# Multiplex Detection of Foodborne Pathogens Using Iron Oxide Nanoparticles and Quantum Dots

## Introduction

In comparison with magnetic microbeads, magnetic iron oxide (IO) nanoparticles are more mobile in complex samples and have faster reaction kinetics, lower mass, higher surface-to-volume ratio, less blocking effect on optical measurement, and lower cost. Thus, the use of IOs may greatly improve immunomagnetic separation of foodborne pathogens, specifically when coupled with an optical detection method. Here we present a development of a multiplex immunoassay by integrating magnetic IO nanoparticles for immunoseparation with quantum dots (QDs) as fluorescent labels for rapid, sensitive, and simultaneous detection of three major pathogenic bacteria, *Salmonella Typhimurium*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*, in food products.

## Materials:

Ocean's iron oxide particle SHS30 (streptavidin surface groups, 30 nm in size), QD530-strep, QD580-strep, QD620-strep (streptavidin functionalized QDs with emission at 530 nm, 580 nm, 620 nm, may be prepared in your own lab or through our customer conjugation service), biotinylated rabbit polyclonal anti-*Salmonella* antibodies (Abs), goat anti–*E. coli* antibodies, and rabbit anti–*L. monocytogenes* antibodies (Biodesign International, Saco, ME), Stock cultures of *Salmonella Typhimurium* (ATCC 14028), *E. coli* O157:H7 (ATCC 43888), and *L. monocytogenes* (ATCC 43251) were obtained from the American Type Culture Collection (ATCC;Manassas, VA) and grown for 18 to 20 h at 37ºC in brain heart infusion broth (Remel, Lenexa, KS), SuperMag™ separator

## Procedures:

1. **Coating of SHS30 with Abs:** Each 20 ul of SHS (1 mg Fe/ml) was washed with 100 ml of PBS (0.01 M, pH 7.4). After magnetic separation, the pellet was resuspended in 20 ul of PBS, and the SHS30 were mixed with 20 ul of biotin-conjugated anti-*Salmonella*, anti–*E. coli*, or anti–*L. monocytogenes* antibodies (0.5 mg/ml) with a rotating mixer for 0.5 h at room temperature. A magnetic field was applied to the samples for 45 min, and the pellet was washed twice with 100 ml of PBS to remove unattached antibodies. SHS30 coated with antibodies were then resuspended in 40 ml of PBS.
2. **Coating of QDs with Abs:** Streptavidin-conjugated QDs with emission wavelengths of 530, 580, and 620 nm were separately coated with anti-*Salmonella*, anti–*E. coli*, and anti–*L. monocytogenes* antibodies, respectively. In this step, 20 ul of each of the three types of QDs were mixed with 20 ul of a relevant biotin-conjugated antibody (0.5 mg/ml) for 30 min. The mixture was shaken on a mixer at room temperature.
3. **Food sample preparation and inoculation:** Chicken carcasses purchased from a local grocery store were individually washed with 100 ml of 0.1% buffered peptone solution in a sterile bag for 1 min. Each 25 g sample of ground beef,

freshcut broccoli, and fresh-cut lettuce (purchased from a local grocery store) was mixed with 225 ml of 0.1% PBS and washed in a stomacher for 1 min. The wash solutions were collected and inoculated with mixed cultures of *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes*. The final inoculation levels of target bacteria wash solutions were $10^0$ to $10^6$ CFU/ml.

4. **Separation of three pathogenic bacteria:** Serial dilutions of the pure cultures of *S. Typhimurium*, *E. coli* O157:H7 or *L. monocytogenes* were prepared in 0.01 M PBS. A 20 ul aliquot of the prepared IO-Ab was mixed with 100 ul of each culture at $10^3$ CFU/ml on a mixer for 45 min at 4ºC. For simultaneous separation experiments, 100 ul of each culture was used to make the 300 ul mixture of the three bacteria, in which the final level was $10^3$ CFU/ml for each culture. A 20 ul aliquot of the prepared IO-Ab for each of three target bacteria (a total of 60 ul) was added into the 300 ul test sample containing the three bacteria and mixed on a rotating mixer for 45 min at 4ºC. The IO-Ab-cell complexes then were collected by a SuperMag™ separator for 45 min and resuspended in 1 ml of PBS. A 100 ul aliquot of the sample was plated on each of three selective media to obtain population estimates for each of the three bacteria. The same level of the original mixed cultures was used as a positive control. The capture efficiency (CE) was calculated with the following equation: CE (%) = $N_c/N_o$ x 100, where $N_c$ is the number of captured cells and $N_o$ is the number of original cells.
5. **Fluorescent detection of three pathogenic bacteria:** After magnetic immunoseparation, the IO-Ab-cell conjugates were mixed with 20 ul of the QDs conjugated with biotinylated anti-*Salmonella*, anti–*E. coli*, or anti–*L. monocytogenes* antibodies with emission wavelengths of 530, 580, and 620 nm, respectively, for 30 min. The QDs were attached to the bacterial cells through the immunoreaction between the antibodies on the QDs and the antigens of bacterial cells, and the IO-cell-QD complexes were formed. Unattached QDs were removed when a magnetic field was applied. The final complexes were washed with PBS twice and resuspended with 150 ml of PBS. The fluorescence intensity of emission peaks produced by the QDs on the IO-cell-QD complexes at various wavelengths was measured with a portable spectrometer (Em =395 nm, Ocean Optics, Inc., Dunedin, FL) to determine the populations of the three bacterial pathogens, which was calculated based on a calibration curve that gives a linear correlation between the change in fluorescence intensity and the logarithmic value of bacterial populations. The change in fluorescence intensity was determined by subtracting the fluorescence intensity value of the control from that of the IO-cell-QD complex. For fluorescence microscopy, the IO-cell-QD conjugate samples were spotted on a glass slide and observed under an Axioplan II imaging microscope (Carl Zeiss, Jena, Germany) equipped with an ORCAER digital camera (Compix, Inc., Cranberry Township, PA).
6. **Transmission electron microscopy of three pathogenic bacteria:** The IO-cell-QD complexes were spotted onto a special 300-mesh grid overnight. The dried samples were directly examined with a JEM-100 CX transmission electron microscope (Jeol, Boston, MA).

**Results and Discussions**

*Individual separation of the three target bacteria*

The capture efficiencies for *L. monocytogenes*, *S. Typhimurium*, and *E. coli* O157:H7 using the IO nanoparticles were 89, 88, and 91% (Fig. 1a), respectively. The immunomagnetic separation using the IO nanoparticles increased the capture efficiency by 1 to 4% compared with that using the 150 nm magnetic nanobeads. However, the separation time with the IO nanoparticles was nine times as long as that using the 150 nm nanobeads.

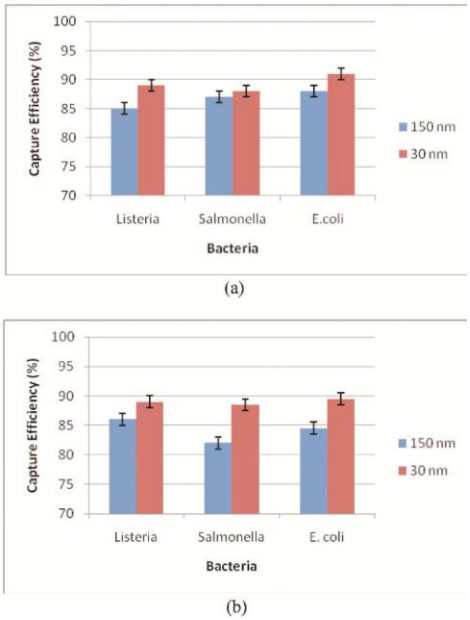


FIGURE 1. Capture efficiency for each of three target bacterial pathogens, *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes*, in pure culture using immunomagnetic nanobeads (MNBs, 150 nm and 30 nm in diameter) for individual (a) and simultaneous (b) separation methods. The streptavidin-bound nanobeads were coated with biotin-labeled anti–*S. Typhimurium*, anti–*E. coli* O157:H7, and anti–*L. monocytogenes* antibodies via biotin-streptavidin binding.

*Simultaneous separation of the three target bacteria*

*L. monocytogenes*, *S. Typhimurium*, and *E. coli* O157:H7 were simultaneously separated, and their capture efficiencies were 89 to 90% for the IO nanoparticles (Fig. 1b). IO nanoparticles specifically and simultaneously captured and separated *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes* at levels as low as 10 CFU/0.1 ml in the various food samples (chicken, beef, and lettuce). The capture efficiency for all three target pathogens in foods was 89 to 90% for IO nanoparticles, which was higher than that obtained with magnetic microbeads with diameters of 1.0 and 2.8 mm.

The TEM images of *L. monocytogenes* cells clearly indicate that the target cells were attached with the antibody coated IO nanoparticles (Fig. 2).

A single cell of *L. monocytogenes* was estimated to be able to bind with 800 to 1,000 IO nanoparticles. In general, nanoparticles provide more opportunities to improve magnetic immunoseparation with better capture efficiency, less interference, and multiplex operation.

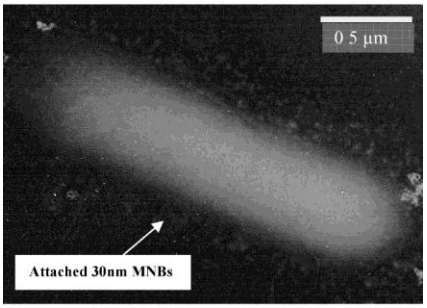


FIGURE 2. TEM images of an *L. monocytogenes* cell attached to the antibody-conjugated magnetic nanobeads (MNBs) of diameter 30 nm. The streptavidin-bound MNBs were coated with biotin-labeled anti–*L. monocytogenes* antibodies via biotin-streptavidin binding.

*Simultaneous detection of the three target bacteria*

Figure 3a shows typical fluorescence spectra measured from 500 to 650 nm for simultaneous detection of the three major foodborne pathogenic bacteria, *S. Typhimurium* (QD530), *E. coli* O157:H7 (QD580), and *L. monocytogenes* (QD620), in pure culture. The background or negative control (PBS) gave fluorescence intensities of 568 to 613 counts, and the mixture of bacterial cultures at 4 to 6 CFU/0.1 ml gave intensities of 776 to 815 counts. The fluorescence intensity increased significantly with increasing populations of 5 to 500 CFU/0.1 ml in the mixed cultures.

The background produced fluorescent peaks at 530, 580, and 620 nm with an intensity of less than 610 counts. For *S, Typhimurium, E. coli* O157:H7, or *L. monocytogenes* populations of 5 CFU/0.1 ml, the samples produced fluorescent peaks with an intensity of 800 counts, which was significantly different from the background counts. The fluorescence intensity also increased with increasing populations of *S. Typhimurium*, *E. coli* O157:H7, or *L. monocytogenes* from 5 to 500 CFU/0.1 ml. Thus, the more bacterial cells in the sample, the more QDs they could bind and the stronger fluorescence they could produce.

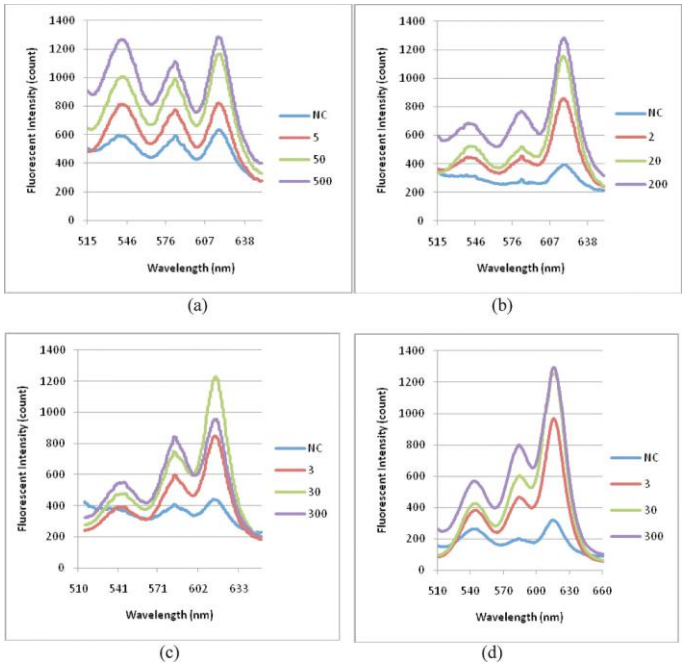


FIGURE 3. Fluorescence spectra measured from 500 to 650 nm for simultaneous detection of three target bacterial pathogens, *S. Typhimurium* (530-nm emission wavelength), *E. coli* O157:H7 (580-nm emission wavelength), and *L. monocytogenes* (620-nm emission wavelength), at 0, $10^0$, $10^2$, and $10^2$ CFU/0.1 ml in (a) pure culture, (b) ground beef samples, (c) fresh-cut lettuce wash samples, and (d) chicken carcass wash samples.

*Simultaneous detection of the three target bacteria in different food samples*

Figure 3b shows typical fluorescence spectra from 500 to 650 nm recorded for simultaneous detection of the three foodborne pathogenic bacteria in ground beef wash solutions. The fluorescence intensities of the negative control were 311, 269, and 391

counts at 530, 580, and 620 nm, respectively (all below 400 counts). At the 2 CFU/0.1 ml level, the peak values of fluorescence intensities for *S. Typhimurium* at 530 nm, *E. coli* O157:H7 at 580 nm, and *L. monocytogenes* at 620 nm were 448, 453, and 845 counts, respectively. With three replicates, the fluorescence intensities of all positive samples are significantly different ($P<0.05$) from that of the negative control.

Figure 3c shows typical fluorescence spectra from 500 to 650 nm recorded for simultaneous detection of the three pathogenic bacteria in fresh-cut lettuce wash solutions. The fluorescence intensities of the negative control were 379, 385, and 407 counts at 530, 580, and 620 nm. At 2 to 3 CFU/0.1 ml, the peak fluorescence intensities of *S. Typhimurium* at 530 nm, *E. coli* O157:H7 at 580 nm, and *L. monocytogenes* at 620 nm were 394, 598, and 846 counts, respectively. For three replicates, the fluorescence intensities of all positive samples at wavelengths of 530, 580, and 620 nm were significantly different ($P<0.05$) from those of the negative controls, whereas the fluorescence intensity at 530 nm was only slightly higher than that of the negative control.

Figure 3d shows typical fluorescence spectra from 500 to 650 nm recorded for simultaneous detection of the three pathogenic bacteria in chicken carcass wash solutions. The fluorescence intensities of the negative controls were 263, 202, and 320 counts at 530, 580, and 620 nm, respectively. At 3 CFU/0.1 ml, the fluorescence intensities of *S. Typhimurium* at 530 nm, *E. coli* O157:H7 at 580 nm, and *L. monocytogenes* at 620 nm were 382, 467, and 966 counts, respectively, which presented more than 100-count changes in fluorescence intensity. For three replicates, fluorescence intensities of samples containing *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes* were significantly different ($P< 0.05$) from that of the negative control. The two curves for 30 and 300 CFU/0.1 ml *L. monocytogenes* at 620 nm were very close, possibly because of the limitation of photon yield with the quantity of QDs used when the higher level of *L. monocytogenes* bound all available QDs. A fluorescent image (x1,000) of *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes* attached with specific antibody-conjugated QDs with emission wavelengths of 530 nm (green), 580 nm (yellow), and 620 nm (red) is shown in Figure 4.

The linear relationships between the change in fluorescence intensity and the log-transformed pathogen population are shown in Figure 5 for the simultaneous detection of the three target bacterial pathogens at levels of 0 to $10^2$ CFU/ml. The linear regression equations are $Y=238.2X+16.2$ ($R^2=0.99$), $Y=192.3X+ 29.2$ ($R^2=0.97$), and $Y= 251.7X+ 23.8$ ($R^2=0.96$) for *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes*, respectively, where Y is the change in fluorescence intensity (counts) and X is the log-transformed bacterial population (log CFU/0.1 ml). The detection limit of $10^0$ to $10^1$ CFU/0.1 ml for this multiplex immunoassay is comparable to that of most rapid detection magnetic immunoseparation methods evaluated in recent years. All $R^2$ values are very high and close,

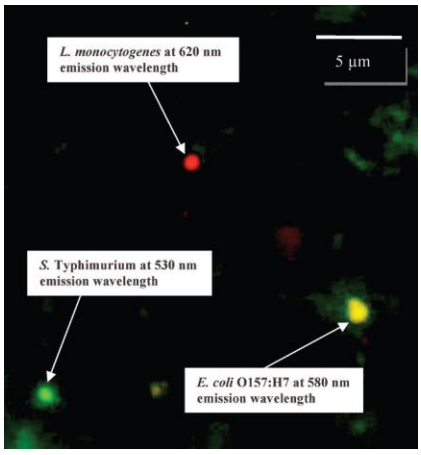


FIGURE 4. Fluorescence microscopy image of *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes* cells attached to antibody-conjugated QDs with emission wavelengths of 530 nm (green), 580 nm (yellow), and 620 nm (red), respectively.

indicating high levels of repeatability and reliability of the multiplex immunoassay. The difference in the slopes of these three regression lines could be caused by (i) the different quantum yields of the QDs used and (ii) the different binding abilities of the specific antibodies used for different target bacteria in this multiplex immunoassay.

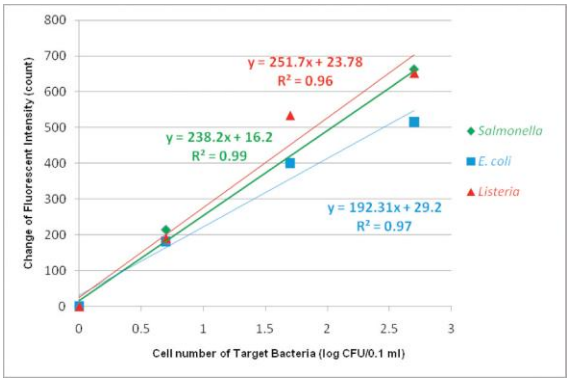


FIGURE 5. Linear relationships of the change in fluorescence intensity versus the log-transformed cell population during the simultaneous detection of three target bacterial pathogens, *S. Typhimurium*, *E. coli* O157:H7, and *L. monocytogenes*, at populations ranging from 0 to $10^2$ CFU/0.1 ml. The changes in fluorescence intensity are the means of five replicates, and the cell populations are those of L. monocytogenes in pure culture.