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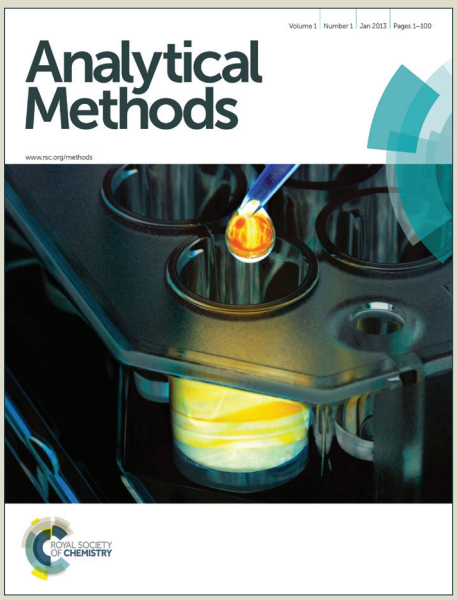

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Quantification of *Salmonella* Typhimurium in liquid food using NanoGene assay

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Abstract

Food-borne pathogen, *Salmonella* Typhimurium was detected and quantified in liquid food samples using NanoGene assay. The NanoGene assay is a recently developed gene quantification assay that employs DNA hybridization in solution and quantum dot nanoparticles. *InvA* gene was used as a functional gene target for the quantification of *S.* Typhimurium in liquid food samples (chicken broth, vegetable broth, and milk). We demonstrated quantification linearity with $R^2 = 0.91$. However a reduced sensitivity suggested that the NanoGene assay might have limited inhibitions to certain food compounds. Food compounds namely glucose, sucrose, L-lysine, casein, minerals, and oil were examined to further identify their respective inhibitory contribution. It was shown that the NanoGene assay was highly resistant to inhibition by carbohydrates. However it was vulnerable to proteins, in particular casein hydrolysate.

Keywords

Bacteria detection; inhibition; NanoGene assay; *Salmonella* Typhimurium; liquid food samples; quantum dot nanoparticles

1. Introduction

Among all the foodborne pathogens, *Salmonella* alone was responsible for ~42,000 cases of food poisoning, ~1.2 million incidents of related illnesses, ~23,000 cases of hospitalizations (35% of total), and ~450 deaths (28% of total).[1, 2] Outbreaks caused by food poisoning are preventable through proper food handling, packaging, sterilizing and rapid monitoring. It is an understatement that it will be ideal to perform near real-time pathogen monitoring at as many junctures along the food supply chain as possible.

Existing pathogen monitoring methods have their own limitations and are either based on culture (e.g., plate counting) or polymerase chain reaction (PCR).[3, 4] These methods provide conclusive and unambiguous results. However they are also time-consuming and laborious.[3] In addition, PCR-based enzymatic methods (i.e., qPCR) suffer from the inhibition by food compounds. Challenges associated with the food matrix and compounds have been the bottleneck for the detection and quantification of pathogens in food samples.[5, 6] Specifically, Rossen et al. reported that pathogen detection could be severely impaired in the presence of food compounds.[7] Therefore it will be beneficial to develop a pathogen detection method that is both rapid and inhibitor resistant to food compounds.

We have previously developed a molecular diagnostic assay (hereafter, NanoGene assay) for *E. coli* O157:H7 detection.[8] The NanoGene assay has two distinct complexes. The first complex is the reference complex that consists of magnetic beads (MBs) coupled with quantum dot nanoparticle (QD_{565}) and a target-specific carrier probe DNA. The second complex is the signaling complex that consists of another target-specific signaling probe DNA and quantum dot nanoparticle (QD_{655}). Unlike PCR assays that depend on enzymatic amplification, the NanoGene assay is based on the DNA hybridization of the target DNA to the two complexes. After hybridization, the complexes are separated from the solution using a magnet and the amount of target DNA is determined by the QD fluorescence ratio of the two complexes (i.e., QD_{655}/QD_{565}).

The NanoGene assay has demonstrated its ability to quantitatively detect *eaeA* gene of *E. coli* O157:H7 in the presence of common PCR inhibitors.[9] It is also capable of working with organic matters laden soils.[10] This has also allowed us to develop the first generation of in-situ pathogen detection system or Gen1-IPDS.[11]

Based on the results from previous studies, it seemed plausible to extend the application of the NanoGene assay to foodborne pathogen monitoring. In this study, we

evaluate the performance of the NanoGene assay by using *Salmonella* Typhimurium as the target pathogen in laboratory media as well as liquid food samples such as chicken broth, vegetable broth, and milk. Specifically we modified the NanoGene assay to target the *invA* gene of *S.* Typhimurium. Note that the *invA* gene has been widely used for the identification of *Salmonella spp.* using PCR or qPCR.[3, 4, 6, 12-16] In addition, we also investigated the impact of six potential inhibiting food compounds (glucose, sucrose, lysine, casein, minerals, and oil) on the performance of the NanoGene assay.

2. Materials and Methods

2.1 Bacterial strain

The bacterial strain used in this study is *Salmonella enterica*; subsp. *enterica*; serovar Typhimurium. Freeze-dried culture of *Salmonella* Typhimurium (ATCC 14028) was revived according to the ATCC's protocol by incubating the cells on Nutrient Agar (Difco Laboratories, Detroit, MI) at 37 °C overnight. Subsequently they were cultivated in Nutrient Broth (Difco) at 37 °C with a gentle agitation. The optical density at 600 nm (OD_{600}) of the bacterial culture was measured to monitor bacterial growth using Spectramax M2 microplate reader (Molecular devices, Sunnyvale, CA).

The bacterial cells attained stationary phase (OD_{600} = ~1.8) after 8 h. The number of bacterial colony forming units (CFUs) was determined by a conventional plate counting method. Based on the colony counts and OD reading of serially diluted culture, it was estimated that bacterial culture with 2×10^8 CFU mL^{-1} would have an OD_{600} of approximately 1.4. The overnight culture was further diluted in fresh nutrient media to obtain the desired cell concentration for spiking into food samples.

2.2 Bacterial culture in food samples

The food samples used for the study were limited to liquids such as milk, chicken broth, and vegetable broth. Commercial UHT milk, chicken broth and vegetable broth were purchased from the retail store. Bacterial culture was grown in nutrient media to an approximate concentration of 2×10^8 CFU mL^{-1}. The bacterial cells (10 mL) were first washed with deionized water and pelleted by centrifugation at 2000 × g for 15 min. Contaminated food samples were prepared by resuspending the cell pellets in 10 mL of clean food sample. In order to create bacterial populations of 2.7 – 7.7 log CFU mL^{-1}, 1 mL of contaminated food sample was serially diluted in 9 mL of clean food sample. Clean food sample was also included in each experiment as a negative control.

2.3 DNA extraction and DNA standards preparation

Prior to gDNA extraction, 9 mL of contaminated food sample was centrifuged (7500 × g, 10 min) and the bacterial cells were washed with 9 mL of 0.9% NaCl. DNA was extracted from both pure bacterial culture and contaminated liquid food samples using MO BIO soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA), in accordance to the manufacturer's instructions. The concentration of the extracted gDNA was determined using ND1000 spectrophotometer (Thermo-Nanodrop Technologies, Wilmington, DE, USA) and its purity was verified by $OD_{260/280}$ and gel electrophoresis. The extracted gDNA was stored at -20 °C until it was ready for use.

Partial *invA* amplicons were produced to construct the standard curve for the NanoGene assay. The *invA* gene encodes an invasion protein, which is required to invade epithelial cells. The invasion is a crucial function of foodborne pathogen.[15, 17] A region spanning 284 bp *invA* amplicons were amplified via PCR with a forward primer, 5′ - TCATC GCACCGTCAAAGGAACC - 3′, and a reverse primer, 5′ - GTGAAATTATCGCCACGTTC GGGCAA - 3′.[15] The PCR was carried out in the 2720 Thermal Cycler (Applied Biosystems,

Foster City, CA) with a 25 μL reaction mixture containing 1.0 μL of 167 ng μL^{-1} gDNA extracted from pure *S*. Thyphimurium culture, 5.0 μL of 5× PCR buffer (Promega, Madison, WI), 2.0 μL of dNTPs (Promega), 0.5 μL of GoTaq® polymerase (Promega), and 0.2 μmol L^{-1} of each PCR primer. The temperature cycle consisted of: an initial denaturation step at 95 °C for 5 min; followed by 35 cycles of denaturation at 95 °C for 1 min; annealing at 60 °C for 1 min; and extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The amplified DNAs were excised from their gel molds and purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA). The concentration and purity of DNAs were determined via the ND1000 spectrophotometer.

2.4 NanoGene assay

Configuration. The NanoGene assay employed in this study was slightly modified from the previously published configuration.[8] The scheme of the NanoGene assay is shown in Figure 1. In order to quantify the amount of *S*. Thyphimurium, *invA* gene was selected as the target DNA. In this case, the reference complex was conjugated with magnetic beads, QD_{565} and 25 bp probe DNA specific to partial *invA* gene (285 bp). The signaling complex consisted of *invA* gene specific signaling probe DNA and QD_{655}. In order to create the reference complex (MB-QD_{565}-probe DNA), 100 μL of the aminated magnetic beads (MB, 2×10^7 beads mL^{-1}, Dynabead M-270, Invitrogen, Carlsbad, CA) were coated with of 8 μL of carboxyl quantum dot nanoparticles (2 μmol L^{-1}, QD_{565}, Invitrogen) by the formation of covalent bonds through ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) coupling. Probe DNA (5′ - amine – CTAGTGAAATTATCGCCACGTTCGG - 3′) for the reference complex was designed for the hybridization of 3′ strand of target DNA. Five hundred pmoles of probe DNAs were conjugated to QD_{565}. Note that QD_{565} were already coated with MB through EDC/NHS covalent coupling. The reference MB-QD_{565}-probe DNA complex was washed with 0.5% and

0.1% of bovine serum albumin (BSA) in phosphate buffer (PB, 0.1 moles L^{-1}, pH 7.4) to effectively block the surface of the MB. Subsequently the reference complex was washed 5 times with PB to remove excess BSA. The signaling complexes were conjugated from 8 μL of carboxyl quantum dot nanoparticles (2 μmol L^{-1}, QD_{655}, Invitrogen) with 160 pmoles of signaling probe. The signaling probe DNA (5′ - GGTTCCTTTGACG GTGCGATGACTA – amine - 3′) was designed for the hybridization of 5′ strand of target DNA. Excess DNA and EDC were removed from the signaling complex (QD_{655}-DNA) by filtration using Amicon Ultra 30,000 MWCO spin filters (Millipore). The reaction mixture was spun at 14,000 rpm for 4 min. Subsequently the concentrated signaling complex was washed for three times and resuspended in 200 μL water after the flow-through was discarded.

DNA hybridization. In order to perform DNA hybridization, both reference and signaling complexes were resuspended using 100 μL of DIG easy Hyb buffer (Roche, Basel, Switzerland) and 5 μL of DI water, respectively. A range of PCR amplicons (*i.e.*, 10 - 10^9 copy number per reaction) were prepared for the construction of standard curve. The genomic DNA extracted from cell spiked food materials were prepared without further amplification. Both PCR amplicons (i.e., standard materials) and gDNA (i.e., spiked target *Salmonella*) as templates were added to a total of 400 μL hybridization reaction. The hybridization between DNA template and both signaling complex and reference complex was performed in the hybridization oven for 8 h at 42 °C. The hybrids were then separated via a MPC®-96S magnet (Life Technologies). Afterwards, the hybridization tubes were washed with the combination of Saline Sodium Citrate (SSC) and Sodium Dodecyl Sulfate (SDS): once with buffer 1 (2× SSC, 0.1% SDS), subsequently once with buffer 2 (0.1× SSC, 0.1% SDS), and three times with buffer 3 (0.1× SSC). The final wash was performed with deionized water prior to the fluorescence measurement.

Fluorescence measurement. The fluorescence of both QDs was measured by the Spectramax M2 microplate reader using a 96-well microplate (Nunc, Roskilde, Denmark). The emission scan was performed from 500 nm to 700 nm to obtain the signals from both QD_{565} and QD_{655}. The maximum endpoint emission was obtained at 570 nm and 660 nm for QD_{565} and QD_{655}, respectively. The excitation was set to 360 nm for both scan and endpoint measurements. All gene quantification results presented in this study were shown as normalized fluorescence (signaling fluorescence QD_{655} per reference fluorescence QD_{565}). The current value corresponding to the limit of detection (LOD) was estimated from the residual standard deviation (SD_{xy}) from standard curve divided by the slope of standard curve and multiplied by 3.3. [18]

2.5 Identification of inhibiting food compounds

Six common food compounds (glucose, sucrose, L-lysine, casein hydrolysate, Calcium ion, canola oil) were identified as potential inhibitors and they were selected from the category of carbohydrate, protein, mineral and lipid: glucose and sucrose as carbohydrate, L-lysine as amino acid, casein hydrolysate for milk protein, Calcium chloride to represent minerals, canola oil representing lipids. Glucose, L-lysine, casein hydrolysate, and $CaCl_2$ were purchased from Sigma-Aldrich. Sucrose was purchased from Fisher Scientific and canola oil was purchased from the retail store. Stock solution was prepared by mixing the food compounds in DI water and diluted to its final concentration. One hundred μL of food compound with various concentrations were directly added to each DNA hybridization reaction. Based on the investigation of the quantity of each food components in food samples (i.e., commercial food information), relevant concentration for the experiment was determined. All spiked *invA* target DNA template was prepared to contain 2.0×10^8 copy numbers. The inhibition effect of food components was shown as the quantification of assay (%). It was calculated from individual

sample fluorescence divided by the negative control fluorescence. Note that the negative control fluorescence was obtained in the absence of inhibitory food compounds.

3. Results and Discussion

3.1 Linearity, sensitivity, and range of quantification

Figure 2A shows the fluorescence scan of QDs from the hybridized complexes. A range of target DNAs (0 – 10^{10} copies) demonstrated almost identical intensities on the reference signal at 565 nm. It indicated that each sample had similar amount of QD_{565} at the reference complex (MB-QD_{565}-probe DNA). At the same time, the florescence intensities at 655 nm increased proportionally with the amount of target DNA copies. It indicated that the signaling DNA from signaling complex (QD_{655}-signaling DNA) was able to hybridize with the target DNA. Figure 2B shows the calibration curve where the normalized fluorescence (i.e., QD_{655}/QD_{565}) is plotted against the *invA* target copy numbers. The quantification result suggested a linearity (R^2 = 0.94) with the LOD of 16 gene copies. The linear range of quantification was 10^2 – 10^8 gene copies per reaction.

The calibration curve shown in Figure 3 corresponded to the *invA* gene quantification using bacterial gDNA spiked in laboratory media. It gave the calibration curve of $y = 0.46\ x + 4.30$, where y is fluorescence signal and x is bacterial concentration in log CFU mL^{-1}. The linear range ($R^2 = 0.91$) of quantification was 3.7 log CFU mL^{-1} to 7.7 log CFU mL^{-1} (5.3×10^3 CFU mL^{-1} – 5.0×10^7 CFU mL^{-1}). The bacterial quantification in laboratory media was successful with a linear range of 3.7 log CFU mL^{-1} through 7.7 log CFU mL^{-1}.

3.2 Quantification of *S.* Typhimurium in liquid foods

Figure 4 shows the *invA* gene quantification results for bacterial gDNA extracted from contaminated food samples. The relative fluorescence in y-axis of Figure 4 was obtained by

subtracting fluorescence of negative control (*Salmonella*-free) from the respective contaminated food samples. Comparing Figure 3 and 4, gene quantification for both chicken broth and vegetable broth (Figure 4, LOQ of 4.3 log CFU mL^{-1}) were very similar to that of laboratory media (Figure 3, LOQ of 3.7 log CFU mL^{-1}). The result indicated the NanoGene assay was capable of bacterial detection and quantification in both chicken and vegetable broth. However, the LOQ (Figure 4, 5.2 log CFU mL^{-1}) of milk sample was one order of magnitude higher than that of chicken and vegetable broth. Therefore it is likely that there are food compounds in milk that are capable of reducing the performance of the NanoGene assay. Similar inhibition in food sample was found in the qPCR assay, which resulted in the sensitivity drop up to 4 log CFU g^{-1}.[6]

3.3 Characterization of NanoGene assay with food inhibitors

The inhibitory effect of individual food components to the NanoGene assay was elucidated and presented in Figure 5. The quantification % obtained by the NanoGene assay was nearly 100% for different concentrations of glucose and sucrose. (Figure 5A and 5B). It suggested that carbohydrate containing foods would not result in diminished performance of the NanoGene assay.

As shown in Figure 5C, L-lysine amino acid exhibited inhibition. Approximately 20% and 40% of inhibition were observed for 130 μg and 500 μg of L-lysine, respectively. Casein hydrolysate exhibited even more inhibition than L-lysine (Figure 5D). It showed 40% inhibition at the lowest test concentration (1.56 μg). Casein at 20 μg resulted in approximately 80% inhibition. The results indicated that NanoGene assay could be compromised by the presence of proteins such as casein hydrolysate.

As shown in Figure 5E, the performance of the NanoGene assay was not affected by the presence of Calcium ion except at the highest concentration (10 mmol L^{-1}). Milk is a

calcium-dominant food. The milk sample used in this study contains 12.5 mg of Calcium per 10 mL, which is equivalent to 30 mmol L^{-1}. It is 15 - 20 times more than vegetable and chicken broths. This result is consistent with the inhibitory result for milk compared to vegetable and chicken broths.

As shown in figure 5F, approximately 4.5% of canola oil resulted in 20% inhibition. Increased amount of canola oil at 18% and 36% of oil resulted in 30% and 80% inhibition respectively. Note that the amount of lipids exceeding 10% is unlikely to be found in real food. For example, the milk sample used in this study contained 1% lipid. Therefore at low amount of lipids, the NanoGene assay's performance would be minimally affected.

The inhibition by protein might be attributed to its interaction with the DNA hybridization buffer. The composition of the hybridization buffer includes salt (e.g., saline-sodium citrate), ionic surfactant (e.g., sodium dodecyl sulfate and N-laurylsarcosine), and blocking agent (e.g., Bovine serum albumin). Salts and surfactant are used to maintain buffer stringency and ionic strength for DNA hybridization. The blocking agent is used to prevent non-specific binding other than complementary hydrogen bonds.[19] These buffer components might have had the undesirable interactions with proteins. The potential interactions are as follows:

(1) Salt was used in the hybridization buffer to increase the efficiency of the DNA hybridization. However they could also result in coagulation in proteins. It is well known that proteins denature or coagulate by heat, pH change, and the addition of surfactant or salt.[7, 20] It is possible that the coagulated and solidified proteins inhibit the DNA hybridization by (i) reducing the salt concentration or (ii) forming a large complex in proximity of the DNA probes.

(2) Surfactants are amphiphilic and they can interact with proteins, which contain multiple functional groups. The interaction between surfactants and proteins could possibly remove the surfactants from the hybridization reaction. This would have reduced the efficiency

of DNA hybridization.

(3) Typical blocking agents are usually protein based compounds. They can effectively prevent non-specific bindings of unwanted targets when used at low concentrations. However the addition of blocking agents into protein-laden food samples (e.g., milk) would excessively increase the amount of proteins available in the reaction. The milk proteins could behave like non-specific binders themselves to the components of NanoGene assay such as magnetic beads. This might result in the inhibition of the NanoGene assay**.**

In summary, we demonstrated the detection of *S.* Typhimurium in liquid food samples by the NanoGene assay without the use of excessive purification/separation processes. The signaling and reference complexes were assembled to target *invA* gene of the *S.* Typhimurium. Among the various types of liquid food samples tested, only milk showed a significant decrease in sensitivity. This was likely due to the significant presence of proteins in milk, which might inhibit the NanoGene assay. Otherwise NanoGene assay performed well for laboratory media, chicken broth and vegetable broth. In other words, the NanoGene assay is suitable for pathogen detection in relatively protein-free food samples. However more work is required to enable the NanoGene assay to work with protein laden food samples as well.

Acknowledgements

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Figure Legends

Figure 1. Scheme of NanoGene assay for *S.* Typhimurium detection

Figure 2. Gene quantifiation with dsDNA amplicons (0 - 10^{10} copies) targeting *invA* gene by NanoGene assay. (A) Fluorescence emission was scanned from 500 nm to 700 nm for varied amount of *S.* Typhimurium *invA* gene. The QDs' peaks at 565 nm and 655 nm indicate internal standard and signal, respectively. (B) Normalized fluorescence was obtained from the fluorescence intensity of QD_{565} and QD_{655} and presented as a calibration curve of *invA* gene quantification ($R^2 = 0.94$).

Figure 3. Quantification of *InvA* gene using genomic DNA of *S.* Typhimurium spiked in laboratory media. Dotted lines depict the range of quantification for S. Typhimurium detection (3.7 through 7.7 log CFU mL^{-1}). The signal and error bars represent mean and standard deviation based on five measurements of fluorescence. The same description regarding the error bars applies to Figure 4 – 5.

Figure 4. Quantitative detection of *S.* Typhimurium spiked in chicken broth, vegetable broth, and milk using extracted genomic DNA.

Figure 5. The inhibitory effect on the gene quantification by individual food components: (A) glucose, (B) sucrose, (C) lysine, (D) casein, (D) Calcium ion, and (F) oil.

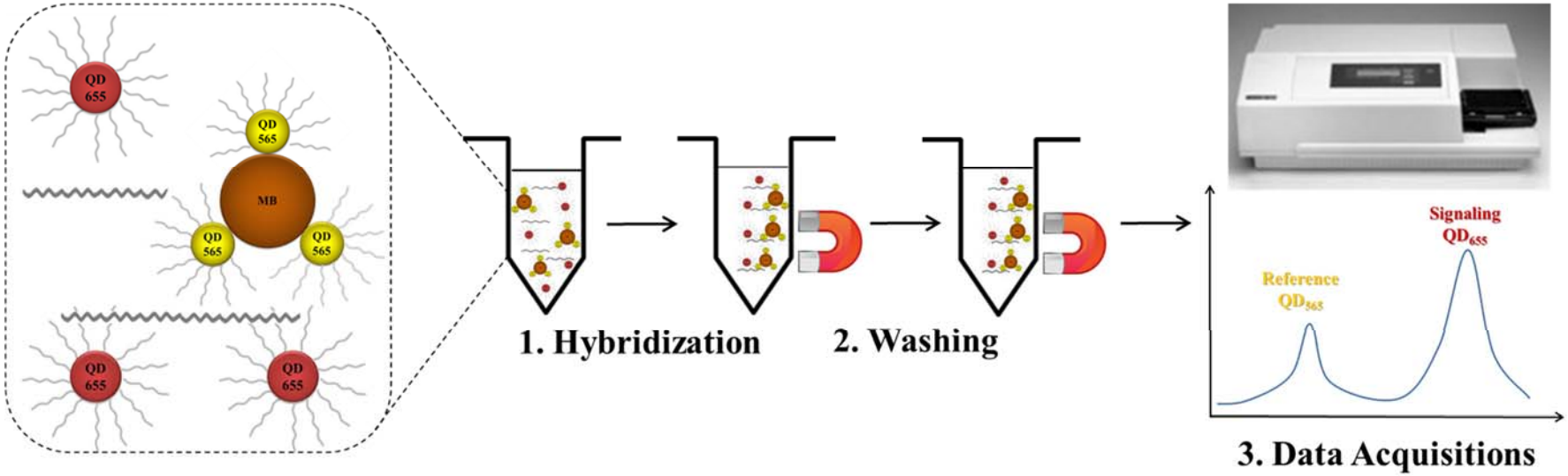
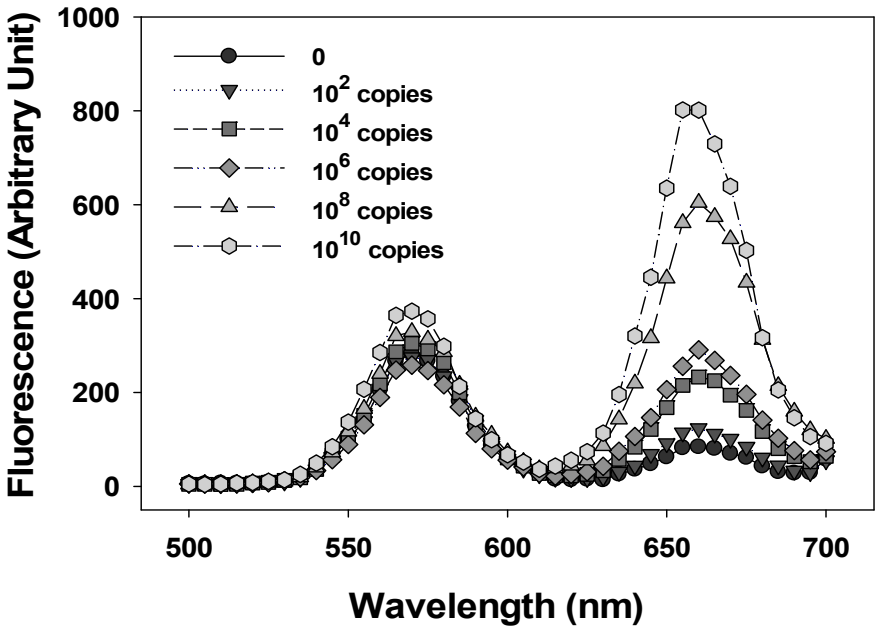


Figure 1. Scheme of NanoGene assay for *S.* Typhimurium detection

(A)



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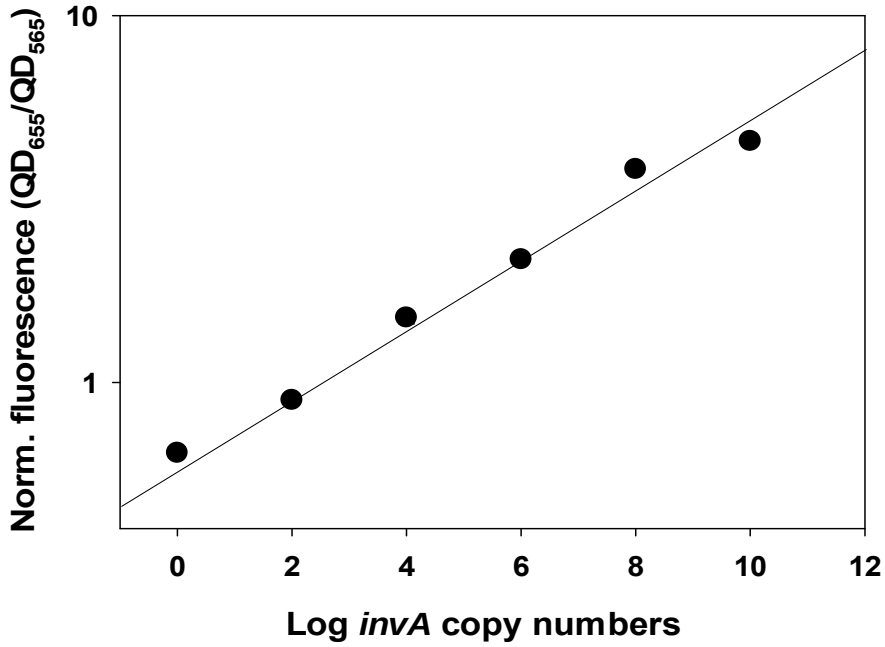

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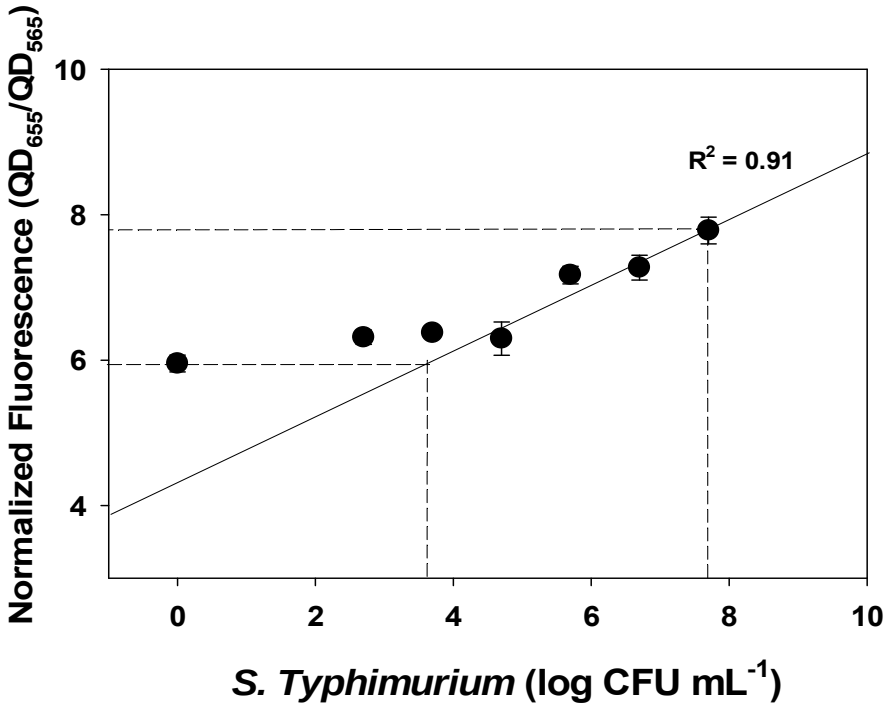

Figure 3.

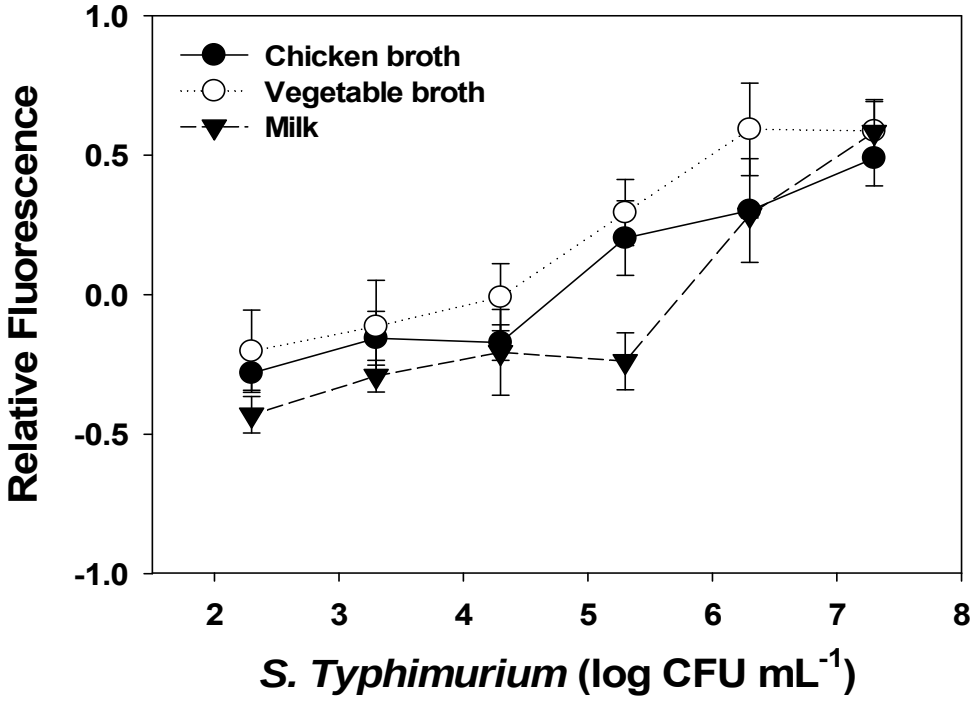

Figure 4.

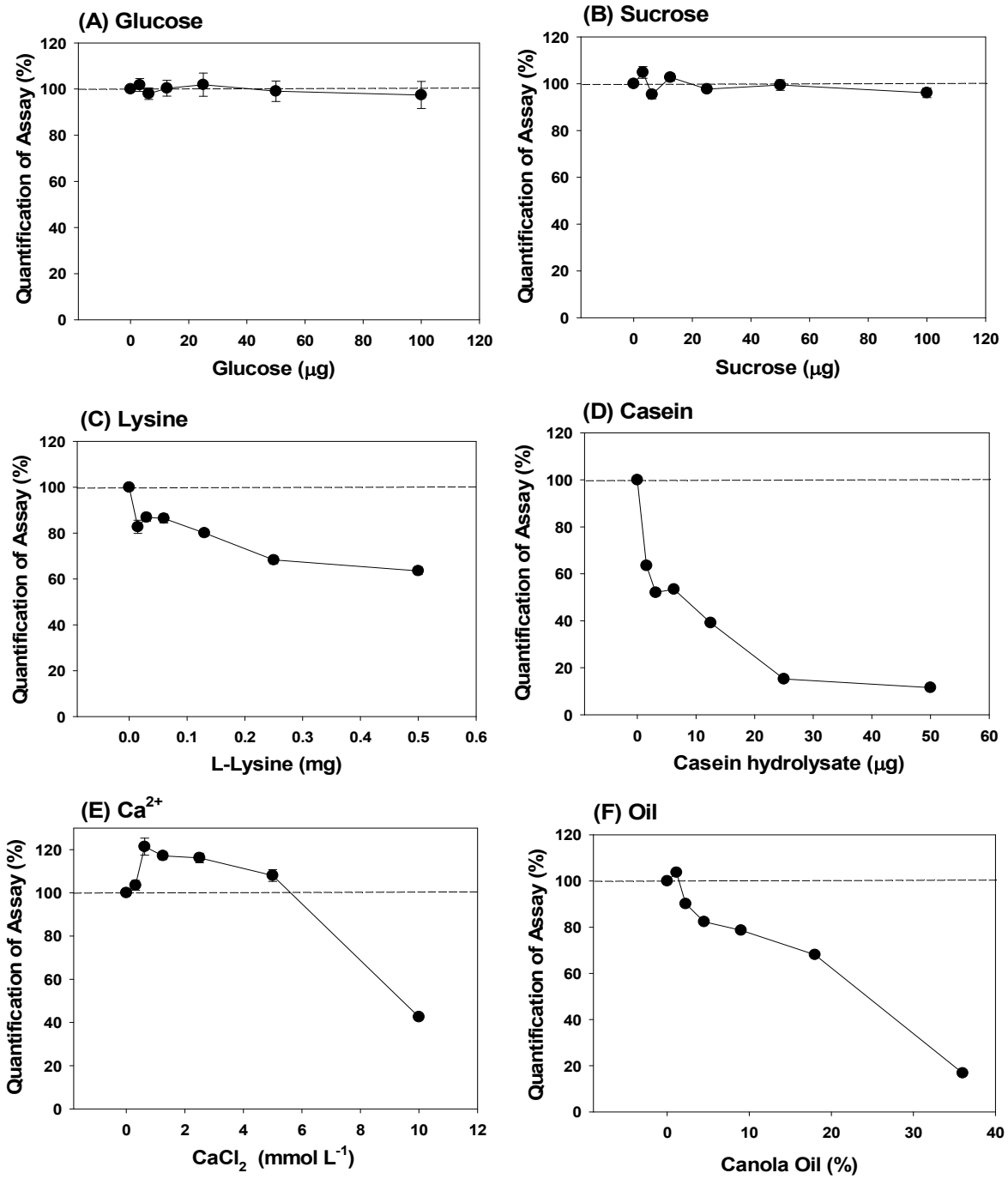

Figure 5.