Sensitive detection of Live *Escherichia coli* by bacteriophage amplification-coupled immunoassay on the Luminex® MAGPIX instrument

Tomotaka Mido[a]*, Eric M. Schaffer[b,c], Robert W. Dorsey[c], Shanmuga Sozhamannan[d,e], E. Randal Hofmann[c,f]

CBRN Detection Technology Section, CBRN Defense Technology Division, Advanced Defense Technology Center, Acquisition, Technology and Logistics Agency (ATLA), Tokyo, Japan[a]; Leidos, Inc., Aberdeen Proving Ground, Maryland, USA[b]; Biosciences Division, U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Grounds, Edgewood, MD, USA[c]; The Tauri Group, LLC, Alexandria, VA, USA[d]; Defense Biological Product Assurance Office, JPM G, JPEO, Frederick, MD, USA[e]; EXCET, Inc. Springfield, VA, USA[f].

Running Head: Bacterial detection via phage on a MAGPIX instrument

#Address correspondence to Edward.R.Hofmann.ctr@mail.mil

*Present address: International Affairs Office, International Cooperation Division, Department of Equipment Policy, Acquisition, Technology and Logistics Agency (ATLA), Tokyo, Japan

Abstract (250 words)

Phages are natural predators of bacteria and have been exploited in bacterial detection because of their exquisite specificity to their cognate bacterial hosts. In this study, we present a bacteriophage amplification-coupled assay as a surrogate for detecting a bacterium present in a sample. The assay entails detection of progeny phage resulting from infection and subsequent growth inside the bacterium present in suspected samples. This approach reduces testing time and enhances sensitivity to identify pathogens compared to traditional overnight plaque assay. Further, the assay has the ability to discriminate between live and dead cells since phages require live host cells to infect and replicate. To demonstrate its utility, phage MS2 amplification-coupled, bead-based sandwich type immunoassay on the Luminex® MAGPIX instrument for *Escherichia coli* detection was performed. The assay not only showed live cell discrimination ability but also a limit of *E. coli* detection of 1×10^2 cells/mL of live cells after a 3-hour incubation. In addition, the sensitivity of the assay was not impaired in the presence of dead cells. These results demonstrate that bacteriophage amplification-coupled assay can be a rapid live cell detection assay compared to traditional culture methods and a promising tool for quick validation of bacterial inactivation. Combined with the unique multiplex bead chemistry afforded by Luminex® MAGPIX platform, the phage assay can be expanded to be an ultra-deep multiplex assay for the simultaneous detection of multiple pathogens using specific phages directed against the target pathogens.

43 **Text (3000 words)**

44 ## Introduction

45 Early diagnosis of an etiological agent is paramount in implementing timely and appropriate
46 countermeasures to prevent fatal consequences. In an outbreak scenario, protecting the patients
47 and preventing further dissemination of the disease relies on early, rapid, accurate and sensitive
48 detection of the infectious agent. This in turn relies on the assay and detection platform used.

49 Currently, four broad categories of biodetection systems are available. 1) Microbiological/
50 biochemical tests, 2) antibody based, 3) nucleic acid based and 4) other methods including mass
51 spec and bioluminescence. The length of assay times and levels of purification of the sample to
52 be tested vary widely with these systems. Conventional microbiological culturing and staining,
53 differential growth of target organisms in selective media require live cells and take time
54 anywhere from 16 hours to several days in some cases, prior to definitive identification of the
55 culprit organisms (1).

56 There are some drawbacks with the antibody or nucleic acid based systems. For example, PCR
57 and nucleic acid sequence-based amplification (NASBA) enrich a single specific piece of DNA
58 or RNA sequence up to 10^6-fold in 20 minutes to a few hours and theoretically have a sensitivity
59 of a single bacterial cell. The PCR methods give rapid, specific detection but are limited by small
60 sample volumes (e.g., 5 μl for PCR). Furthermore, substances in the sample matrix may inhibit
61 the PCR reaction and the steps used to concentrate the sample to obtain enough templates for
62 PCR may concentrate the inhibitors as well. Immunoassays are based on the concept that any
63 compound that is capable of triggering an immune response can be targeted as an antigen and
64 have been used not only for all types of agents including spores, toxins, and viruses. In general,

65 PCR is much more sensitive than immuno-assays (1). These NASBA and immunoassays cannot
66 discriminate between live and dead target pathogens.

67 There is another paradigm that takes advantage of phages for bacterial detection. Phages are
68 bacterial viruses and are specific to each bacterial species they infect and sometimes, even strains
69 of a given species. The kinetics of interaction between bacteria and their cognate phages is
70 comparable to that of antigen-antibody interaction, making them highly suitable for bacterial
71 detection (2, 3). In addition, the phage-bacterial specificity has evolved over millions of years
72 making them as good as or even better than antigen-antibody specificity. The specificity is
73 attributed to a receptor on the surface of phage that interacts with a receptor on the bacterial
74 surface and this pair is unique. This specificity has been used to develop phage-typing schemes
75 for bacterial species and strains (4-9). Moreover, the cost incurred in producing a phage-based
76 detection reagent is relatively inexpensive compared to the antigen-antibody based reagents. In
77 addition, phages can be useful in deciphering viability of a bacterial pathogen in the sample and
78 furthermore, replication of phage inside the bacterium leads to an amplification of the detection
79 signals thus increasing the sensitivity of the assay.

80 A number of detection systems exploiting phage-bacterial specificity have been developed for
81 different bacteria (10). One of the earliest phage based detection systems involved incorporation
82 of *lux* genes in a mycobacterial phage genome. Expression of the *lux* genes in susceptible
83 mycobacterial cells emitted luminescence signals captured by a handheld Polaroid camera device
84 termed “bronx-box” (11). Similar approaches have been taken for construction of recombinant
85 phages for the detection of *Bacillus anthracis* and *Yersinia pestis* (12, 13). Another elegant
86 fluorescence technique, designed to detect deadly *E. coli O157:H7* bacteria, relied on
87 introducing green fluorescent protein (*gfp*) gene via a bacteriophage. Expression of phage-

encoded *gfp* inside the bacterium emits fluorescence that can be measured in a flow cytometer (14). These methods involved extensive genetic manipulation and relatively expensive fluorescent measurement instruments. There are other limitations to this approach: a) level of expression of LUX/GFP is dependent on the phage promoter that controls its expression; b) low photostability of GFP permits fluorescence measurement only for a few seconds to a minute under normal microscopic conditions and therefore, renders the quantitative fluorescence assay difficult in GFP expressed cells. In order to improve the sensitivity and potential for multiplexing, phage-quantum dot assays for rapid high-sensitive detection of bacterial pathogens have been described (15, 16). Although phage-quantum dot approach has certain advantages in multiplexing and increased sensitivity, appropriate instruments for measuring multiplex fluorescence signals are not available and thus are not field deployable.

Previous studies have demonstrated the utility of phage amplification coupled-detection assay in a simple platform such as lateral flow immunoassay (LFI) and showed reasonable detection limits (17, 18). Recently, a magnetic bead coupled to phage tail fiber protein has been used as a sensitive tool for detection of *Salmonella* cells (19). Here, we have harnessed phage features with the multiplex capability of MAGPIX platform to develop a phage amplification coupled assay to detect viable bacteria. These features are: 1) The exclusivity of phage infection of live cells; 2) Phage growth following infection resulting in an exponential increase of progeny particles by several orders of magnitude thereby increasing the sensitivity of the assay; 3) The relatively rapid nature of the assay compared to traditional plaque assay or even conventional culture methods. 4) An unparalleled multiplex capability offered by MAGPIX platform because of its unique bead chemistry (20). As a proof of concept, we describe a MAGPIX bead based sandwich type immunoassay, hereafter referred as phage MAGPIX assay, using phage MS2 and anti MS2

antibodies as a surrogate for the detection of *E. coli*. Infection, subsequent replication and growth of MS2 inside *E. coli* present in a sample results in the release of increased number of (several orders of magnitude) progeny MS2 particles, which are captured by the anti MS2 antibody coupled to MAGPIX beads. A secondary (detector) anti-MS2 antibody is added to the complex followed by an additional incubation with streptavidin-coated phycoerythrin (SAPE). The resulting fluorescence of the complex is measured and reported as an indicator of the specific bacteria present in the sample.

Results

Determination of assay linearity of phage MS2 based MAGPIX immunoassay

The capture sandwich immunoassay for MS2 phage on the MAGPIX platform was developed using polyclonal anti MS2 antibodies. In the assay, target antigen (MS2) is captured by antibodies (anti MS2 antibodies) coupled on the surface of beads, followed by quantification of the bead bound complex by labeled antibodies. Thus, an increase in progeny phage; i.e., phage amplification can be correlated with amplification in fluorescence signal. The concept of using MAGPIX instrument for this assay is illustrated in Figure 1.

The phage MAGPIX assay, as an indirect measurement of bacterial detection, is incumbent upon detection of progeny phages rather than the input phage used to initiate infection. Therefore, the input phage concentration should be low enough (below the detection limits of the instrument) so that upon phage amplification there is high enough phage titer to result in significant signal amplification that can be detected. Also, it should not be too low, in which case the assay would require longer incubation times to produce high enough phage titers that would generate measureable fluorescence signal intensities. In order to determine the appropriate initial MS2

concentration, the linearity of the MS2 MAGPIX assay was assessed. A dose response curve of the assay was generated by serial dilution of MS2 in LB media and measuring the median fluorescence intensity (MFI). A clear linearity of signal intensity was seen at phage concentrations ranging from 1×10^6 pfu/ mL to 1×10^9 pfu/ mL (Figure 2). Thus, initial MS2 concentration to assess the signal amplification in the assay based on phage replication was determined to be 1×10^6 pfu/mL.

Determination of the live cell discrimination ability of phage MAGPIX immunoassay

In order to evaluate the utility of MS2 amplification-coupled assay for *E. coli* detection, initially, live cell discrimination ability of the assay was assessed. Live cells or heat inactivated *E. coli* cells at a concentration of 1×10^6 cells/mL were infected with MS2 at a multiplicity of infection of 1 (1×10^6 pfu/mL) and incubated for 18 hours and the resulting phage particles were analyzed by MS2 MAGPIX immunoassay. The results showed that live cells infected with MS2 amplified fluorescence signal intensity almost 1000 fold at the end of the incubation period whereas dead cells or live cells without the addition of MS2 did not (Figure 3) indicating the ability of the assay to detect live cells as opposed to dead cells.

Establishing the limit of detection and incubation time for the MS2 MAGPIX assay

Having established the live bacterial detection using the MS2 MAGPIX assay, next we investigated the sensitivity (limits of detection of *E. coli*) of the assay, and the minimal incubation time required for making a positive call in the assay. Signal amplification (as indicated by MFI) of the assay in the presence of varying concentrations of live *E. coli* (0-10^6 cells/ml) was followed from 0 to 3 hours of incubation. The results indicated that with increasing concentrations and incubation times there was a corresponding increase in the signal intensities

(Figure 4). Furthermore, the MFI was increased significantly in samples containing *E. coli* at 1×10^2 cells/mL after 3-hour incubation and thus establishing a limit of detection for this assay. The limit of detection is defined by a signal greater than the mean background MFI plus three standard deviations. Higher concentrations of *E. coli* (10^5-10^6 CFU/ml) produced signal intensities that allowed detection in shorter incubation times; i.e., 1 hour where almost 9-fold increase was observed with 10^5 cells/ml compared to 10^3 cells/ml.

Determination of MS2 phage binding selectivity between live and dead cells using MAGPIX assay

When bacterial samples are inactivated, it is difficult to assess complete inactivation by NASBA or immunoassay because dead bacteria still contain nucleic acids or immunoassay targets (epitopes) that are reactive to the respective assays. On the other hand, since phage amplification-coupled assay require live bacteria, it can be a valuable tool for validation of complete inactivation. However, incomplete inactivation may result in samples containing both live and dead cells. In this case, phage can potentially bind to both dead and live cells. Phage binding to dead cells can competitively inhibit binding to live cells (i.e., reducing the number of phages available for infecting live cells) and thereby reduce the sensitivity of the assay. To investigate if MS2 can selectively bind to live cells in the presence of dead cells, MS2 phage MAGPIX assay was performed in the presence varying concentrations of live cells (0-10^6 cells/ml) or live and dead cells at the same concentrations (0-10^6 cells/ml) for one hour. The results indicated that the signal intensities (as MFI) between the groups are very comparable indicating that the presence of dead cells did not affect the infection, replication and growth of live bacteria present in the sample (Figure 5). This result indicates that MS2 selectively bound to only living cells in the presence dead cells.

178

179 **Discussion**

180 An ideal bioagent detection technology/platform would have the following desirable properties:
181 The detection assays must have the potential for rapid, reproducible, high-sensitive (detection at
182 very low concentrations of the agent), high-specific (high true positive/true negative and low
183 false-positive/ low false-negative) detection of agents (conventional as well as uncharacterized or
184 genetically modified agents) directly from complex matrix samples with minimal false results,
185 capable of detecting low concentrations of target agents without interference from background
186 materials. Also, the platform to conduct the assays should be user-friendly, portable and a point
187 of care or field device that is capable of detecting multiple agents simultaneously in a high
188 throughput manner (processing hundreds of samples) in any matrix type (clinical or
189 environmental). Additionally it is highly desirable to have a flexible technology with an open
190 architecture; amenable for a plug and play format to develop new assays rapidly and, above all,
191 the technology should be inexpensive. Although several of the currently available commercial
192 detection platforms provide many of the desired features, no one system can satisfy all of these
193 criteria.

194 Many of the current rapid detection technologies are based either on PCR or some form of an
195 immunoassay such as lateral flow immunoassay. The major drawback of these methods is that
196 they cannot discriminate whether the suspected sample contains live or dead bacteria. This
197 discrimination is critical especially in a biothreat/ biosurveillance scenarios not only for making
198 correct courses of action but also for verifying if decontamination activities were successful.
199 Also, in a clinical or point of care/field setting such a discrimination ability and phage mediated
200 signal amplification will be very useful for determining antibiotic sensitivity rapidly. For

example, in slow growing bacteria like *Mycobacterium* spp such an approach has been used to test antibacterial susceptibility in the field (11). Also, these technologies are limited in their potential for multiplexing and high throughput analysis. To address these two gaps, as a proof of concept, we developed a phage based live agent detection assay using MAGPIX instrument. Phage MS2 is an *E. coli* male specific phage because of its specificity to infect only strains that carry the F pilus (21). MS2 has a burst size of 5000-10000 per infected cell (22) and a short burst time of 30 minutes (23). In this study, MS2 amplification-coupled MAGPIX immunoassay was performed to demonstrate utility of bacteriophage amplification-coupled immunoassay for *E. coli* detection. Essentially, in this assay, progeny particles resulting from infection of bacteria in a sample are detected and reported as an indicator of the pathogen present. We found a sensitivity of 1 X10^4 cells/ml of *E. coli* and selective binding to live cells and no inhibition in the presence of dead bacteria after a one hour incubation. This limit of detection is quite comparable to published immunoassays for *E. coli* detection (24, 25). The limit of detection of this assay is dependent on the burst size of a given phage in its specific bacterial host. Phages with high burst will yield large number of progeny particles that will yield correspondingly high signal intensity in the MAGPIX assay. However, for phages with low burst size, a large number of bacteria should be present during initial infection, in order to yield high enough signal intensity above the background to make a positive call. The optimal multiplicity of infection needs to be determined for each phage-bacterium combination since the initial bacterial load in each sample may vary. Intuitively, it would seem phage-based assays are not available for other types of agents such as spores, viruses and toxins. However, using M13 like phages, one can pan for phages with peptide displays that bind specifically to these biothreats and add that to the MAGPIX panel of assays.

223 Despite these limitations, our future efforts will focus on developing a phage multiplex MAGPIX
224 assay panel to target bacterial, viral and toxin threats.

225 **2. Materials and Methods**

226 **2.1. Phage stock**

227 MS2 phage and *E. col* strain C-3000 (ATCC 15597) used in this study were obtainedfrom
228 Brouns lab, Delft University of Technology (NL). The phage stock was prepared from the seed
229 stock in small scale liquid culture using established laboratory procedures (26)

230 **2.2. MAGPIX immunoassay development**

231 Polyclonal antibodies provided by Defense Biological Product Assurance Office (DBPAO);
232 Rabbit anti- MS2 (Rab α-MS2: ABE#120, J-291100-02) were used to develop the capture
233 sandwich assay on the MAGPIX platform (Luminex, Austin, TX). Antibodies were immobilized
234 on MagPlex carboxylated microspheres (Luminex, Austin, TX) at 5pg antibody/ microsphere
235 using the carbodiimide coupling protocol provided by the manufacturer. To conduct the coupling,
236 0.1 M Sodium Phosphate monobasic (pH6.2±0.2) was used for washing beads and incubation.
237 Microspheres were then washed and resuspended in 0.01M PBS (pH7.4±0.1) with
238 0.05%Tween20. Antibodies immobilize on microsphere were used as the capture in the assay.
239 Antibodies were also biotinylated using a 30-fold molar excess of BT-LCLC-NHS (Thermo
240 Scientific, Rockford, IL) as the detection element with a streptavidin-phycoerythrin conjugate
241 (SAPE) serving as the tracer molecule.

242 **2.3. MAGPIX immunoassay procedure**

2500 microspheres per well of antibody coated microspheres were incubated for 1 hour with samples. Following incubation, the sample was, washed, and then incubated with 4µg/mL biotinylated antibody for 30 minutes. The microspheres were washed again and then incubated with 4µg/mL SAPE to generate the fluorescent complex. After a final wash and re-suspending in 100µL of wash buffer, the assay results were evaluated using the MAGPIX instrument. Incubations were performed at room temperature, at 800rpm, and protected from light. Samples were washed twice with 100µL of wash buffer (0.01M PBS with 1%BSA and 0.05%sodium azide, pH7.4).

2.4. MS2 amplification-coupled MAGPIX immunoassays

MS2 amplification-coupled MAGPIX immunoassays were performed on the MAGPIX platform. Prior to MAGPIX immunoassay, MS2 and *E.coli* (Delft University of Technology, NL) diluted in LB media were incubated at 37 °C shaking at 300 rpm for respective time. E-coli concentration (cells/mL) was calculated by OD_{600} based on the formula as OD_{600} of 1.0 = 8×10^8 cells/mL. To prepare near 100% of live cells, live E-coli used in this study was incubated to keep OD_{600} below 1.0 after inoculating. When dead cells were used, *E.coli* was inactivated by heating over 95°C in water bath for 15 min prior to the OD_{600} measurement. Incubated samples were analyzed by MAGPIX assay.

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Appendixes

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Figure 1. Illustration of bacteriophage amplification-coupled immunoassay for bacterial detection on MAGPIX instrument. (A) Phage infection of a bacterial cell and subsequent growth inside the bacterium results in host cell lysis and release of progeny phage in large numbers. (B) Antibody coupled on the surface of magnetic bead captures input and progeny phage. (C) Addition of detector antibody followed by (D) an incubation with SAPE. The fluorescence emitted by the final complex is measured in the MAGPIX instrument and reported as relative fluorescence units.

Figure 2. Assay linearity of MS2 MAGPIX immunoassay detection. Sandwich type MAGPIX immunoassay for MS2 detection was performed at respective MS2 concentrations. Vertical axis

342 shows the median fluorescence intensity (MFI). Values are average of two independednt
343 measurements of two replicates each.

344

345 Figure 3. Validation of live cell discrimination ability of MS2 amplification coupled assay
346 MS2 amplification-coupled MAGPIX immunoassay for *E. coli* detection was performed.
347 Samples were incubated for 18 hours prior to analysis on the MAGPIX instrument. Vertical axis
348 shows the median fluorescence intensity (MFI). The data in this figure is based upon 2 separate
349 run of 2 replicates.

350

351 Figure 4. Determination of the limit of detection and incubation time for MS2 phage MAGPIX
352 assay. Signal intensity of MS2 amplification-coupled MAGPIX immunoassay for *E. coli*
353 detection was tracked from 0 to 3 hours. Samples containing 1.0×10^6 PFU/mL of MS2 and live
354 cells at varying concentrations from 0-1 x 10^6 CFU/ml were incubated for indicated times prior
355 to analysis on the MAGPIX instrument. Vertical axis shows the median fluorescence intensity
356 (MFI) and each data point is the average of 2 replicates of 2 separate assays.

357

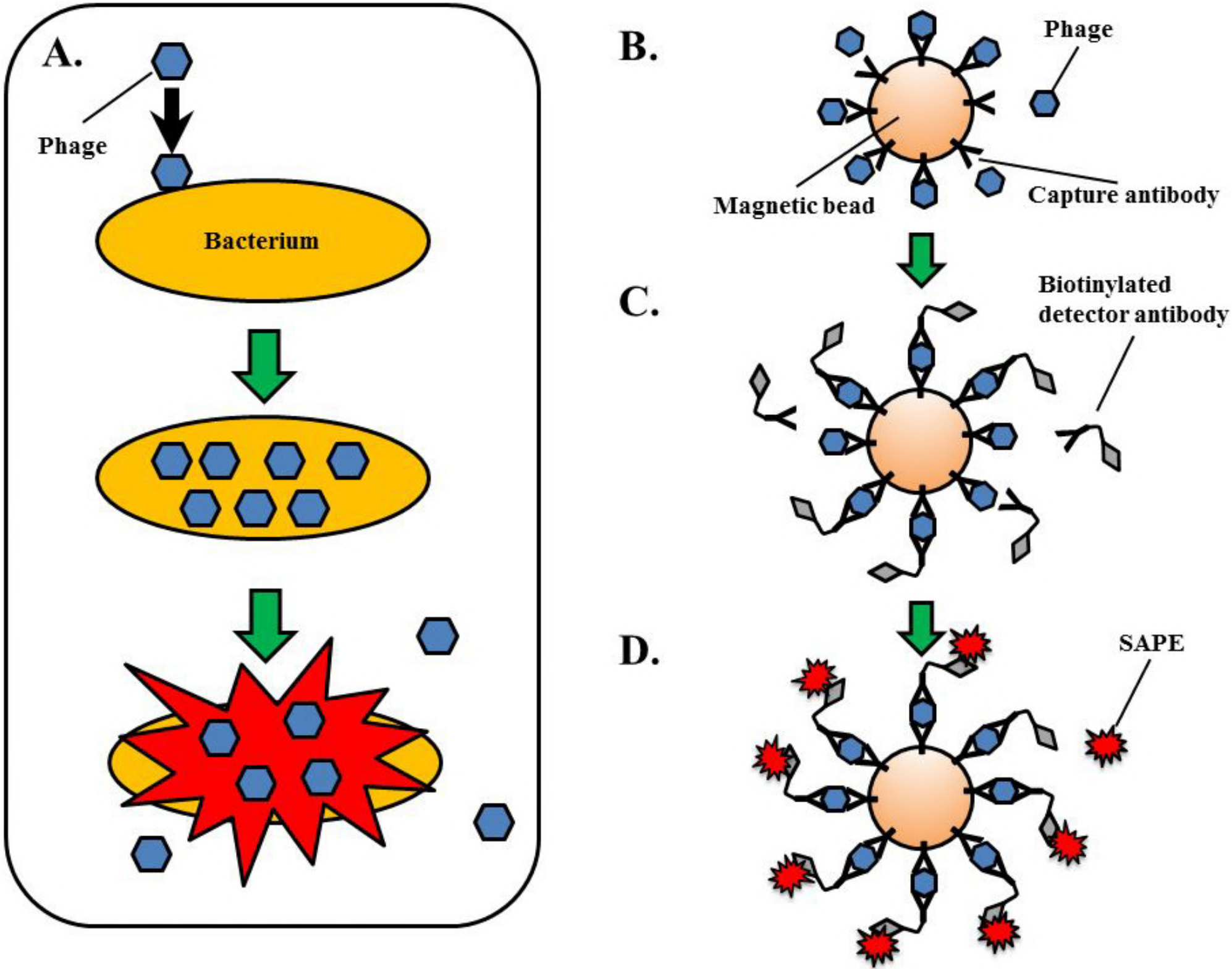
358 Figure 5. Determination of MS2 phage binding selectivity between live and dead cells using
359 MAGPIX assay. Signal intensity of MS2 amplification-coupled MAGPIX immunoassay for *E.*
360 *coli* detection was compared between sample containing only living cells and sample containing
361 live cells and dead cells. Samples containing 1.0×10^6 pfu/mL MS2 and live cells at respective
362 concentration were incubated with/without 1.0×10^6 cells/mL inactivated cells for 1 hour prior to

363 the immunoassay. Vertical axis shows the median fluorescence intensity (MFI). The values

364 represent average of two separate runs with 2 replicates each.

365

366


A.
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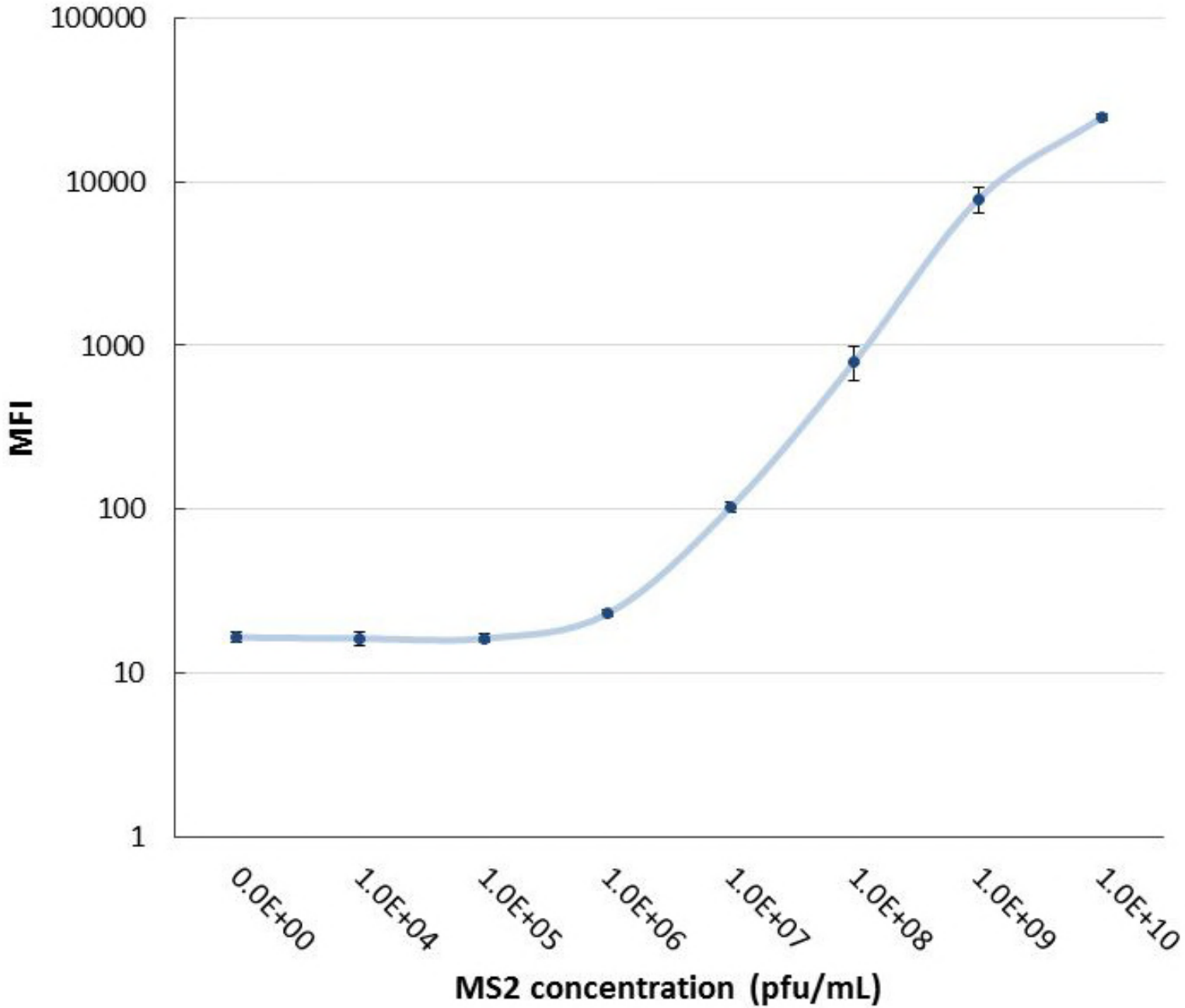
B.
Phage
Magnetic bead
Capture antibody
C.
Biotinylated
detector antibody
D.
SAPE

Figure 2


100000
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MFI
0.0E+00
1.0E+04
1.0E+05
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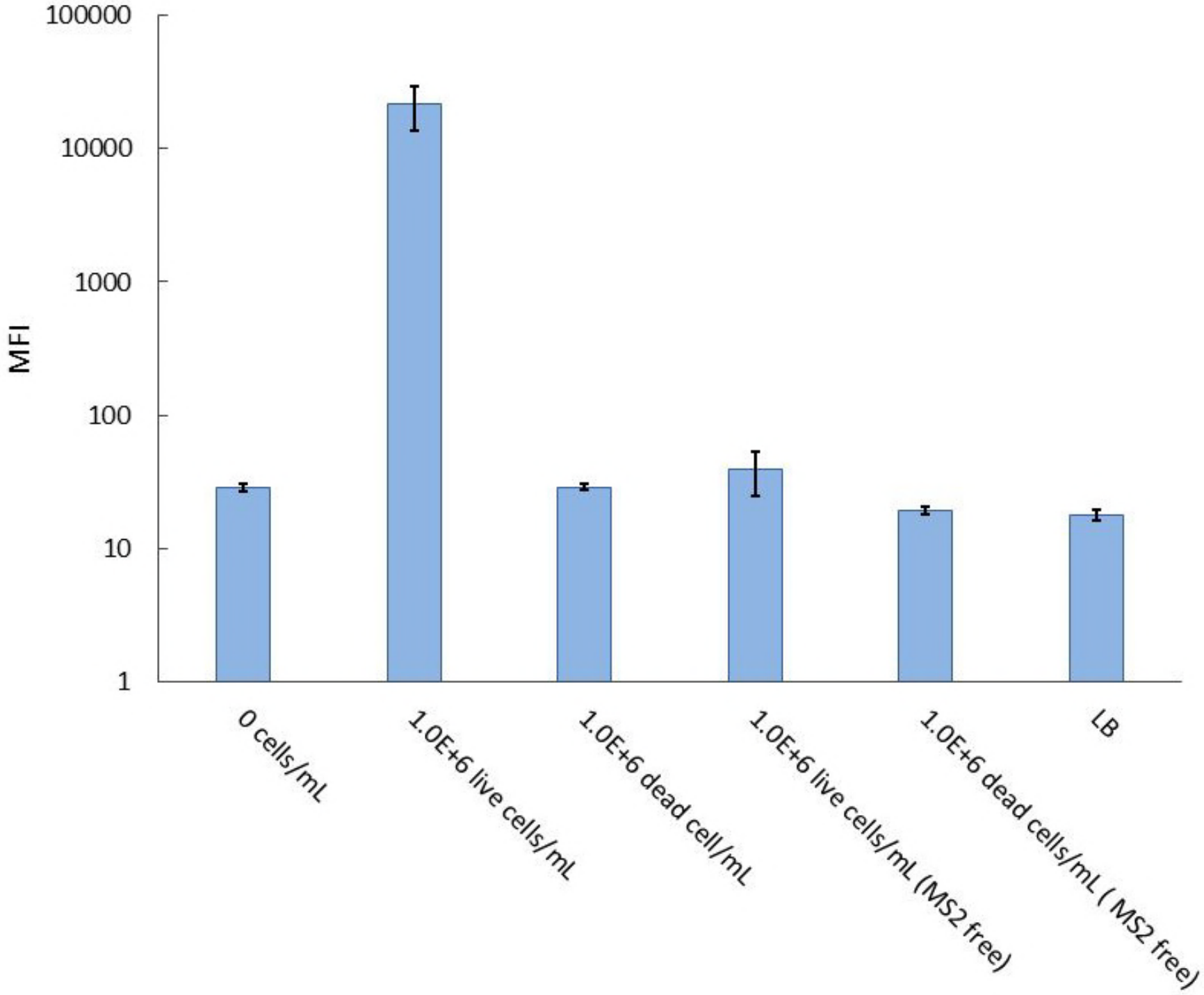
1.0E+10
MS2 concentration (pfu/mL)

Figure 3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10000
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100
10
1
MFI
0 cells/mL
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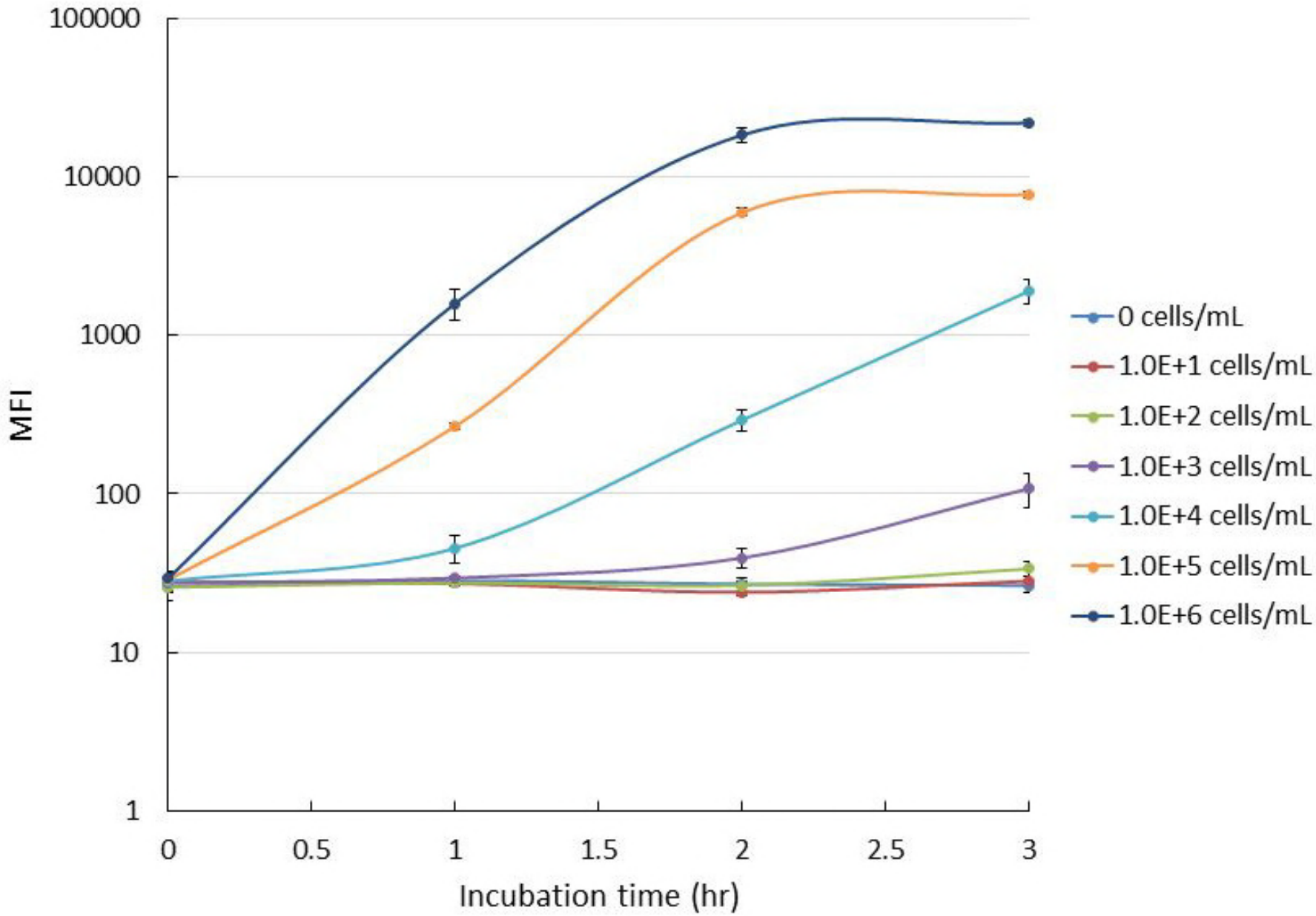
1.0E+6 dead cell/mL
1.0E+6 live cells/mL (MS2 free)
1.0E+6 dead cells/mL (MS2 free)
LB

Figure 4


100000
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10
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MFI
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1
1.5
2
2.5
3
Incubation time (hr)
0 cells/mL
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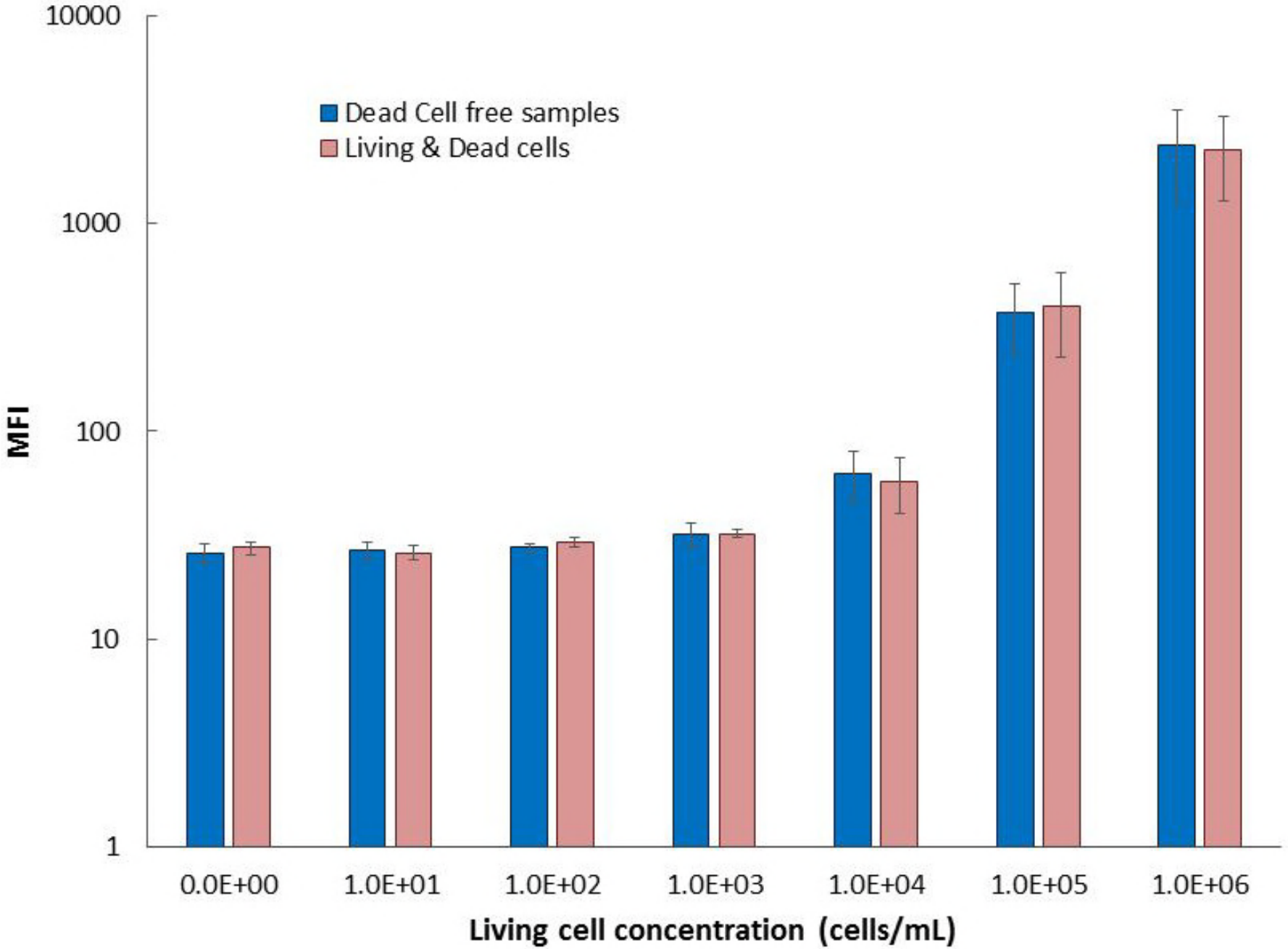
1.0E+2 cells/mL
1.0E+3 cells/mL
1.0E+4 cells/mL
1.0E+5 cells/mL
1.0E+6 cells/mL

Figure 5


10000
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MFI
Dead Cell free samples
Living & Dead cells
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1.0E+04
1.0E+05
1.0E+06
Living cell concentration (cells/mL)