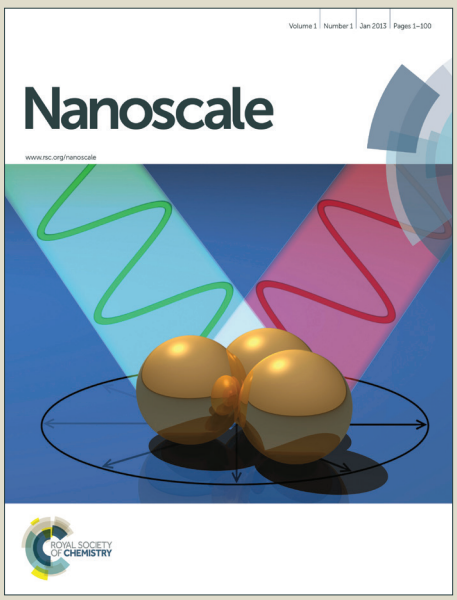
Nanoscale

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/nanoscale

Simultaneous Direct Detection of Shiga-toxin Producing *Escherichia coli* (STEC) Strains by Optical Biosensing with Oligonucleotide-functionalized Gold Nanoparticles

Irwin A. Quintela[1], Benildo G. de los Reyes[2], Chih-Sheng Lin[3], and Vivian C.H. Wu[1]*

[1] School of Food and Agriculture, The University of Maine, Orono, ME 04469-5735, USA,

[2] School of Biology and Ecology, The University of Maine, Orono, ME, 04469-5735, USA,

[3] Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 30005, Taiwan

* Corresponding author. Tel.: +1 207 581 3101; Fax: +1 207 581 1636

E-mail address: vivian.wu@umit.maine.edu

Abstract

A simultaneous direct detection of Shiga-toxin producing strains *E. coli* (STEC; "Big Six" – O26, O45, O103, O111, O121, and O145) as well as O157 strains by optical biosensing with oligonucleotide-functionalized gold nanoparticles (AuNPs) was developed.

Initially, conserved regions of *stx* genes were amplified by asymmetric polymerase chain reaction (asPCR). Pairs of single stranded thiol-modified oligonucleotides (30-mer) were immobilized onto AuNPs and used as probes to capture regions of *stx*1 (119-bp) and/or *stx*2 (104-bp) genes from STEC strains. DNA samples from pure cultures and food samples were sandwich hybridized with AuNP-oligo probes at optimal conditions (50^{o}C, 30 min). A complex was formed from the hybridization of AuNP-probes and target DNA fragment that retained initial red color of the reaction solutions. For non-target DNA, a color change from red to purplish-blue was observed following an increase in salt concentration, thus providing the basis of simultaneous direct colorimetric detection of target DNA in the samples. Enrichment and pooling systems were incorporated to efficiently process large number of food samples (ground beef and blueberries) and detection of live targets.

The detection limit was < 1 log CFU/g, requiring less than 1 h to complete after DNA sample preparation with 100% specificity. Gel electrophoresis verified AuNP-DNA hybridization while spectrophotometric data and transmission electron microscope (TEM) images supported color discrimination based on the occurrence of molecular aggregation. In conclusion, the significant features of this approach took advantage of the unique colorimetric properties of AuNPs as a low-cost and simple approach yet with high specificity for simultaneous detection of STEC strains.

***Keywords*:** gold nanoparticle; optical biosensing; STEC; Shiga-toxin

1. Introduction

The emergence of Shiga toxin-producing strains of *E. coli* (STEC) as a major foodborne pathogen has posed serious public health concerns [1]. Epidemiological evidence shows that specific STEC serotypes account for a large number of serious infections such as hemorrhagic colitis and hemolytic-uremic syndrome [2, 3].

O-antigens on the surface of *E. coli* are significant virulence factors that are targets of both innate and adaptive immune systems, playing a major role in pathogenicity. The antigenic specificity of the strain is based on the O-antigens that determine the O-serogroups [4]. The top six O-groups ("Big Six" - O26, O45, O103, O111, O121, and O145) share common epidemiological and virulence properties and have been determined by the Centers for Disease Control and Prevention (CDC) to be the causative agents of 71% of non-O157 STEC diseases [3-6]. STEC strains release two putative potent cytotoxins called Shiga toxins (Stx1 and Stx2), encoded by *stx*1 and *stx*2 genes, respectively [7, 8]. There are more than 100 serotypes of STEC that carry either or both *stx*1 and *stx*2 [9]. With its low infectious dose, STEC strains can cause damages to the intestinal linings, seizures, respiratory and kidney failures, and paralysis of susceptible patients due to Shiga toxins [10]. Recently, the top six serogroups have been declared by the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA FSIS) to be adulterants in raw beef [11]. Aside from beef, STEC are also transmitted through a wide array of foods including dairy products and fresh produce such as lettuce and spinach [12, 13]. One of the most extensive STEC outbreaks occurred in Germany on May 2011 with 3,816 cases, 845 of which had HUS and 54 deaths were recorded [14].

To efficiently detect STEC contamination in food and environmental samples, the assay should be easy, quick and accurate. The traditional culture method using Sorbitol MacConkey

agar is relatively inexpensive, effective, and widely used based on the absence of sorbitol fermentation. However, the long turnaround time and non-specificity due to the emerging non-O157 STEC strains that ferment sorbitol limit its use [9]. Conventional PCR assays are flexible in terms of detection and identification of multiple target genes in one reaction. However, most first-generation PCR assays have cumbersome protocols for detecting the amplified products and numerous PCR assays lack sequence specific identification of the amplified genes [15]. None-portable PCR systems make the application difficult in the on-site testing. Discriminating viable from non-viable cells after PCR is another challenge because DNA is always present whether the cell is dead or alive [16].

Optical biosensors have received substantial interest for bacterial pathogen detection due to its simplicity, sensitivity, and selectivity. Target recognition usually generates electrical or optical signals, which can ultimately report the presence of the target materials [17]. Gold nanoparticles (AuNPs) are emerging novel colorimetric reporters for the detection of various substances such as nucleic acids, proteins, and antibodies due to its excellent optical, physical and chemical properties such as high dispersity in solution, ease of synthesis, low cost, and extremely high extinction coefficients (hundreds to thousand larger than those of organic dyes)[18]. AuNPs are spherical particles with a typical diameter of 2 − 50 nm that exhibit distinctive properties known as Surface Plasmon Resonance (SPR) or the collective electron oscillations which give intense red color and allows shifting to blue color upon aggregation of AuNPs [19, 20]. This color change offers a suitable platform for colorimetric sensing of any specific analyte of interest [21]. The sensitivity and selectivity of AuNPs based colorimetric detection are comparable to fluorescent detection methods [22]. Though immunoassays and other molecular based biosensing methods have been widely studied to rapidly detect and identify foodborne bacterial pathogens,

many of these methods are designed for detection of a single species of pathogens in one assay [23]. Therefore, a simultaneous method that will detect and identify STEC strains from food and environmental samples is highly desirable.

Our aim was to develop a method for simultaneous direct detection of STEC strains by an optical biosensing method with oligonucleotide-functionalized AuNPs and DNA sandwich hybridization. To our knowledge, this is the first report of a simultaneous and highly sensitive optical biosensing detection method for the STEC strains that express and produce either *stx*1 or *stx*2 or both.

2. Experimental methods

2.1. Bacterial cultures preparation

Twenty STEC strains representing seven serogroups, O26:H11 HH8, O26:H11 SJ1, O26:H11 SJ2, O26:H2 TB285, O45:H2 SJ7, O45:H2 05-6545, O45:H2 96-3285, O103:H2 GG7, O103:H25 SJ11, O103:H11 SJ12, O111:H8 EE5, O111:NM SJ13, O111:H- 94-0961, O121:H19 SJ18, O121:H19 96-1585, O145:NM SJ23, O145:H28 07865, O145:H- 94-0491, O157:H7 (ATCC 35150), O157:H7 (ATCC 49835) and *S.* Typhimurium H3379, were obtained from the U.S. Department of Agriculture – Agricultural Research Service, Eastern Regional Research Center (Wyndmoor, PA, USA) and from the Pathogenic Microbiology Laboratory at the University of Maine . Bacterial cells were stored in cryogenic beads with Brucella broth and glycerol (CryoSavers; Hardy Diagnostics, Santa Maria, CA, USA) at −80°C. Frozen culture beads were activated by incubating overnight at 37°C in Brain Heart Infusion (BHI) broth (Neogen, Lansing, MI, USA). Revived cells were re-inoculated to new BHI broth under the same

conditions to prepare the working cultures. Viable cell counts during the experiments were obtained by plating on MacConkey Agar with Sorbitol (Neogen).

2.2. Primers and probes designs

Two pairs of *stx* specific primers [For(*stx*1-1-F)- (5'–CATCGCGAGTTGCCAGAATG– 3') and Rev(*stx*1-1-R)- (5'–AATTGCCCCCAGAGTGGATG– 3'); For(*stx*2-5-F)- (5'–GTATAC GATGACGCCGGGAG– 3') and Rev(*stx*2-5-F)- (5' –TTCTCCCCACTCTGACACCA– 3') were designed using NCBI Primer Blast to amplify the conserved regions of *stx*1 (119-bp) and *stx*2 (104-bp) genes. Primer sequences were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Oligonucleotide probes were manually designed based on sequence complementarity with the target regions. Hybridization conditions for each probe with target *stx* region were initially optimized and confirmed by digesting the hybridization products with S1 Nuclease (Promega, Madison, WI, USA). Probes were modified with thiol-linked tags [HS-$(CH_2)_6$] at the 5' or 3' terminus, [P1-30-119AS-SH - (5'– CCGGACACA TAGAAGGAAACTCATCAGATG – 3'-$(CH_2)_6$-HS) and P2-30-119AS-SH - (HS-$(CH_2)_6$ – 5'–TTTATTGTGCGTAATCCCACGG ACTCTTCC – 3'); P1-30-104AS-SH - (5'-ATTCGCCCC CAGTTCAGAGTGAGGTCCACA - 3'-$(CH_2)_6$-HS) and P2-30-104AS-SH - (HS-$(CH_2)_6$ – 5'–CCTCTCCCCGATACTC CGGAAGCACATTGC – 3') (Eurogentec, San Diego, CA, USA).

2.3. DNA extraction and asymmetric PCR

Genomic DNA from pure bacterial cultures (concentration levels at 1 – 3 CFU/ml) was harvested using DNeasy Blood & Tissue Kit according to manufacturer's protocol (Qiagen GmbH, Hilden, Germany); 2 µl of DNA samples from the final elution step were used as templates for asymmetric PCR (asPCR). All DNA samples were stored at -20^{o}C until use.

The optimized asPCR conditions that amplified the 119-bp target region of *stx*1 and 104-bp region of *stx*2 had an initial denaturation at 95°C for 2 min; 35 cycles of 30 sec denaturation at 95°C, annealing at 56°C for 30 sec, elongation at 72°C for 30 sec and final extension at 72°C for 5 min. The same protocol was used for genomic DNA extraction and amplification of target regions from different food enrichments.

2.4. Synthesis of AuNPs

An average of 13 nm diameter of AuNPs was synthesized using citrate reduction method with slight modifications as previously described [24]. Briefly, 25 ml of 2.5 mM of chloroauric acid solution was brought to 130°C with constant stirring. Sodium citrate (3 ml of 38.3 mM) was rapidly injected to the boiled chloroauric acid. The solution was moderately stirred for another 10 min at room temperature. Then, the freshly prepared AuNPs was characterized by measuring its absorption spectra (400 – 700 nm wavelength) using a microplate reader spectrophotometer (BioTek Power Wave XS, Winoskii, VT, USA).

2.5. Functionalization of AuNPs with thiol-modified oligonucleotide probes

AuNPs solution was transferred to centrifuge tubes wrapped with aluminum foil. Each thiol-modified oligonucleotide probe (20 µM, 20 µl) was added to the AuNPs solution (20 µM, 980 µl) and kept in water bath at 37°C for 24 h[24]. Increasing amount of salt buffer solution (0.05 – 1.0 M NaCl in 10 mM Na_2HPO_4, pH 7.4) was added at six different time points for the next 48 h to steadily immobilize the probes onto the surface of AuNPs. The excess unbound probes were washed from the functionalized AuNP solution by centrifugation at 19,530 × *g* for 30 min. The resulting clear supernatant was carefully removed while the remaining dark red oily residue was dispersed in 0.05 M NaCl in 10 mM Na_2HPO_4, pH 7.4. Final absorption spectra was measured

(400 – 700 nm wavelength) using a microplate reader spectrophotometer (BioTek Power Wave XS, Winoskii, VT, USA). Functionalized AuNP solution was stored at 4^{o}C until further use.

2.6. AuNP optical biosensing for STEC strains

Figure 1 presents the principles of the AuNPs optical biosensing based on DNA sandwich hybridization using functionalized AuNPs. AuNP-probes (50 µl each) and its corresponding target and non-target asymmetric PCR products were denatured separately and iced for 5 min. Denatured probes and PCR products were mixed together and incubated at 50^{o}C for 30 min to complete the hybridization process. The reaction solutions were kept at room temperature for 5 min before the color challenge test.

Salt solution (1 M NaCl in 10 mM Na_2HPO_4, pH 7.4, in the final reaction) was added to each reaction solution for examination of color reaction. For quantitative analysis, absorption spectra was measured (400 – 700 nm wavelength) before and after adding salt solution using a microplate reader spectrophotometer (BioTek Power Wave XS, Winoskii, VT, USA). For verification of successful DNA and AuNP-probes hybridization, reaction solutions (20 µl each) were run in gel electrophoresis (3% agarose gel and ethidium bromide) prior to adding salt solution.

2.7. Transmission electron microscopy (TEM)

Philips CM10 TEM (Philips, Eindhoven, Netherlands) operating at 80kV was used to characterize AuNPs. Briefly, 2 µl of each thiol-modified AuNPs samples (with or without bacterial sample) was deposited onto a carbon-coated grid (200 mesh). The grids were then allowed to air dry at room temperature for 30 – 45 min before viewing under TEM for analysis.

2.8. Detection of STEC from food samples

2.8.1. Pooling enrichment

Ground beef and blueberries were used to assess the application of the developed AuNP optical biosensing in STEC contaminated food matrices. Pooling and enrichment systems (Figure 2) were incorporated to efficiently process a large number of food samples (50 samples) at the shortest possible time and ensure detection of viable STEC cells. The initial microbial contamination level in food samples before enrichment was < 1 log CFU/g, which was achieved by 10-fold serial dilution and confirmed by the plate count method with MacConkey Agar with Sorbitol (Neogen).

2.8.2. Ground beef

Ground beef was purchased from a local retail store and used to prepare inoculated samples. The procedures for identifying STEC in the USDA/Food Safety and Inspection Service (FSIS) Microbiology Laboratory Guidebook (MLG) 5.05 were adapted with slight modification. Briefly, 50 samples (25 g each) were collected and grouped into five; each group had 10 samples. Freshly grown cells of STEC strains - O26:H11 HH8, O26:H11 SJ1, O26:H11 SJ2, O26:H2 TB285, O45:H2 SJ7, O45:H2 05-6545, O45:H2 96-3285, O45:H2 SJ7, O45:H2 05-6545, O45:H2 96-3285, O103:H2 GG7, O103:H25 SJ11, O103:H11 SJ12, O111:H8 EE5, O111:NM SJ13, O111:H- 94-0961, O121:H19 SJ18, O121:H19 96-1585, O145:NM SJ23, O145:H28 07865, O145:H- 94-0491, O157:H7 (ATCC 35150) and O157:H7 (ATCC 49835) were spiked randomly at 0 – 9 CFU/g (< 1 log CFU/g) contamination levels. Each sample was transferred into individual stomacher bag (Blender Bag, Fisher Scientific, Wilmington, DE, USA). A volume of 225 ml of modified Tryptic Soy Broth (mTSB) with 8 mg/l novobiocin and casamino acids (Neogen) was added and then homogenized using a stomacher for 2 min. All pre-enriched samples were incubated at 42 ± 1^{o}C for 6 h.

Fifty ml aliquot was collected from each post-enriched sample and pooled together as shown in Figure 2. To remove solidified fats and debris, 1 ml aliquots from each of the five pooled samples were centrifuged at low speed (125 × *g*) for 10 min. Supernatant was then filtered through 5 µm pore size membrane (Acrodisc, Pall Corporation, Cornwall, UK). Genomic DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's protocol.

2.8.3. Blueberries

Blueberries were supplied by a local farmer in Maine. Briefly, 50 samples (25 g each) were collected and grouped into five; each group had 10 samples. Freshly grown cells of same STEC strains as used in the ground beef inoculation were spiked individually to 2 groups at 0 – 9 CFU/g (< 1 log CFU/g) contamination levels. Briefly, 50 samples (25 g each) of blueberries were prepared in stomacher bag (Blender Bag, Fisher Scientific, Wilmington, DE, USA). Then, 225 ml of mTSB with 8 mg/l novobiocin and casamino acids (Neogen) was added and mixed (Rotomix Orbital Shaker, Dubuque, IA, USA) at 2 × *g* for 2 min. Similar to ground beef, all pre-enriched samples were incubated at 42 ± 1^{o}C for 6 h.

From each post-enriched sample, 50 ml was collected and pooled together as shown in Figure 2. Genomic DNA was extracted from each these five blueberry pooled samples using DNeasy Blood & Tissue Kit (Qiagen) as described by the supplier.

2.9. AuNP optical biosensing on food samples

After genomic DNA extraction and target amplification, procedures for AuNP optical biosensing of various STEC pure culture strains (described in Section 2.6) were applied to food samples in duplicates using 96-well microplate. In addition, 1 log CFU/ml contamination level for both positive (STEC O145:NM) and negative (*S.* Typhimurium H3379) controls and blank

(nuclease-free water) were included. AuNP optical biosensing was conducted in parallel with conventional PCR and gel electrophoresis for direct comparison.

2.10. Statistical analysis

The experimental data were analyzed using JMP software. Each experiment was repeated three times to test the reproducibility and stability of the AuNP optical biosensors. Mean ± standard deviation (S.D.) absorbance reading of reaction mixtures after adding salt-buffer solution in experiments were compared by a two-tailed Student's *t*-test. *P*-values less than 0.05 were considered to be statistically significantly different.

3. Results and Discussion

3.1. Functionalization of AuNPs with thiol-modified oligonucleotide probes

AuNPs are unique kinds of hybrid materials due to their characteristic optical properties that allow convenient signal transduction for biosensor applications facilitated by the colorimetric changes of dispersed and aggregated particles. AuNPs obtained by the citrate reduction method appear as monodispersed globular structures with a diameter of about 10 – 15 nm, stabilized by weakly bound citrate anions [20, 25]. AuNPs with approximately 13 nm diameter absorb green light, which corresponds to a strong absorption band (surface plasmon band) at ~520 nm in the visible light spectrum; therefore solutions of AuNPs appear red in color [26]. The surface of AuNPs can be tailored by the functionalization of ligands (oligonucleotides and proteins) to specifically bind with its target. The freshly synthesized AuNPs synthesized in the present study were functionalized with thiol-modified oligonucleotides.

Increased salt-buffer concentration during the immobilization of thiol-modified oligonucleotide on the surface of AuNPs reduces electrostatic repulsion between negatively-

charged strands due to a screening effect permitting more oligonucleotides to approach the particle surface [27]. Thiol modification on the 5' end of one probe and 3' end of the other probe provides stable chemical bonds between the thiol group and gold atoms efficiently replacing citrate ions on the surface of AuNPs and enhancing immobilization [10, 25].

Successful immobilization of thiol-modified oligonucleotide probes caused shift of 3 – 5 nm on its absorbance peak of AuNP solution without noticeable change in color. Optimization steps in the experiment were performed by adjusting the amount and volume ratio of thiol-modified oligonucleotide and AuNPs during the immobilization process. Washing of functionalized AuNPs after immobilization step was also incorporated in the protocol to ensure that unbound thiol-probes were eliminated in the final AuNP-probe solution prior to DNA sandwich hybridization. These steps have significantly reduced the background noise and greatly enhanced the color reactions of samples after subjecting to an increased salt concentration. The optimized final concentration of functionalized AuNPs was 20 nM (Supplementary information).

3.2. AuNPs optical biosensing for STEC strains in pure cultures

DNA samples from different log concentrations of cells (1 – 3 log CFU/ml) were used for asPCR. The specificity of the assay was demonstrated by precise amplification of *stx* genes from STEC strains of different serogroups. By using asPCR, single stranded amplicons were generated corresponding to the highly-conserved *stx* target regions (119-bp/*stx*1 and 104-bp/*stx*2).

The AuNP optical biosensing was developed and applied to the amplified regions of *stx*1/*stx*2 genes from various STEC strains. Twenty STEC strains representing the "Big Six" and O157 serogoups were tested with the negative control (non-target, *S.* Typhimurium H3379) and blank (nuclease-free water instead of DNA). In a 96-well microplate, it was observed that the DNA sample reactions from various STEC strains remained were red in color whereas those

containing *S.* Typhimurium H3379 and blank solutions developed a purplish-blue color after the addition of salt (1 M, final reaction concentration). This approach allowed simultaneous visual comparison of solutions before and after the salt induced AuNP-probes aggregation. Purification steps of amplified products were found not to be necessary since 35 cycles of asPCR provided an adequate amount of detectable single-stranded DNA.

As shown in Figure 3A, AuNP optical biosensing targeting *stx*1 gene of STEC O26: H11 and *stx*2 gene of STEC O111:H19 strains, hybridization of AuNP-probes with both target regions retained its initial red color. This was due to the formation of AuNP-probes network complexes that stabilized AuNPs even in an increased salt concentration. The negatively-charged phosphate groups of DNA backbone of the newly formed AuNP-probes complexes enhanced interparticle electrostatic repulsion force. This repulsion force effectively counteracted both van der Waals attraction force of AuNPs and high ionic strength of salt solution [28]. Moreover, the formation of DNA duplex from DNA sandwich hybridization may have provided a buffering effect to the reaction solution, thereby stabilizing AuNPs and prevented it from aggregating in an increased salt environment [24]. For the negative control reaction solution containing AuNP-probes and non-complementary sequences, a changed from red to purplish-blue color was observed. DNA sandwich hybridization did not occur in the negative control, thus, only a weak repulsion force between AuNPs was present which made it prone to destabilization. As salt solution was added, the net ionic strength of the negative reaction solution increased that reduced and neutralized the existing weak interparticle repulsion charge of AuNPs. The increased in salt concentration ultimately resulted to the aggregation of AuNPs and change in color of the negative control samples.

Spectrophotometric data curve presented in Figure 3B illustrates the broadening of peaks towards longer wavelength for the non-target and blank reaction solutions due to particles aggregation after adding salt solution. For target samples, the absorbance curves after adding salt (1 M in final reaction) peaked at a lower absorbance compared to its initial absorbance due to the additional volume of salt solution which diluted the reaction solutions. However, it did not alter the trend of color reactions between target and non-target samples. The extent of this shift depends mainly on particle and aggregate sizes, density, fractal dimensions, whereas the rate of change of the spectral shift depends on aggregate size and growth rate [20]. Figure 3D shows the successful DNA sandwich hybridization between AuNP-probes and target DNA on gel electrophoresis, while the negative control and blank reaction did not show any band. This figure presents results that perfectly correlate with the results of AuNP optical biosensing assay reactions as described. In this study, an aggregation parameter was derived to quantify the variation of integrated absorbance of samples after adding salt solution. The absorbance ratio (625 nm/525 nm) of the non-target samples and blank from two longer wavelengths, as presented in Figures 3C, 4C, 4D and 5B clearly shows significant difference at $P < 0.05$ as compared to the target samples. These results confirmed the occurrence of salt induced AuNP-probes aggregation that allowed discrimination between STEC and non-STEC strains.

Figures 3E, 3F and 3G show TEM images of AuNP-probes in various reaction solutions after an increased salt concentration (1M in final reaction). Figure 3E is an image of AuNP-probes and non-target DNA from *S.* Typhimurium H3379 (1 log CFU/ml) showing the aggregation of AuNPs as interparticle electrostatic repulsion is relieved by increasing salt concentration. Both Figure 3F (STEC O26 – *stx*1 assay) and Figure 3G (STEC O111 – *stx*2 assay) present successfully hybridized AuNPs-probes with complementary target sequences in an

311 increased salt concentration reaction solution. The images show well dispersed and highly
312 stabilized AuNPs.
313 To further demonstrate the reproducibility and stability of the assay, the STEC strains
314 carrying both *stx* genes can be tested. Figure 4 presents a successful detection of both *stx*1 and
315 *stx*2 genes from STEC O145:NM targeting in a range of 1 – 3 logs CFU/ml bacterial source.
316 Figure 5 shows simultaneous detection of s*tx*1 and s*tx*2 genes from STEC O45:H2, STEC
317 O103:H11, STEC O121:H19 and STEC O157:H7 at 1 log CFU/ml pure culture set-up.
318 Earlier results (data not shown) demonstrated 100% specificity of the assay in
319 simultaneous and direct detection of asPCR products from 9 log CFU/ml STEC cell
320 concentration while employing *S.* Typhimurium and nuclease-free water as the negative control
321 and blank, respectively. These results provided the basis to challenge the sensitivity of the assay
322 by using lower cell concentrations (1 – 3 logs CFU/ml) for DNA preparation. Based on the
323 results shown in Figures 3 – 5, the limit of detection (LOD) of the assay is 1 log CFU/ml.
324
325 **3.3. AuNP optical biosensing on food samples**
326 STEC is a rapidly emerging threat as a stubborn foodborne pathogen and together with *E.*
327 *coli* O157:H7, it has been implicated with hemorrhagic colitis and potentially deadly systemic
328 sequelae in humans [29]. However, STEC detection in most end-product testing is primarily
329 focused on STEC O157:H7, and screening of other serogroups or strains is often neglected [30].
330 The applicability of the simultaneous detection of STEC strains with the developed AuNP
331 optical biosensing method was evaluated in ground beef and blueberries. Prevalence of STEC
332 contamination in these food samples was also assessed.

Combined with a robust sampling plan and shortened enrichment procedures, the new AuNP optical biosensing method was effective in detecting contaminants in ground beef and blueberries that were experimentally inoculated with various concentrations of STEC cells with < 9 CFU/g. Enrichment and pooling systems were incorporated to efficiently process large number of food samples at the shortest possible time and ensure detection of viable STEC cells. In the present study, 6 h enrichment was determined by plate count method (data not shown) to be the shortest period that would ensure growth of STEC strains to > 1 log CFU/g. Modified TSB with 8 mg/l novobiocin and casamino acids (Neogen) was used to enrich food samples with low levels of STEC and to increase the probability of harvesting and extracting genomic DNA for asPCR.

Two out of five post-enriched pooled samples (data not shown), either ground beef or blueberries, tested positive for the presence of STEC strains by the AuNP optical biosensing in a 96-well microplate. A total of forty individual post-enriched samples which were components of positive pools were then tested simultaneously. Duplicate reaction samples in a 96-well microplate after adding salt are shown in Figure 6. Individual STEC strain may carry one or both types of *stx* genes commonly contaminating ground beef and other food items including leafy greens, thus duplicate samples ensure greater detection coverage [9]. In total, 33 samples were positive for *stx*1 and 25 samples for *stx*2 from both ground beef and blueberries samples. Thirty-seven samples were positive for both *stx* genes while 22 samples showed negative detection.

With the blank and negative control (*S.* Typhimurium) turned purplish-blue after adding salt solution, all positive individual samples along with the positive control (STEC O145:NM) were differentiated with ease. Color change for blank and non-target samples was observed within 1 – 2 min after adding salt solution. Without the aid of any complicated instrument,

conducting visual examination to simultaneously discriminate negative and positive reactions from the samples offers flexibility and rapid detection[31]. AuNP optical biosensing was compared with conventional PCR and gel electrophoresis showing similar results. Reference to the concentration of STEC strains inocula, the data presented confirmed < 1 log CFU/g detection limit for the AuNP optical biosensor in food samples. This detection limit is very significant based on the previous study that the infectious dose of STEC strains such as STEC O157:H7 is < 10 cells [10]. The actual DNA sandwich hybridization steps and color challenge test require less than 1 h to complete. Shorter turnaround detection period while accommodating multiple samples translates convenience and automation [32]. This feature makes this newly developed optical biosensing advantageous over the other common approaches especially for screening STEC in food samples when rapid testing with immediate results is highly sought. In the present study, the detection subjects come from the amplified DNA (*stx* genes) of pre-enriched STEC cells in pure culture or inoculated samples. It was not intended to provide a precise correlation between the value 625 nm/525 nm ratio and the original concentration of STEC (Supplementary information).

Currently, numerous methods utilizing AuNPs for detection are available as discussed and reviewed by several researchers. AuNP-based optical biosensing detection method still continues to be a promising tool for rapid and specific foodborne pathogen detection. It has been presented in this study the unique and stable properties of AuNPs that can be exploited for simultaneous pathogen detection while considering the ease of its synthesis and as an inexpensive material. The advantageous features of this approach successfully challenged a superior detection limit in pure culture and actual food samples. More importantly, this study has incorporated simplified sampling approach and enrichment method that made the entire protocol

379 turnaround time to be less than a day and ensured the viable cell detection. All steps established
380 in this study require simple equipment and do not need highly skilled staff.
381
382 **4. Conclusions**
383 In this study, a direct and simultaneous detection of STEC strains by an optical
384 biosensing method using oligonucleotide-functionalized AuNPs was developed. This approach
385 allowed a simultaneous visual discrimination and identification of STEC DNA samples
386 following DNA sandwich hybridization with highly specific thiol-modified probes immobilized
387 on the surface of AuNPs and optimum salt concentration (1 M in final reaction). To demonstrate
388 the stability and reproducibility of this optical biosensing, the method was applied to artificially
389 inoculate pooled and individual ground beef and blueberries samples. The results showed that the
390 new biosensing assay is highly sensitive and stable for simultaneous and rapid detection of the
391 STEC strains carrying *stx* gene(s). The detection limit was superior (< 1 log CFU/g) requiring
392 less than 1 h to complete after DNA sample preparation and 100% specificity. Gel
393 electrophoresis confirmed AuNP-DNA hybridization while spectrophotometric data and
394 transmission electron microscope (TEM) images supported the color discrimination based on the
395 occurrence of molecular aggregation. . The significant features of this approach took advantage
396 of the unique colorimetric properties of AuNPs as a low-cost, specific, and simple approach for
397 simultaneous detection of STEC strains, elevating the potential of nanobiosensing for rapid
398 detection of multiple foodborne pathogens to a different stage.
399
400
401

Acknowledgements

This research was supported by USDA NIFA-AFRI Competitive Grant (Award # 2011-68003-30420) and the Maine Agricultural and Forest Experiment Station at the University of Maine with external publication number 3680. This work is based upon research supported in part by Hatch Grant number ME08562-10 from the USDA National Institute of Food and Agriculture. The authors would like to thank Dr. Pina Fratamico, ERCC, ARS-USDA for providing the STEC strains, Ms. Ai Kitazumi for her assistance during the initial part of the experiment and Ms. Fang-Yuan Yeh (Cammy) for her input on the final layout of the figures.

411 **Figure Legends**

412 **Figure 1.** A schematic diagram of simultaneous direct detection of various STEC strains using
413 AuNP optical biosensing assay targeting *stx*1 and *stx*2 genes at optimized conditions (37^{o}C for
414 30 min). (**A**) For target DNA set-up, a successful DNA sandwich hybridization between AuNP-
415 probes (Probe 1 and Probe 2) and target DNA occurs due to its high complementarity. Following
416 an increased salt concentration, AuNP-probes and target DNA complexes remain stable and
417 retain its original red color. (**B**) For non-target DNA (including blank) set-up, successful
418 hybridization does not occur but instead, salt-induced AuNP aggregation followed after
419 increasing its salt concentration. The color of reaction solutions with salt-induced aggregated
420 AuNPs shifts from the original red to purplish-blue. Color differentiation facilitates direct
421 detection of positive and negative samples. (**C**) For simultaneous direct detection of various
422 STEC strains using a 96-well microplate, each sample was prepared in duplicates for both *stx*1
423 and *stx*2 assays. (**D**) A TEM image of oligonucleotide-modified AuNPs, 275,000 X
424 magnification.
425
426 **Figure 2.** The principles of rapid enrichment of STEC cells in food samples after artificial
427 inoculation, pooling and AuNP optical biosensing assay. (**A**) Simultaneous direct detection of
428 various STEC strains from ground beef (25 g) and blueberries (25 g) starts from the shortened
429 pre-enrichment using 225 ml modified Tryptic Soy Broth (mTSB) with 8 mg/l novobiocin and
430 casamino acids at 42 ± 1^{o}C for 6 h to ensure detection of viable STEC cells. (**B**) Pooling of post-
431 enriched samples is performed to accommodate large number of food samples. (**C**) Amplified
432 samples generated by asymmetric PCR are simultaneously tested using AuNP optical biosensing
433 assay. (**D**) If pooled sample is tested negative, then all of its individual samples are declared

negative. (**E**) However, positive pooled sample requires testing of all its individual post-enriched samples. Simultaneous AuNP optical biosensing assay is conducted in a 96-well microplate; gel electrophoresis and spectrophotometric data confirm successful DNA sandwich hybridization.

Figure 3. Simultaneous direct detection of *stx*1 gene from STEC O26:H11 strain and *stx*2 gene from STEC O111:H19 strain. In this pure culture set-up, each strain carries only one type of *stx* gene. (**A**) AuNP optical biosensing assay before and after adding salt solution (50 µl, 1 M NaCl-Na_2HPO_4 final reaction). (**B**) Absorbance curve of representative samples before and after adding salt (1 M NaCl-Na_2HPO_4 in final reaction) was generated showing the formation of new absorbance peaks at longer wavelengths of non-target and blank samples. (**C**) Absorbance ratio of samples at wavelengths 625 nm and 525 nm after adding salt (1 M NaCl-Na_2HPO_4 in final reaction) shows significance difference at $P < 0.05$ between STEC strains, non-target and blank samples which validated 1 log CFU/ml as the limit of detection (LOD). B = blank; NT = non-target *S.* Typhimurium H3379 (1 log). (**D**) Gel electrophoresis was conducted to verify the occurrence of a successful DNA sandwich hybridization between AuNP-probes and the target gene. Gel bands are shown at the expected 119-bp (*stx*1) and 104-bp (*stx*2) regions, respectively. All samples were in log CFU/ml concentration. (**E**) A TEM image of AuNP-probes and non-target DNA from *S.* Typhimurium H3379 (1 log CFU/ml) clearly shows aggregation of AuNPs as interparticle electrostatic repulsion force is relieved by increasing salt concentration. (**F**) STEC O26 1log CFU/ml – *stx*1 assay and (**G**) STEC O111 1log CFU/ml – *stx*2 assay present successfully hybridized AuNPs-probes with complementary target sequences in an increased salt concentration reaction solutions. AuNP-probes are obviously linked with each other but not

456 tightly bound. The last two images both show well dispersed and highly stabilized AuNPs.
457 Inserts on the upper right corner of each image are the actual AuNPs reaction solutions.
458
459 **Figure 4.** Simultaneous and direct detection of s*tx*1 and *stx*2 genes from STEC O145:NM at 1
460 log CFU/ml, 2 log CFU/ml and 3 log CFU/ml pure culture set-up. STEC O145:NM carries both
461 *stx*1 and *stx*2 genes. (**A**) AuNP optical biosensing assay for *stx*1 gene. (**B**) AuNP optical
462 biosensing assay for *stx*2 gene. Absorbance ratio of samples at wavelengths 625 nm and 525 nm
463 after adding salt (1 M NaCl-Na_2HPO_4 in final reaction) is presented in for *stx*1 assay (**C**) and for
464 *stx*2 assay (**D**), indicating significance difference at $P < 0.05$ between STEC strain, non-target
465 and blank samples. B = blank; NT = non-target *S.* Typhimurium H3379. All samples were in log
466 CFU/ml concentration.
467
468 **Figure 5.** Simultaneous direct detection of both *stx*1 and *stx*2 genes from STEC O45:H2, STEC
469 O103:H11, STEC O121:H19 and STEC O157:H7 at 1 log CFU/ml pure culture set-up. Each
470 strain is carrying both genes. (**A**) AuNP optical biosensing assay before and after adding salt
471 solution (50 µl, 1 M NaCl-Na_2HPO_4 final reaction). (**B**) Absorbance ratio of samples at
472 wavelengths 625 nm and 525 nm after adding salt (1 M NaCl-Na_2HPO_4 in final reaction)
473 indicating significance difference at $P < 0.05$ between STEC strains, non-target and blank
474 samples. B = blank; NT = non-target *S.* Typhimurium H3379. All samples were in 1 log CFU/ml
475 concentration.
476
477 **Figure 6.** Application of the newly-developed AuNP optical biosensing assay on artificially
478 inoculated food samples. Individual ground beef (20 samples) and blueberries (20 samples) from

positive post-enriched pooled samples, in duplicates, were simultaneously tested for STEC strains in a 96-well microplate. The figure shows the reaction solutions following an increased in salt concentration (1 M NaCl-Na_2HPO_4 in final reaction). Wells A1-A3 and E1-E3 contain blank, negative (*S*. Typhimurium H3379) and positive controls (STEC O145:NM), respectively. In total, 33 samples showed positive detection for *stx*1, 25 samples for *stx*2 and 37 samples for both *stx*1and *stx*2. Twenty-two samples showed negative detection for *stx*1and *stx*2.

References

1. D. G. Newell, M. Koopmans, L. Verhoef, E. Duizer, A. Aidara-Kane, H. Sprong, M. Opsteegh, M. Langelaar, J. Threfall, F. Scheutz, J. v. der Giessen and H. Kruse, *Int. J. Food Microbiol.*, 2010, 139, Supplement, S3-S15.

2. A. W. Paton and J. C. Paton, *J. Clin. Microbiol.*, 2002, 40, 271-274.

3. F. Wang, L. Jiang, Q. Yang, W. Prinyawiwatkul and B. Ge, *Appl. Environ. Microbiol.*, 2012, 78, 2727-2736.

4. C. DebRoy, E. Roberts, A. M. Valadez, E. G. Dudley and C. N. Cutter, *Foodborne Pathog. Dis*, 2011, 8, 651-652.

5. J. T. Brooks, E. G. Sowers, J. G. Wells, K. D. Greene, P. M. Griffin, R. M. Hoekstra and N. A. Strockbine, *J. Infect. Dis.*, 2005, 192, 1422-1429.

6. N. Kalchayanand, T. M. Arthur, J. M. Bosilevac, J. W. Schmidt, R. Wang, S. D. Shackelford and T. L. Wheeler, *J. Food Prot.*, 2012, 75, 1207-1212.

7. J. E. Blanco, M. Blanco, M. P. Alonso, A. Mora, G. Dahbi, M. A. Coira and J. Blanco, *J. Clin. Microbiol.*, 2004, 42, 311-319.

8. W. M. Fedio, K. C. Jinneman, K. J. Yoshitomi, R. Zapata, C. N. Wendakoon, P. Browning and S. D. Weagant, *Int. J. Food Microbiol.*, 2011, 148, 87-92.

9. T. E. Grys, L. M. Sloan, J. E. Rosenblatt and R. Patel, *J. Clin. Microbiol.*, 2009, 47, 2008-2012.

10. V. C. Wu, S.-H. Chen and C.-S. Lin, *Biosensors Bioelectron.*, 2007, 22, 2967-2975.

11. G. E. Tillman, J. L. Wasilenko, M. Simmons, T. A. Lauze, J. Minicozzi, B. B. Oakley, N. Narang, P. Fratamico and W. C. J. R. Cray, *J. Food Prot.*, 2012, 75, 1548-1554.

12. Y. Arakawa, T. Sawada, K. Takatori, K.-I. Lee and Y. Hara-Kudo, *Biocontrol Sci.*, 2011, 16, 159-164.

13. A. Gill, A. Martinez-Perez, S. McIlwham and B. Blais, *J. Food Prot.*, 2012, 75, 827-837.

14. J. Bergan, A. B. Dyve Lingelem, R. Simm, T. Skotland and K. Sandvig, *Toxicon*, 2012, 60, 1085-1107.

15. U. Reischl, M. T. Youssef, J. Kilwinski, N. Lehn, W. L. Zhang, H. Karch and N. A. Strockbine, *J. Clin. Microbiol.*, 2002, 40, 2555-2565.

16. O. Lazcka, F. J. D. Campo and F. X. Muñoz, *Biosensors Bioelectron.*, 2007, 22, 1205-1217.

17. V. Velusamy, K. Arshak, O. Korostynska, K. Oliwa and C. Adley, *Biotechnol. Adv.*, 2010, 28, 232-254.

18. J. Du, B. Zhu, X. Peng and X. Chen, *Small*, 2014.

19. S. M. Shawky, D. Bald and H. M. E. Azzazy, *Clin. Biochem.*, 2010, 43, 1163-1168.

20. D. Aili, P. Gryko, B. Sepulveda, J. A. G. Dick, N. Kirby, R. Heenan, L. Baltzer, B. Liedberg, M. P. Ryan and M. M. Stevens, *Nano Lett.*, 2011, 11, 5564-5573.

21. K. Saha, S. S. Agasti, C. Kim, X. Li and V. M. Rotello, *Chem. Rev.*, 2012, 112, 2739-2779.

22. W. Zhao, W. Chiuman, M. A. Brook and Y. Li, *ChemBioChem*, 2007, 8, 727-731.

23. L. Yang and Y. Li, *Analyst*, 2006, 131, 394-401.

24. S.-H. Chen, K.-I. Lin, C.-Y. Tang, S.-L. Peng, Y.-C. Chuang, Y.-R. Lin, J.-P. Wang and C.-S. Lin, *IEEE Trans. NanoBiosci.*, 2009, 8, 120-131.

25. V. Chegel, O. Rachkov, A. Lopatynskyi, S. Ishihara, I. Yanchuk, Y. Nemoto, J. P. Hill and K. Ariga, *J. Phys. Chem. C*, 2011, 116, 2683-2690.

26. W. Zhao, M. A. Brook and Y. Li, *ChemBioChem*, 2008, 9, 2363-2371.

27. R. Jin, G. Wu, Z. Li, C. A. Mirkin and G. C. Schatz, *J. Am. Chem. Soc.*, 2003, 125, 1643-1654.

28. W. Zhao, W. Chiuman, J. C. Lam, S. A. McManus, W. Chen, Y. Cui, R. Pelton, M. A. Brook and Y. Li, *J. Am. Chem. Soc.*, 2008, 130, 3610-3618.

29. D. J. Bolton, *Foodborne Pathog. Dis*, 2010, 8, 357-365.

30. S. Derzelle, A. Grine, J. Madic, C. P. de Garam, N. Vingadassalon, F. Dilasser, E. Jamet and F. Auvray, *Int. J. Food Microbiol.*, 2011, 151, 44-51.

31. M. Lin, H. Pei, F. Yang, C. Fan and X. Zuo, *Advanced Materials*, 2013, 25, 3490-3496.

32. V. Jasson, L. Jacxsens, P. Luning, A. Rajkovic and M. Uyttendaele, *Food Microbiol.*, 2010, 27, 710-730.

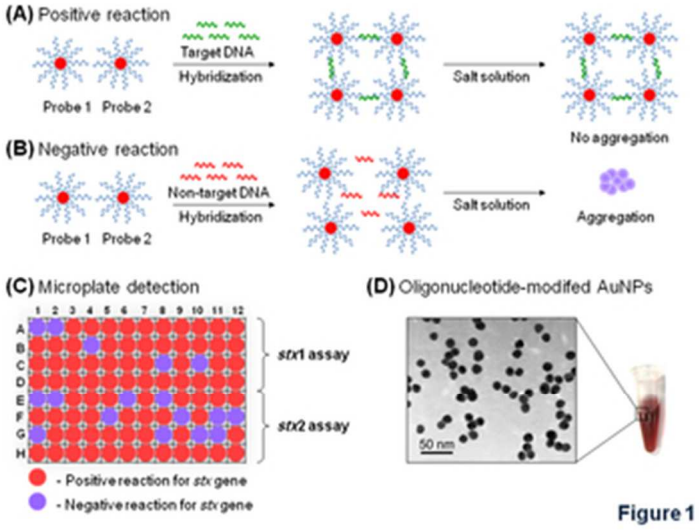


Figure 1

30x22mm (300 x 300 DPI)

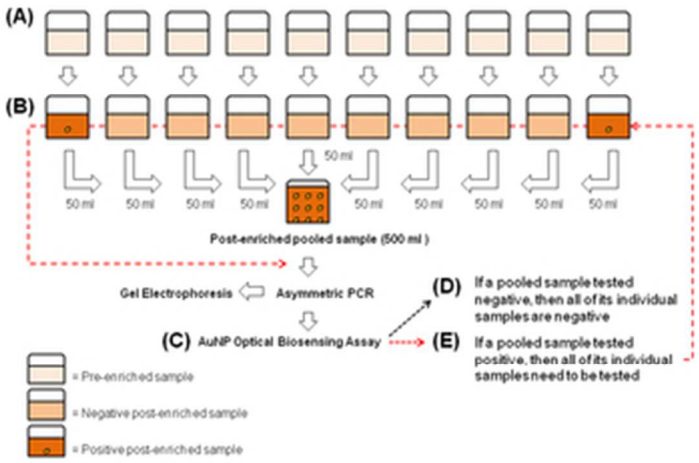


Figure 2

30x22mm (300 x 300 DPI)

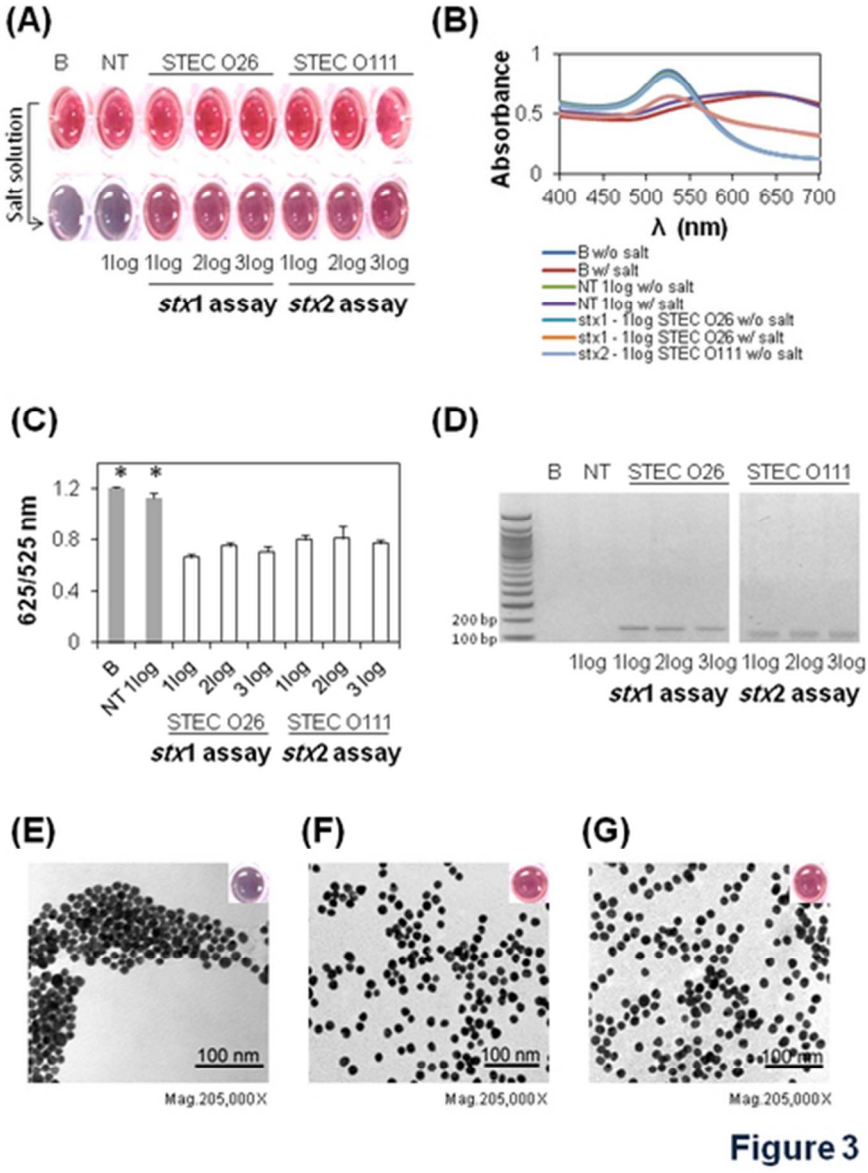


Figure 3

40x54mm (300 x 300 DPI)

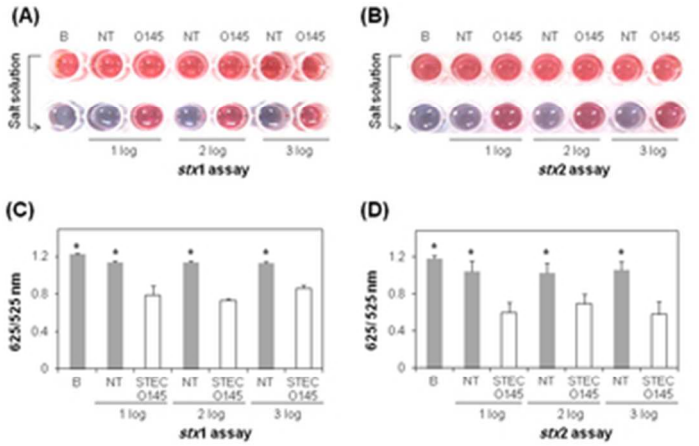


Figure 4

30x22mm (300 x 300 DPI)

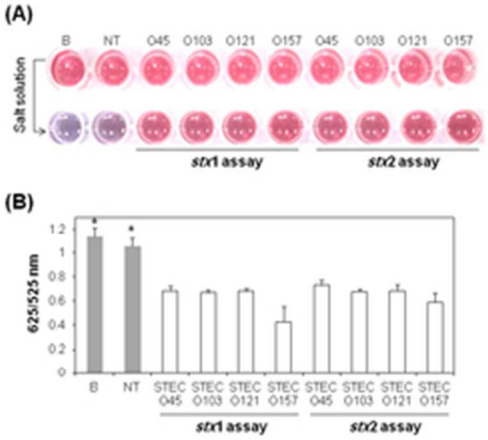


Figure 5

30x22mm (300 x 300 DPI)

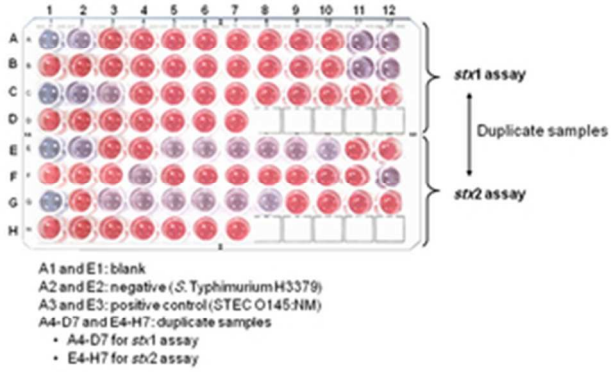


Figure 6

30x22mm (300 x 300 DPI)