

Technical Note

Bioreagents Based On Inert Magnetic Particles As Powerful Diagnostic Tools For Microbial Quantification

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A rapid and sensitive quantification process for Escherichia coli was developed using inert magnetic nano-particles (IIMPs) for the directed immobilization of antibodies. The protocol consisted of the incubation of 1-20 mL samples with polyclonal anti-E. coli immunomagnetic particles in a suitable capturing medium. The resulting E. coli-immunomagnetic complexes were easily isolated by a simple magnet, washed, and incubated with a second polyclonal anti-E. coli labelled with horseradish peroxidase (HRP); the final immunomagnetic bead-E. coli- HRP anti-E. coli complexes, in a sandwich format, were again isolated by the magnet and washed. The final protocol took less than 60 minutes to complete and had a detection limit of 1 CFU of E. coli per mL in aqueous samples spiked with known microbial concentrations. Negative controls were introduced. Microbial counting of E. coli was made by an independent laboratory, using standard microbiological method. No previous enrichment or concentration steps were made.

INTRODUCTION

For many years, antibody-antigen interaction has been used to design immuno-magnetic bioseparation techniques and biosensors. Performance of immuno-reagents depends on availability of suitable antibodies and magnetic solid surfaces. Significant improvements in the manufacture of magnetic particles attracted the interest on their use as supports of antibodies. Homogeneity in the shape and size of particles contributes to perform identically in a suspension, with respect to sedimentation and kinetics of binding to antibodies. Super-paramagnetism is other physical property very interesting to design easy-handling diagnostic tools based on particles. As soon as a magnetic field is removed, once the magnetic particles are separated by a magnet, these particles should not attach to each other through inter-magnetic force but should go directly back into a suspension.

However, chemical composition of the particle surface is critically important for their successful use like robustness reagents to design in vitro test with clinical or environmental relevance. Chemical properties of the surface must lead to a proper orientation of antibodies and a reduced non-specific binding. Considerable attention has been given to this problem by the manufactures of magnetic particles. An inert surface that does not bind to biological elements other than the specific binding molecule and the target for immuno-magnetic capture is desirable. For example, progressive adsorption of immobilized antibody on the support surface (only several days at low ionic strength) or non-specific binding may yield a false result (1). Proper orientation and time-stability of the antibodies on the inert surface allows an optimized ratio of active antibody/particle, leading to a long-

time and non-expensive generation of immunomagnetic particles. With the scientific support of National Research Council (CSIC, Spain), the company Biótica, Bioquímica Analítica, S.L. design protocols to modify these immunomagnetic particles, and also is developing rapid techniques based on this kind of immunomagnetic particles. Immunomagnetic particles are usually used in small scale for separation of nucleic acids, target cells and like parts of the procedures for the determination of selected analytes (ELISA and related techniques). Up to date the use in small scale prevails, so the full potential of immunomagnetic particles has not been fully exploited. A major concern for microbiology diagnostics is the lack of sensitivity associated to small sample volumes. The sample volume used in PCR-based techniques ranges from < 1 to 20 μ L; also in ELISA-based techniques it ranges 25 to 100 μ L. However, legal requirements are often one cultivable microorganism per mL of sample, so reduction of the sample volume strongly restricts the test sensitivity (2). The losses of immunomagnetic particles during their handling in the analytical process are a greater problem for the user to conciliate a sensitive microbial detection with the scale of working volume. Further development has been made by Biótica, S.L. in order to prevent these losses, so it has been possible increasing the scale of sample volume up to 1000 times.

MATERIALS AND METHODS

Culture of Bacteria

Lyophilized strain of *E. coli* was requested to Spanish Type Culture collection (CECT) from the University of Valencia (Spain), and was reconstituted by Microbiology Department of Servyeco, S.L. (Castellón, Spain), credited laboratory of water analysis. After 24 hours-incubation, growing bacteria are transferred from the plate culture to a liquid medium. The bacterial suspension is diluted with 150 mM phosphate buffer pH 7,0. Cell concentrations were obtained ranging 1 to 100000 cfu/mL.

Preparation of immunoparticles

Super-Paramagnetic Particles [EM1-100/40 (COOH-modified) or M2-070/60 (NH₂-modified)] supplied by Estapor® Microspheres (Merck Chimie SAS., France), were washed by repetitive centrifugation and suspension. Cleaned particles were treated to obtain reactive surfaces to immobilize antibodies. Chemically modified antibody (anti *E. coli*) was immobilized on the particles following protocol designed by Biótica, S.L. In all cases, the immobilized anti *E. coli* was determined quantifying the difference in protein concentration in the supernatant before and after immobilization, using the Bradford method. After immobilization of the antibody, immunomagnetic particles (IMPs) were treated with carbohydrate-based polymer and inert IMPs (IIMPs) were obtained.

Capture of *E. coli*

For first experiment, 100 μ L of IIPMs were incubated 15 min with 1 mL of aqueous samples, each one of them with different concentrations of *E. coli*, ranging from 100 to 100000 cfu/mL. Sample without *E. coli* is the negative control. For second experiment, 25 μ L of IIMPs were incubated 10 min with 20 mL of aqueous samples, each one of them with different concentrations of *E. coli*, ranging from 1 to 1000 cfu/mL. In all cases, capture and concentration of *E. coli* - IIMPs complexes were achieved by the use of neodymium iron boron planar magnet (Aimanz, S.L., Barcelona, Spain). Repetitive magnetically concentration and release (removing the magnet) is applied for washing steps. No loss of IIMPs was observed if recovery buffer of Biótica is applied. Recovering buffer of Biótica was not applied in the first experiment. Finally the total volume is reduced to 1-2- mL, once the capturing event occurred. *E. coli* - IIMPs complexes were incubated with a second anti *E. coli* labelled with horseradish peroxidase (HRP) in a suitable buffer. After washing steps, labelled sandwich complexes were obtained.

Activity measurements

Horseradish peroxidase (HRP) activity was determined using H₂O₂ as the oxidizing substrate and ABTS as the reducing substrate. Activity was spectrophotometrically followed (Biomate 3, Thermoelectron Corporation) by recording the increase in the absorbance at 405 nm.

Chemicals

All chemicals used were laboratory grade, obtained from commercial suppliers.

RESULTS AND DISCUSSION

Capture of *E. coli* is illustrated in figure 1. Briefly, 20 μ L of IIMPs were added to 1mL - *E. coli* culture., Direct microscopy observation of the captured bacteria was

made. Between 2-4 cells per particle was observed in the experimental conditions. Capturing ratio depends on particle size (3). Moreover, 200 μ L of the *E. coli* sample was cultured before capturing event (figure 1, plate A). After capturing event, 200 μ L of the last supernatant from washing step was also cultured (figure 1, plate B). Finally the 20 μ L of IIMPs recovered after washing was also cultured (figure 1, plate C). A major proportion of the highly concentrated culture of *E. coli* is captured by the IIMPs, appearing in plate C. A minor proportion of *E. coli* appears in plate B. Because our objective is to develop friendly-user assay technique, initial work focused on the preparation of inert immunomagnetic particles (IIMPs) and also on the design of an easy and adjusted protocol.

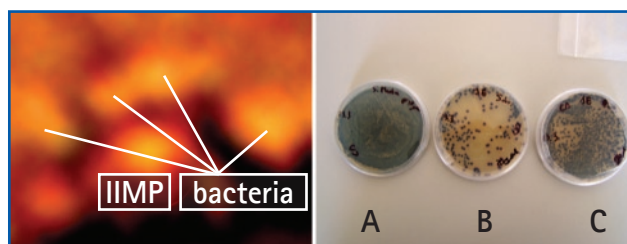


Figure 1. Direct microscopy image showing captured *E. coli* cells on the IIMPs. Comparison between plate counts before and after capturing event illustrated viability of performing separation of *E. coli*, prior to adjust analytical protocol (explanation in the text).

The data for the first experiment suggest that with 1mL incubation for capturing event the detection limit could be 100 *E. coli* cells per mL (figure 2), without previous enrichment or concentration step. Enrichment step is habitually in magnetic particles-based techniques (4, 5, and 6). Experiments were conducted to improve detection limit, taking into account legal restrictions for a major number of microbiological applications. In a second experimental series, a correct prevention of losses of magnetic particles allowed to increase sample volume, to reach a detection limit as low as 1 *E. coli* cell per mL (figure 3).

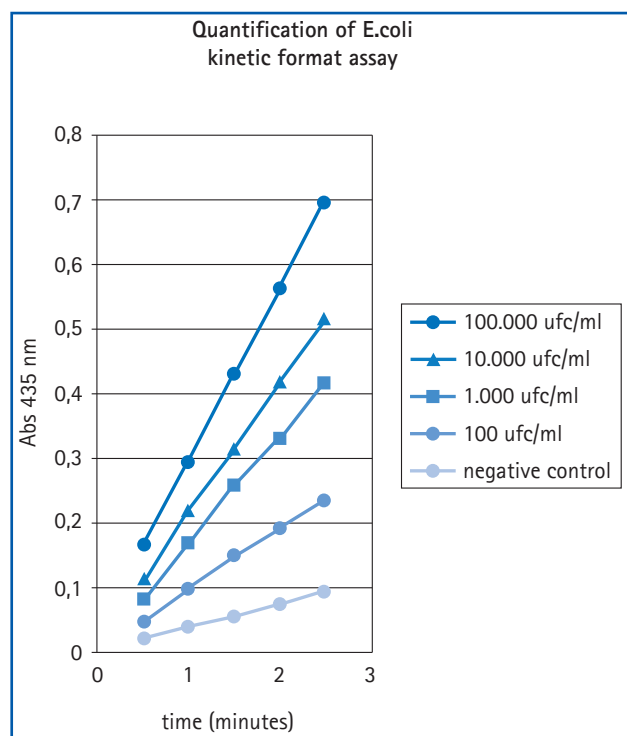


Figure 2. Results of IIMPs assays for serial dilutions of *E. coli* ranging from 100000 to 100 cfu/mL. Detection limit was 100 cfu/mL. 100 μ L of IIMPs in 1 mL of *E. coli* sample was used, with non-conditioned capturing buffer.

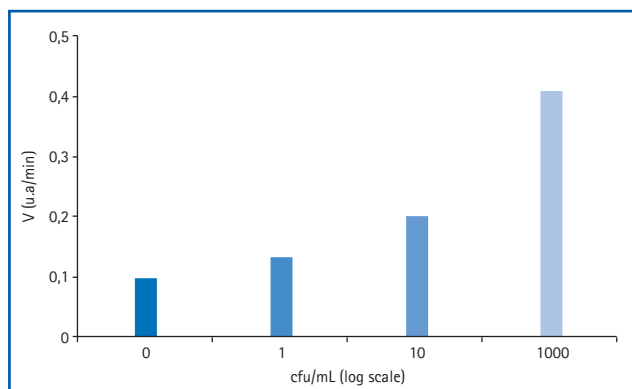


Figure 3. Sensitivity for detection of *E. coli*, using Estapor® magnetic particles of processed by Biótica, S.L. Cells of freshly cultured *E. coli* were diluted in a specific buffer solution avoiding loss of Estapor® magnetic particles. Bacterial suspensions in 20 mL were captured with 25 µL of inert immunomagnetic particles (IIMPs). Captured bacteria were washed with buffer, using a magnet. After washing, the pellet is suspended and incubated in a 1mL solution of a second anti *E. coli* labelled with HRP. Finally, the pellet is suspended in the assaying media for HRP activity determination. The increase of absorbance at 405 nm vs. time was recorded and reaction rate (V) calculated.

Combining optimized surface properties of commercially available magnetic particles and preventing the drain of immunomagnetic composites by a suitable capturing medium, it has been possible to reach good detection limit for microbiological diagnostics, reducing the volume of magnetic particles and also the ratio of antibody/support. Wider adoption of particle-based techniques as a routine practice will not be possible only developing low-cost methodologies with reasonable accuracy and limit of detection. Moreover, user-friendly approach should be incorporated to design user protocol. Approach of this study takes only 1-hour to complete detection, without prior enrichment step of the sample. Inert surface prepared for direct immobilization of antibodies in a proper orientation, and also easy recoveries of particles in major volumes were critical.

Replacing antibody against other bacteria, the developed approach could become good immunomagnetic techniques for diagnostic purposes.

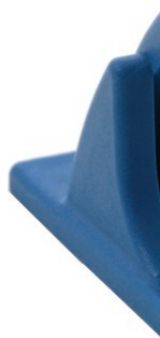
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