upland, CA) combined with the KDS Legato™ 200 series syringe pump from KD Scientific Inc. (Holliston, MA) was used to dispense the test and control line. Electrochemical measurements were performed with a LC-4C

amperometric controller (BAS Bioanalytical Systems Inc., U.S.) and Autolab PGSTAT Eco-chemie, using magneto-electrodes based on graphite-epoxy composite (m-GEC) as working electrodes (Pividori and Alegret, 2005).

2.2. Chemicals and biochemicals

Dynabeads streptavidin magnetic beads (Prod. N° 112.06) (streptAv-MPs) were purchased from Invitrogen Dynal AS (Oslo, Norway). InnovaCoat® GOLD 40nm Streptavidin gold nanoparticles (streptAv-AuNPs) were purchased from Innova Biosciences (Cambridge, UK). Anti-digoxigenin (11214667001) (antiDIG), anti-fluorescein (11426320001) (antiFLU), anti-digoxigenin-POD (11426346910) (antiDIG-HRP) and anti-fluorescein-POD (11426346910) (antiFLU-HRP) were purchased from Roche Diagnostics. The buffer solutions were prepared with milliQ water and all other reagents were in analytical reagent grade (supplied from Sigma and Merck). The composition of these solutions is described in Supp. data.

2.3. Preparation of the lateral-flow strips

The streptAv-AuNPs were diluted 8 times in conjugate diluting buffer and embedded in the glass fiber conjugate pads. The cellulose fiber sample pads were then soaked into sample pad buffer. The pads were then dried for 3 h at RT. The antibodies and the positive control biotinylated reporter were dispensed on the nitrocellulose membrane and were then dried at RT for 1 h. Finally, the strips were assembled in the usual way on the adhesive backing card.

2.4. Bacterial strains, growth conditions and DNA extraction

The bacterial strains *Salmonella enterica serovar* Typhimurium (ATCC® 700720™) and *E. Coli* O157:H7 (clinical isolate supplied by Hospital of Bellvitge, Barcelona, Spain) were grown in Luria Bertani (LB) broth or agar plates for 18 h at 37 °C. The lysis of the bacteria, DNA extraction and purification was performed according to the kit manufacturer (DNeasy Tissue and Blood Kit, Qiagen). The extraction and purification efficacy was evaluated by spectrophotometric analysis as UV absorption at 260 nm.

2.5. Oligonucleotides sequences

The oligonucleotides were obtained from TIB- Molbiol GmbH (Berlin, Germany). These primers were selected for the amplification of *yfiR* (375 bp) and *eaeA* (151 bp) gene

fragments specific to *S. Typhimurium* and *E. coli*, respectively (Kawasaki et al., 2005). Each set of primer was double tagged in 5′ end with BIO/FLU and BIO/DIG (Table 1).

Preferred position for Table 1

2.6. Quadruple-tagging PCR

The quadruple-tagging PCR was achieved by a set of four tagging primers for the amplification of the *yfiR* (375 bp), and *eaeA* (151 bp) genes, being the primers for each set labelled with FLU/BIO and DIG/BIO coding for *Salmonella* (S) and *E. coli* (C), respectively (Table1). During the amplification, the amplicons were labelled at the same time with BIO/FLU for *Salmonella* (S) and BIO/DIG for *E. coli* (C). The PCR was performed by using 100 ng of chromosomal DNA of each microorganism in the PCR mixture. Moreover, the negative controls were also included, in which no DNA template was added to the PCR mixture. The Expand High Fidelity PCR System kit (Roche Molecular Biochemicals) was used for performing the PCR reaction in a thermal cycler (Product Nº 2720, Applied Biosystems, Life Technologies Corporation) (as shown in Table S1 and Figure S1, Supp. Data). The performance of the quadruple-tagging PCR were analysed with conventional agarose gel electrophoresis on 4% agarose gel containing 0.5 x Tris-acetate-EDTA (TAE) and ethidium bromide staining, using HinfI digested ϕ174 DNA as a molecular weight marker, as shown in Figure S2.

2.7. Simultaneous detection of *Salmonella* and *E. coli* by quadruple-tagging PCR and electrochemical magneto-genosensing assay

The detailed procedure for the simultaneous detection of *Salmonella* and *E. coli* is described in Supp. data and schematically shown in Figure 1. Briefly, after the quadruple-tagging PCR, the product was divided in two separated reaction chambers. The common BIO-tag was used for the immobilization of the amplicons on streptavidin-magnetic particles (streptAv-MPs), while the FLU and DIG-tags allowed the labelling by the specific antibodies, antiFLU-HRP and antiDIG-HRP, coding for *Salmonella* and *E. coli*, respectively. The procedure comprised the following steps: (i) immobilization and preconcentration of the tagged amplicons on streptAv-MPs, based on the BIO-tag of the amplicons and (ii) incubation with the electrochemical reporters, in detail AntiFLU–HRP, and AntiDIG–HRP coding for *Salmonella* (FLU-tag), and *E. coli* (DIG-tag), respectively, in two different incubation chambers; (iii) magnetic actuation by an array of two working electrodes (one coding for *E. coli*, while the other for *Salmonella*), which

contain a small magnet (m-GEC) (Pividori and Alegret, 2005); (iv) amperometric readout using the m-GEC electrodes polarized at -0.100V (vs. Ag/AgCl), under enzyme saturation conditions in PBSE buffer, upon the addition of hydroquinone (1.81 mM) and hydrogen peroxide (4.90 mM). More details about the amperometric determination are provided in Supp. data (Figure S3). Further characterization of the magneto electrodes (including reproducibility of the construction, renewal and reusability, and stability are also detailed in Supp. data, Figures S4, S5 and S6. The steady-state current was used for the electrochemical signal plotted in further results shown in Figs. 2 and 3. In order to determine the LODs, a calibration curve was performed with increasing amount of *E. coli* and *Salmonella* amplicon. The specificity of the assay was performed by challenging all possible combinations, including i) the binary combinations (S/C), and ii) the single combinations (S; C), as well as a negative control.

Preferred position for Figure 1

2.8. Simultaneous detection of *Salmonella* and *E. coli* by quadruple-tagging PCR and nucleic acid lateral flow assay

The procedure for the simultaneous detection of *Salmonella* and *E. coli* by NALF is schematically described in Figure S7, Supp. data. Different amounts of the tagged amplicons were diluted in 150 μL of running buffer and the mixed was added to the sample pad (Figure S7, panel b1). After 5 min, 100 μL of running buffer were added in order to drag the remaining streptAv-AuNPs to the absorbent pad. The streptAv-AuNPs thus reacted with the common BIO-tag of the amplicons from *E. coli* and *Salmonella* (Figure S7, panel b2). As the products moved along the strip, the streptAv-AuNPs/amplicons were captured by the specific antibodies (antiDIG coding for *E. coli* and antiFLU coding for *Salmonella*) in separated location on the strip (Figure S7, panel b3). A valid test was considered when the remaining streptAv-AuNPs reacted with a biotinylated reporter used as a positive control at the control line. The visual readout was thus achieved as well as the interpretation of the results (Figure S7, panel b4, Supp. Data). For the quantification of the optical signal, the images were taken with FastGene FAST Digital System and the resulting images were processed with ImageJ software (NHI). In order to determine the LODs, a calibration curve was performed with increasing amount of *E. coli* and *Salmonella* amplicon. The specificity of the magneto-genosensors coding for each bacteria, *Salmonella* (S) and *E. coli* (C) was performed by challenging all possible combinations, including i) the binary combinations (S/C), and ii) the single combinations (S;C), as well as a negative control. To come up with the

stability and repeatability study, nine strips were prepared and evaluated along 15 days. They were protected from light and moisture by wrapping them in aluminum foil and kept in a zip plastic bag with silica gel. Four of them were kept at room temperature (RT), while other two were kept at 4ºC.

3. Results and Discussion

3.1. Quadruple-tagging PCR

The end-point amplicons studied by agarose gel electrophoresis are shown in Figure S2 (Supp. Data). Two separated bands, related to each fragment target gene, in detail *S. enterica yfiR* gene fragment (375 pb) and *E. coli eaeA* gene fragment (151 bp) were observed in lane 4, corresponding to the expected amplicon size when compared with the molecular weight markers in lane 5 (in base pairs). The single combinations are shown in lanes 2 and 3. The negative control with no DNA template is also shown in Fig. S2, lane 1. This control is mandatory since it can detect the presence of primer secondary structures (including hairpins, self and cross dimmers, produced by inter and intramolecular interactions between the primers), which can adversely affect primer template annealing, by reducing the availability of primers to the reaction leading to poor or no yield of the product. Moreover, such undesirable products carrying tags can also be non-specifically amplified, leading to false positive results. As expected, no undesirable amplification in the negative control was observed. Moreover, each target bacteria produced a specific band relative to its correspondent amplicon, showing the PCR outstanding specificity. The relative intensities of the bands can be correlated with the individual performance of each set of primers, and the amplicon length, since as higher the amplicon length is, greater the signal will be, due to ethidium bromide staining (Kawasaki et al., 2005).

3.2. Simultaneous detection of *Salmonella* and *E. coli* by quadruple-tagging PCR and electrochemical magneto-genosensing assay

The LOD of the method was firstly calculated by serial dilution of the tagged-amplicon in single combinations, and the results are shown in Figure 2. For *E. coli*, the amplicon ranging from 0.0 to 0.52 ng was processed by using antiDig–HRP as electrochemical reporter (while keeping the *Salmonella* in 0.0 ng). In the case of *Salmonella*, the amplicon ranging from 0.0 to 0.74 ng was processed by using antiFLU-HRP as electrochemical reporter (while keeping the *E. coli* in 0.0 ng). In both cases, the electrochemical signal was fitted using a nonlinear regression (Four Parameter logistic Equation– GraphPad Prism Software) (R^2=0.9973 and 0.9927 for *E. coli* and

Salmonella, respectively). The LOD was calculated for *E. coli*, by processing the negative control samples (n=6) obtaining a mean value of 0.540 μA with a standard deviation (SD) of 0.052. The cut-off value was then determined with a one-tailed t test at a 95% confidence level (t=2.015), giving a value of 0.645 μA. The LOD was found to be 0.083 ng in 140 μL of sample (0.59 pg μL^{-1}). The LOD was calculated for *Salmonella*, by processing the negative control samples (n=6) obtaining a mean value of 0.455 μA with SD of 0.052. The cut-off value was then determined with a one-tailed t test at a 95% confidence level (t=2.015), giving a value of 0.561 μA. The LOD was found to be 0.105 ng in 140 μL of sample (0.75 pg μL^{-1}).

Preferred position for Figure 2

Figure 3 shows the simultaneous detection of *E. coli* (Figure 3, panel A) and *Salmonella* (Figure 3, panel C) by quadruple-tagging PCR followed by electrochemical magneto-genosensing on streptAv-MPs. The LOD of the method was calculated in three replicates of each serial dilution of the tagged-amplicon in the binary combinations. For *E. coli*, the amplicon ranging from 0.0 to 0.52 ng was processed by using antiDig–HRP as electrochemical reporter (while keeping the *Salmonella* in 0.74 ng). On the contrary, for *Salmonella*, the amplicon ranging from 0.0 to 0.74 ng was processed by using antiFLU-HRP as electrochemical reporter (while keeping the *E. coli* in 0.52 ng). In both instances, the electrochemical signal was fitted using a nonlinear regression (Four Parameter logistic Equation– GraphPad Prism Software) (R^2=0.9958 and 0.9910 for *E. coli* and *Salmonella*, respectively). The LOD were calculated as above, obtaining similar values than those for the single combinations shown in Figure 2: 0.092 ng in 140 μL of sample (0.66 pg μL^{-1}) for *E. coli* (when *Salmonella* is also present at high concentration level), and 0.164 ng in 140 μL of sample (1.17 pg μL^{-1}) for *Salmonella* (when *E. coli* is also present at high concentration level), highlighting the robustness and specificity of the method. However, the specificity of the magneto-genosensors coding each bacteria, *Salmonella* (S) and *E. coli* (C), was further studied in three replicates by challenging all possible combinations of the amplicons, including i) the binary combinations (S/C), ii) the single combinations (S; C), as well as the negative control, with the two electrochemical reporters (antiFLU-HRP and antiDIG–HRP, respectively). As observed in Figure 3, panels B and D, each of the electrodes only detected one of the two pathogen, even in the presence/or absence of the other one. For instance, in the electrode coding for *E. coli* (Figure 3, panel B), the mean value for the electrochemical signal obtained for *E. coli* (C) (13.47 μA, CV% 3.0), is almost the same when *Salmonella* is also present (S/C) (13.35 μA, CV% 3.8), while

when *E. coli* is absent (negative control) (0.58 μA, CV% 7.8), the signal is equal even in the presence of *Salmonella* (S) at high concentration level (0.51 μA, CV% 6.0) (Figure 3, panel B). Similar results were obtained in the case of *Salmonella* (Figure 3, panel D), since the mean value for *Salmonella* (S) (7.53 μA, CV% 4.3), is almost the same when *E. coli* is also present (S/C) (7.68 μA, CV% 2.5), while when *Salmonella* is absent (negative control) (0.45 μA, CV% 4.6), the signal is equal even in the presence of *E. coli* (C) (0.49 μA, CV% 8.1), highlighting the specificity of both, the quadruple-tagging PCR, as well as the electrochemical detection. Hence, the results suggest that this approach was able to clearly distinguish between the two different bacteria and their single and binary combinations, with outstanding repeatability. The stability of the magneto-genosensing approach is determined by the stability of the reagents (PCR mix, antibodies and strepAv-MPs), that should be kept at 4ºC as recommended by the manufacturers. As the m-GEC electrode is not biologically-modified, they were storage at RT. Further details about reproducibility of the construction, renewal and reusability, and stability of the m-GEC electrodes are provided in Supp. data, Figures S4, S5 and S6.

Preferred position for Figure 3

3.3. Simultaneous detection of *Salmonella* and *E. coli* by quadruple-tagging PCR and nucleic acid lateral flow assay

The detection of *Salmonella* and *E. coli* by quadruple-tagging PCR followed by NALF was performed as schematically shown in in Figure S7 (supp. Data). The total assay time is less than 15 min. The results of the tests can either be estimated with the naked eye or by measuring the intensity of the red bands with the software ImageJ. The LOD of the method was calculated by serial dilution of the tagged-amplicon in single combinations, and the results are shown in Figure 4, panel A. For *E. coli*, the amplicon was ranging from 0.0 to 103 ng while for *Salmonella*, from 0.0 to 143 ng. The relative areas obtained by processing the images were fitted using a nonlinear regression (Four Parameter logistic Equation– GraphPad Prism Software) (R^2=0.9952 and 0.9958 for *E. coli* and *Salmonella*, respectively). The NALF approach was able to visually detect (signalling by arrows in Figure 4) as low as 5.2 ng in 150 μL of sample (LOD = 34 pg μL^{-1}) for *E. coli* and 14.3 ng in 150 μL of sample (LOD = 95 pg μL^{-1}) for *Salmonella*.

Preferred position for Figure 4

The Figure 4, panel B, shows the specificity study for the simultaneous detection of *E. coli* and *Salmonella*, in single and binary combination, with an amount of amplicon of 51 ng of *E.coli* and 71 ng of *Salmonella,* as well as the negative control. As can be observed, the negative control only provided signal in the control line, as expected. No cross-reaction of the signal-generating system (strept(Av)-AuNPs) were thus observed with the specific antibodies (antiDIG coding for *E. coli* and antiFLU coding for *Salmonella*) located on the test lines of the strip. Furthermore, for samples containing exclusively the *Salmonella* amplicon, only the antiFLU test line provided a positive signal. No cross-reaction of the BIO/FLU double-tagged *Salmonella* amplicon was thus observed with the antiDIG antibody located in the test line coding for *E. coli*. Similarly, for samples containing only the BIO/DIG double-tagged *E. coli* amplicon, no cross-reaction was observed in the antiFLU test line coding for *Salmonella*. Finally, the binary combination provided signals in both test lines, coding for *Salmonella* and *E. coli.* Hence, the results suggest that this approach was able to clearly distinguish between the different bacteria and their single and binary combinations.

The stability of the strips was evaluated along 15 days, by keeping the strips protected from light and moisture, at RT and at 4ºC. The results for the binary combination containing 51 ng of *E.coli* and 71 ng of *Salmonella* are shown in Figure 5. The interday stability study during 15 days, showed CV% of 12.3, 12.7 and 7.8 % for *E. coli*, *Salmonella* and the positive control line, respectively (n=9). The repeatability of the NALF assay was study with the strips recently prepared (numbered as 1, 2 and 3, Figure 5), showing CV% of 4.5, 7.3 and 8.8 %, for *E. coli*, *Salmonella* and the positive control line, respectively, suggesting also a good reproducibility in the preparation of the strips (n=3). Furthermore, no significant differences in signals were observed when the strips were storage at RT or 4ºC. The CV% for the strips (n=3) kept for 1 week at RT (Nº 4 and 5) and 4ºC (Nº 6), were 5.4, 5.9 and 7.8 % for *E. coli*, *Salmonella* and the positive control, while also similar CV% (7.1, 3.0 and 5.3 %) were obtained when the strips were kept for 2 weeks (nº 7, 8 and 9). The results are also similar to the strips recently prepared (Nº 1, 2 and 3), suggesting that they can be storage either at RT or 4ºC without any loss in the activity, at least for 15 days. Although it is known that the biological reagents can be housed in the lateral flow strips at RT without loss of activity before an expiration date (for instance, the commercial available pregnancy tests), further studies should be done for longer storage period. Finally, it is important to highlight that none of the NALF assays performed in this work provided invalid results, since in all cases the line corresponding to the positive control was observed.

Preferred position for Figure 5

4. Conclusions

Concerns about food safety have increased in more affluent societies. The Center for Disease Control and Prevention estimates that only in the United States each year roughly 48 million people get sick from a foodborne illness. Contamination can occur during production, processing, distribution or preparation. For this reason, it is extremely important the detection at any point of the food chain production with rapid and reliable techniques. Beside this, it is important to highlight the burden of foodborne diseases in the developing world, where the facilities and equipments for the detection of pathogens combined with the lack of availability of essential medicines and supplies for treatment make increase the case fatality rate. According to World Health Organization, billions of people are at risk and it is estimated that 1 in 10 people fall ill every year and 420000 die as a result of consuming contaminated food. Recent guidelines published by WHO recommend that diagnostic devices for developing countries to be ASSURED being this acronym defined by (A) Affordable, (SS) Sensitive, Specific, (U) User-friendly, (R) Rapid and Robust, (E) Equipment free, and (D) Deliverable to those who need it. In this work, two methods (electrochemical genosensors and NALF) following these recommendations are compared, demonstrating to be promising candidates for the detection of *Salmonella* and *E. coli* at low-resource settings. The specificity was studied obtaining outstanding results with both methods, being able to clearly distinguish between the different bacteria and their single and binary combinations. Among the two methods, it has to be highlighted the simplicity, low cost and the rapidness of lateral flow. Qualified personal is not required and the results can be read with the naked eye in less than 15 min unlike the electrochemical magneto genosensor which the time of assay is 2 hours. Nevertheless, the electrochemical magneto genosensor showed a higher sensitivity and noticeable improved limits of detection, being the LODs as low as 83 pg of *E.coli* PCR amplicon (0.59 pg μL^{-1}) and 105 pg for *Salmonella (*0.75 pg μL^{-1}) compared with 5.2 ng (34 pg μL^{-1}) and 14.3 ng (95 pg μL^{-1}) for *E.coli* and *Salmonella,* respectively, visually detected by the NALF approach. Furthermore, the electrochemical magneto genosensor provides quantitative results. Although both methods require PCR for amplification, reliable thermocyclers that are cheap, portable and operated with batteries are now in the market, which can easily be adapted in resource-constrained settings to meet the demands for ASSURED diagnosis recommended by WHO.

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Figure captions

Table 1. Sequences of the set of primers for the quadruple-tagging PCR amplification for the simultaneous detection of *Salmonella* and *E. coli*.

Figure 1. Schematic representation of the simultaneous detection of *Salmonella* and *E. coli* by (A) Quadruple-tagging PCR followed by either (B) Electrochemical magneto-genosensing on streptAv-MPs or (C) Nucleic acid lateral flow.

Figure 2. Electrochemical responses for the electrochemical magneto genosensing at amplicon amounts in single combinations ranging from (■) 0.0 to 0.52 ng of *E. coli* amplicon (while keeping the *Salmonella* in 0.0 ng) using 60 µg AntiDIG–HRP and (▲) 0.0 to 0.74 ng of *Salmonella* amplicon (while keeping the *E. coli* in 0.0 ng) using 60 µg AntiFLU-HRP. The error bars show the standard deviation for n = 3. The negative controls are also shown (n=6).

Figure 3. Electrochemical responses for the simultaneous electrochemical magneto genosensing at amplicon amounts in binary combinations ranging from (■) 0.0 to 0.52 ng of *E. coli* amplicon (A) (while keeping the *Salmonella* at high amount of 0.72 ng) using 60 µg AntiDIG–HRP and (▲) 0.0 to 0.72 ng of *Salmonella* amplicon (C) (while keeping the *E. coli* in 0.52 ng) using 60 µg AntiFLU-HRP. Panel (B) and (D) show the specificity study for i) the binary combinations (S/C), and iii) the single combinations (S; C), challenged towards (B) 60 µg AntiDIG-HRP coding for *E. coli* and (D) 60 µg AntiFLU–HRP coding for *Salmonella*. In all cases (B and D), an amplicon amount of 0.52 and 0.72 ng, respectively for *E. coli* and *Salmonella*. The error bars show the standard deviation for n=3. The negative controls are also shown (n=3).

Figure 4. (A) Results obtained for the NALF at amplicon amounts in single combinations ranging from (■) 0.0 to 103 ng of *E. coli* amplicon (while keeping the *Salmonella* in 0.0 ng) and (▲) 0.0 to 143 ng of *Salmonella* amplicon (while keeping the *E. coli* in 0.0 ng). The corresponding images of the strips are also shown. (B) Results obtained in single and binary combination, with an amount of amplicon of 51 ng of *E.coli* and 71 ng of *Salmonella*.

Figure 5. Stability and repeatability study of the NALF, for the binary combination containing 51 ng of *E.coli* and 71 ng of *Salmonella*. The corresponding images of the strips are also shown. n=9.

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504

TABLE 1

STRAIN	GENE	PRIMER SEQUENCE (5'-3')	TYPE	5'-LABELS	SIZE (bp)
S. enterica	*yfiR*	GTCACGGAAGAAGAGAAATCCGTACG	Forward	Fluorescein	375
		GGGAGTCCAGGTTGACGGAAAATTT	Reverse	Biotin	
E. coli	*eae*A	GGCGGATAAGACTTCGGCTA	Forward	Digoxigenin	151
		CGTTTTGGCACTATTTGCCC	Reverse	Biotin	

Figure 1

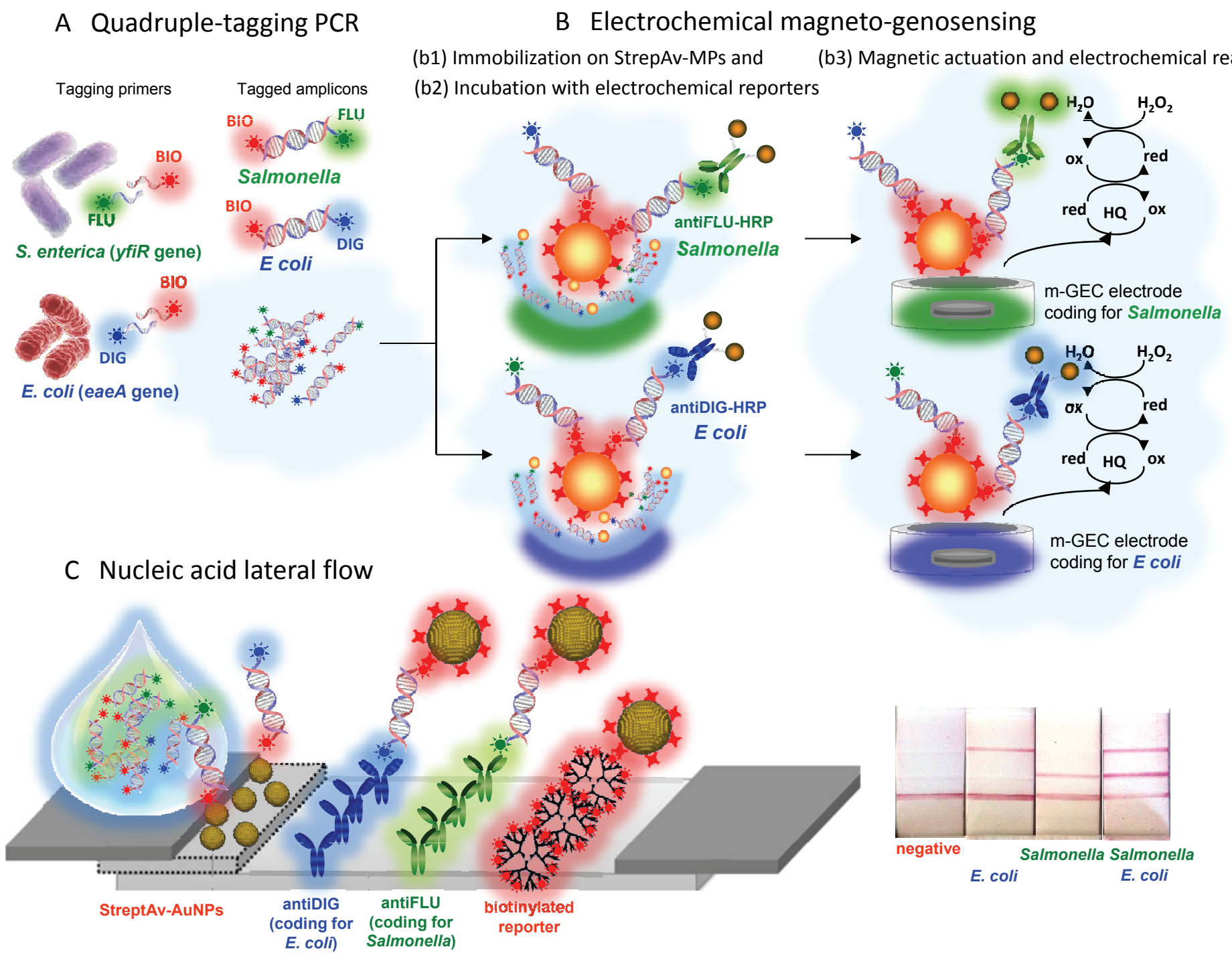

A Quadruple-tagging PCR
Tagging primers
Tagged amplicons
BIO
FLU
S. enterica (yfiR gene)
Salmonella
DIG
E coli
E. coli (eaeA gene)
B Electrochemical magneto-genosensing
(b1) Immobilization on StrepAv-MPs and
(b2) Incubation with electrochemical reporters
(b3) Magnetic actuation and electrochemical readout
antiFLU-HRP
antiDIG-HRP
H2O
H2O2
ox
red
HQ
m-GEC electrode coding for Salmonella
m-GEC electrode coding for E coli
C Nucleic acid lateral flow
StreptAv-AuNPs
antiDIG (coding for E. coli)
antiFLU (coding for Salmonella)
biotinylated reporter
negative
Salmonella
E. coli
Salmonella
E. coli

Figure 2

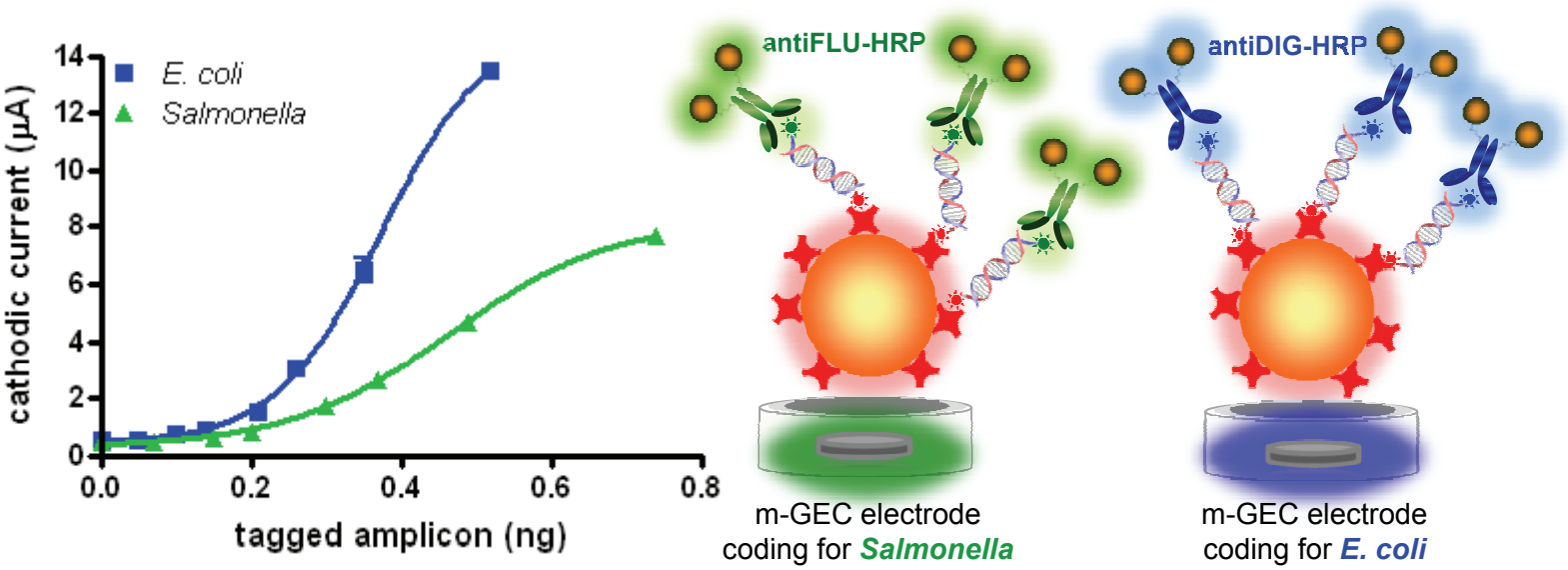

E. coli
Salmonella
cathodic current (µA)
tagged amplicon (ng)
0
2
4
6
8
10
12
14
0.0
0.2
0.4
0.6
0.8
antiFLU-HRP
antiDIG-HRP
m-GEC electrode coding for Salmonella
m-GEC electrode coding for E. coli

Figure 3

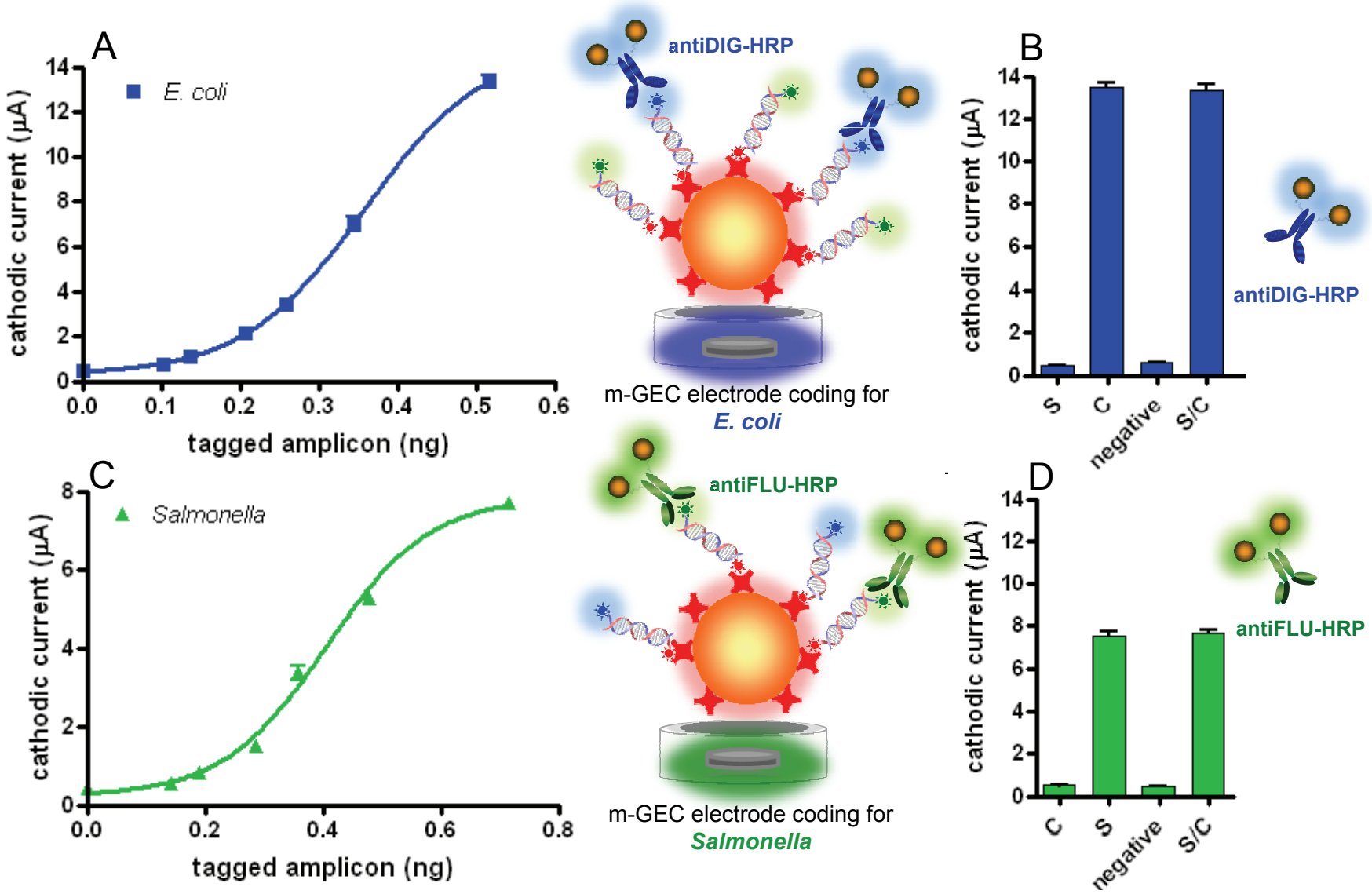

A
E. coli
cathodic current (μA)
tagged amplicon (ng)
antiDIG-HRP
m-GEC electrode coding for
E. coli
B
cathodic current (μA)
S
C
negative
S/C
antiDIG-HRP
C
Salmonella
cathodic current (μA)
tagged amplicon (ng)
antiFLU-HRP
m-GEC electrode coding for
Salmonella
D
cathodic current (μA)
C
S
negative
S/C
antiFLU-HRP

Figure 4

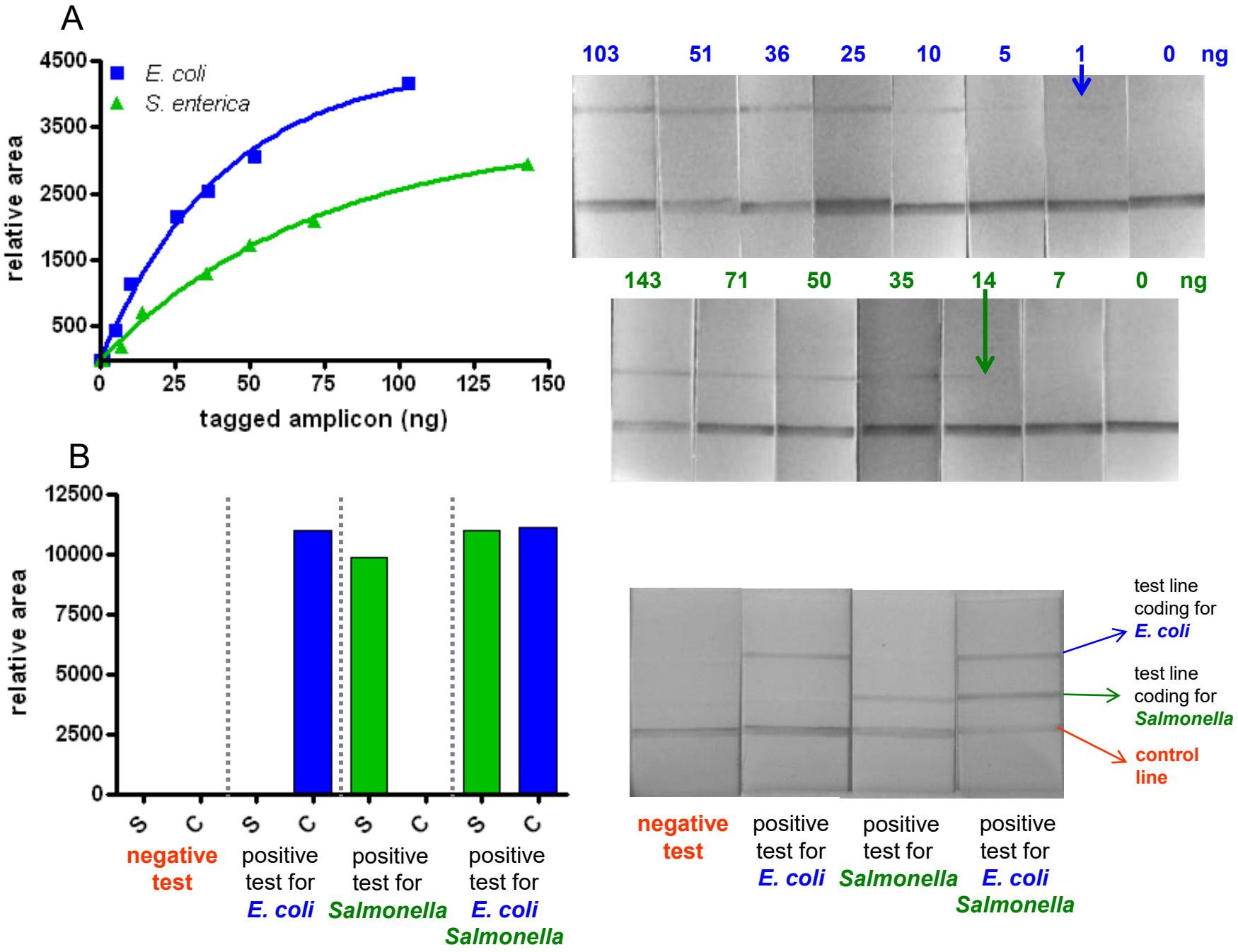
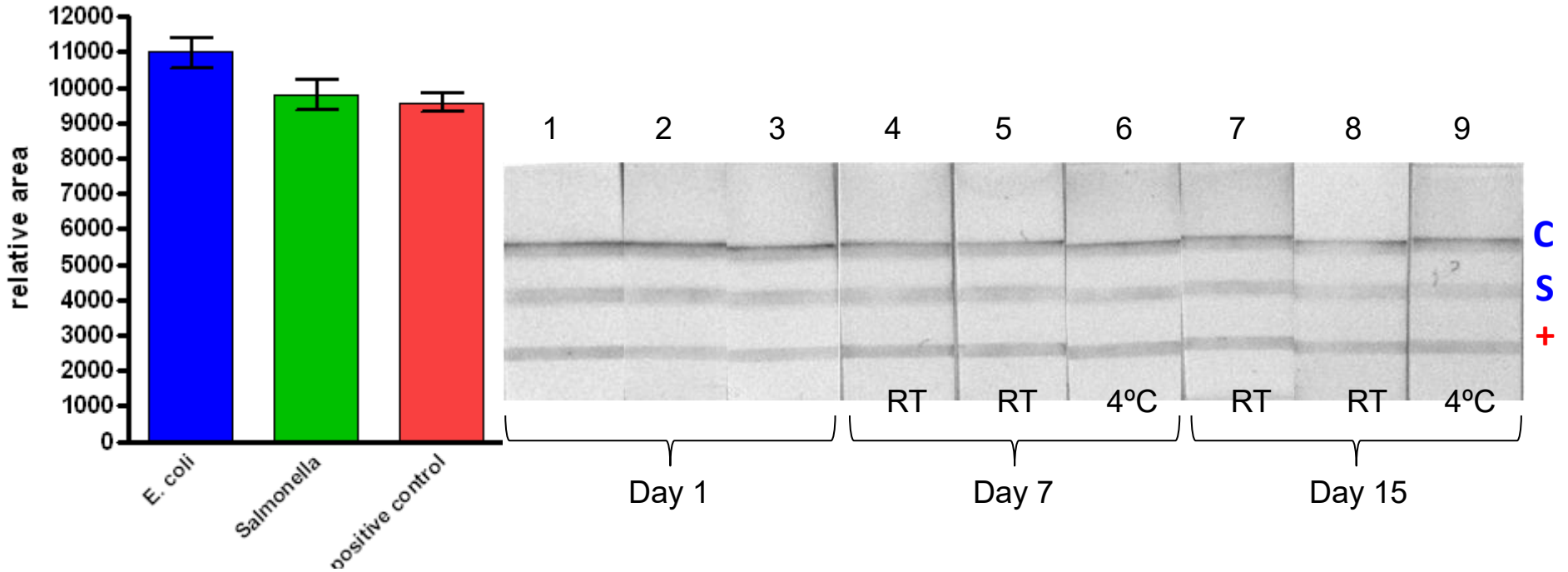

A
4500
3500
2500
1500
500
relative area
E. coli
S. enterica
0
25
50
75
100
125
150
tagged amplicon (ng)
103
51
36
25
10
5
1
0
ng
143
71
50
35
14
7
0
ng
B
12500
10000
7500
5000
2500
0
relative area
S
C
negative test
positive test for E. coli
positive test for Salmonella
positive test for E. coli Salmonella
test line coding for E. coli
test line coding for Salmonella
control line
negative test
positive test for E. coli
positive test for Salmonella
positive test for E. coli Salmonella

Figure 5


relative area
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
E. coli
Salmonella
positive control
1
2
3
4
5
6
7
8
9
RT
RT
4ºC
RT
RT
4ºC
Day 1
Day 7
Day 15
C
S
+

Supplementary Material

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