ISSN 0026-2617, Microbiology, 2020, Vol. 89, No. 1, pp. 13–27. © Pleiades Publishing, Ltd., 2020.
Russian Text © The Author(s), 2020, published in Mikrobiologiya, 2020, Vol. 89, No. 1, pp. 17–33.

REVIEWS

Modern Approaches to Differentiation of Live and Dead Bacteria Using Selective Amplification of Nucleic Acids

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Received May 7, 2019; revised July 1, 2019; accepted September 29, 2019

Abstract—Specific amplification of nucleic acids is a convenient and quick alternative to the culture-based method of detecting bacterial cells. However, conventional PCR and other amplification reactions can not differentiate between live bacteria and dead or dormant ones, and are also capable of amplifying DNA that persists for a long time and in a cell-free state. Several methods have been developed in order to establish the viability of microorganisms by amplification of specific sequences of nucleic acids, both those controlled by changing temperatures and isothermal ones. For some of them, DNA modified by phenanthridine dyes serves as a target, and simultaneous use of monoazides of ethidium and propidium was shown to be preferable for the purpose. For other methods, the targets are directly RNA molecules or their cDNA copies. Pre-rRNA detection seems to be the most preferable approach, due to the presence of these types of RNA exclusively in living cells.

Keywords: microbial viability, live and dead bacteria, PCR, RT–PCR, NASBA (Nucleic Acid Sequence Based Amplification), Molecular Viability Testing (MVT), ethidium monoazide (EMA), propidium monoazide (PMA), pre-rRNA

DOI: 10.1134/S0026261720010038

In applied microbiology, a topical issue is the need for reliable, highly sensitive, and specific assays for detection of viable microorganisms in various media, both for diagnostics of infectious diseases, and for evaluation of the sanitary state of the environment (e.g., water, air, or foodstuffs). The culture-based approach, although currently considered the gold standard, still has a number of important shortcomings that are, in particular, related to a considerable duration of the analysis. Moreover, a large number of microorganisms of medical and/or veterinary significance, such as nearly all currently known obligate and facultative intracellular parasites or anaerobic bacteria, are unculturable under normal conditions. Accordingly, immediately after the advent of polymerase chain reaction (PCR), this method was adopted by microbiologists to detect target microorganisms by amplification of specific DNA sequences in the analyzed specimens.

Modern amplification-based approaches to differentiation of live and dead bacterial cells started developing in the mid 1990s, when it was proposed to use boundary regions of rRNA and pre-rRNA as targets. In the early 2000s, this research direction was further boosted due to introduction of ethidium monoazide (EMA), which permeates only dead bacterial cells and intercalates within DNA molecules, thus preventing their amplification by PCR (Nogva et al., 2003). This review will summarize and analyze the considerable body of experience that has been accumulated during the fifteen years since the approach to differentiating viable and dead bacteria by means of PCR with phenanthridine dyes (EMA, as well as propidium monoazide, PMA) was proposed; in addition, it considers other methods of nucleic acid amplification employed for differentiation of live and dead bacteria. However, before discussing these techniques, we will briefly describe the viability states in bacteria.

Currently, several major forms of bacterial viability state are recognized: apart from spores, there are at least three different states: live, dead, and dormant bacteria. The latter group is frequently referred to as viable but nonculturable (VBNC) cells. For instance, bacteria may acquire the VBNC status as a result of exposure to bacteriostatics or sublethal concentrations of antibiotics, other bactericides, or disinfectants. A study by Davey (2011) recognized an even greater number of grades between live and dead cells. That is, a living cell with active metabolism (1) may become a living cell with decreased metabolic activity (2), a cell with decreased RNA levels (3), and then an intact cell without signs of metabolic activity (4). The subsequent viability stages involve compromised membrane integrity (5), DNA degradation (6), and finally, formation of cell debris (7). It is specifically pointed out that the so-called point of no return after which it becomes impossible to restore the viability of bacteria exposed to stress factors is unknown, as well as the exact point of bacterial death (Davey, 2011). Moreover, they may vary depending on bacterial species and their environment. The viability states in bacteria, including dormant cells, are comprehensively discussed in several reviews (Kell et al., 1998; Barer and Harwood, 1999; Oliver, 2010; Pinto et al., 2013).

DIFFERENTIATING LIVE AND DEAD BACTERIA BY AMPLIFICATION OF RNA OR DNA FRAGMENTS WITHOUT THEIR PRELIMINARY MODIFICATION

Since the invention of PCR, this method has been extensively applied to detect bacteria in diverse environments. The very first work aimed at detection of toxicogenic *Escherichia coli* was performed at the University of Kuwait and employed the initial low-efficient PCR variant using the Klenow fragment of *E. coli* DNA polymerase I (Olive et al., 1988). The level of the assay sensitivity was higher than 1000 bacteria, which could be detected using 24 PCR cycles and subsequent hybridization of the 299-bp-long amplicon with a labeled DNA probe. Subsequently, the method was improved to allow *E. coli* detection by electrophoresis after 30 cycles of amplification with thermostable DNA polymerase (Olive, 1989). In the same period, it was shown that PCR could be employed for practical purposes of detecting pathogenic bacteria (Bernet et al., 1989; Dutilh et al., 1989; Rosa and Schwan, 1989). The possibility to detect disease-causing bacterial agents that are unculturable in vitro was considered particularly promising. For instance, PCR was used to detect *Mycobacterium leprae*, the causative agent of lepra (Hartskeerl et al., 1989). In another study published several years later, viable *M. leprae* cells were detected by amplifying two DNA fragments of different sizes: 571 and 285 bp (Jamil et al., 1993).

Fairly soon, it became clear that DNA molecules could be preserved in dead bacteria and even in the free form in the environment, which was why their detection by PCR did not provide the crucial information as to whether the specimen in question actually contained viable microorganisms capable of affecting other living organisms, including humans, or the environment. Therefore, RNA molecules with lifetimes that are significantly shorter than that of DNA were considered more appropriate targets for detection by PCR in combination with reverse transcription (RT–PCR). For instance, it was shown that PCR amplification of a 650-bp-long cDNA fragment encoding the *mip* RNA of *Legionella pneumonia* could be used to differentiate between live and dead bacteria, as well as those in the VBNC state (Bej et al., 1991). Subsequently, this group utilized the same approach to differentiate live and dead *Vibrio cholerae* cells (Bej et al., 1996). An RT–PCR assay employing several mRNA species as targets to ensure sensitivity gradation was shown to be efficacious for detection of live *Listeria monocytogenes* cells in meat products (Klein and Juneja, 1997). It was reported that RT–PCR using mRNA targets was appropriate to differentiate between several species of live bacteria (Sheridan et al., 1998; Lleò et al., 2000). Moreover, Sheridan et al., (1998) also noted that RT–PCR targeted at detection of 16S rRNA molecules was unsuitable for specific differentiation of viable bacteria, since the presence of significant amounts of mature rRNA distorts the results. At the same time, it was reported that rRNA was successfully used for differentiation of live and dead cells of *E. coli* and *Staphylococcus aureus* (McKillip et al., 1998). To eliminate false positive results caused by amplification of genomic DNA instead of mRNA during detection of viable microorganisms, it was proposed to perform RT–PCR only after repeated DNase treatments of RNA specimens resulting in complete disappearance of target amplicons in conventional PCR (Kobayashi et al., 2009).

For a number of reasons, RT–PCR is not a perfect technique of detecting viable bacteria. In particular, it is necessary to remove DNA from the specimens using DNase treatment, which is a difficult task itself due to the presence of residual amounts of these nucleic acids. There is a risk of partial or even complete disruption of target RNA as a result of DNase contamination with RNase. It should also be taken into account that PCR has an extremely high sensitivity, which is why it is critically important that these bacterial detection assays be thoroughly controlled both for false positive results due to working zone contamination or other causes and for false negative results, for instance, caused by the presence of various kinds of PCR inhibitors in the specimens (Chemeris et al., 2011, 2012a, 2012b).

Apart from RT–PCR, microbial viability state can be assessed by detecting bacterial RNA using a technique termed NASBA (Nucleic Acid Sequence Based

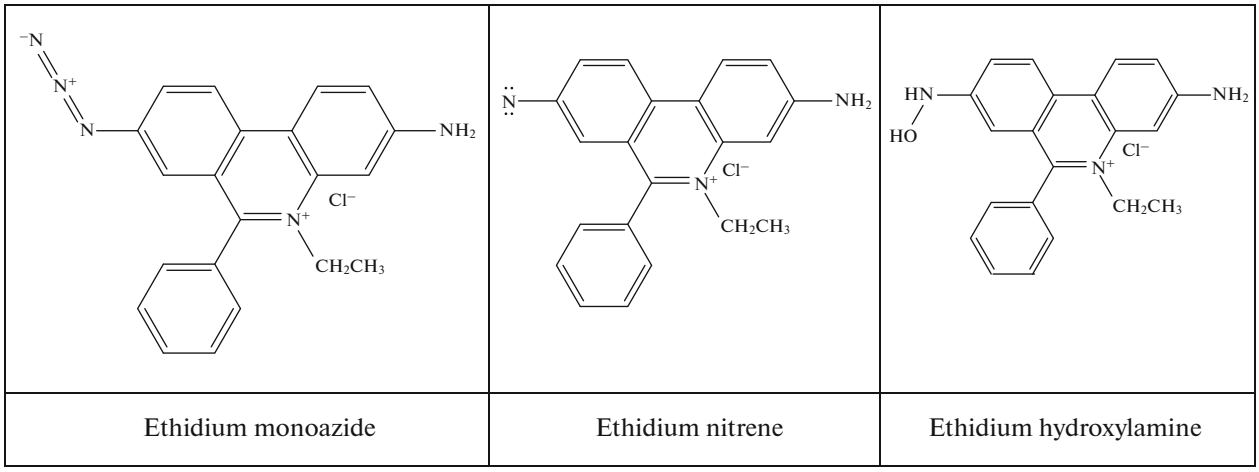


Fig. 1. Structural formulas of ethidium monoazide and the products of its photolysis.

Amplification): isothermal amplification that does not involve the initial dsDNA chains. Its utility has been demonstrated in several bacterial species (Vliet et al., 1994; Simpkins et al., 2000), in particular, using real-time monitoring of amplification results (Fykse et al., 2007). A study that compared PCR, RT–PCR, and NASBA assays for differentiation of live and dead bacteria showed that NASBA had the highest sensitivity (Birch et al., 2001). Along with NASBA, mRNA in live *Mycobacterium tuberculosis* cells was detected using the technique of reverse transcriptase strand displacement amplification (RT–SDA), which also enables amplification under isothermal conditions (Hellyer et al., 1999).

It was shown that RT–PCR targeting a fragment of intact pre-rRNA can distinguish antibiotic-resistant *M. tuberculosis* cells from the sensitive ones (Cangelosi et al., 1996). Moreover, by selecting primers in such a way that one of them anneals to a site of mature 16S rRNA, and the other one, to a fragment removed during rRNA processing, it was possible to detect pre-rRNA molecules that have just appeared in the course of transcription. However, it was only fifteen years later that a basically new technique of differentiating live and dead bacteria ultimately termed Molecular Viability Testing (MVT) was proposed (Cangelosi et al., 2010; Weigel et al., 2017). This molecular assay of bacterial viability employs RT–PCR for selective amplification of an intact pre-rRNA sequence with primers for cDNA synthesis and amplification designed so as to anneal to a fragment of mature rRNA and to a pre-rRNA fragment that exists only not long. The authors claim that this technique is the most accurate means of assessing microbial viability state, since it is known that dormant cells do not synthesize new pre-rRNAs and do not retain those synthesized previously, whereas addition of nutrients can induce a rapid transformation of dormant cells into metabolically active live ones, which is accompanied by appearance of new pre-rRNAs. Moreover, the suitability of MVT based on detecting intact pre-rRNA by means of RT–PCR for differentiation of microbial viability states was shown for several slow- and fast-growing bacterial species of different phylogenetic groups: *M. tuberculosis*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *S. aureus*. It is noted that MVT provides a more accurate estimate of bacterial viability state than the widespread techniques based on detecting bacterial DNA after exposure to phenanthridine dyes (Cangelosi and Meschke, 2014). Nevertheless, it is this latter group of methods that will be comprehensively discussed below.

METHODS OF DIFFERENTIATING LIVE AND DEAD BACTERIA BASED ON VIABILITY PCR USING PHENANTHRIDINE DYES

DNA modification with phenanthridine dyes was proposed based on the fact that ethidium monoazide (3-amino-8-azido-5-ethyl-6-phenylphenanthridinium chloride) can permeate the compromised membranes of dead bacterial cells, bind to their DNA, and induce fluorescence that may be detected using flow cytofluometry; thus, dead microorganisms can be counted (Riedy et al., 1991). Importantly, it was considered that exposure to visible light made DNA/EMA complex irreversible and changes some DNA properties. Previous research established also that light causes transformation of EMA that intercalated DNA molecules into extremely reactive ethidium nitrene (Knowles, 1972), which interacts with DNA, while free EMA in the presence of water undergoes photolysis and produces relatively inert ethidium hydroxylamine (Graves et al., 1981); the structural formulas of these compounds are shown on Fig. 1.

The principal idea of this approach is as follows: after exposure to visible light, EMA that has permeated the damaged membrane of dead bacterial cells in the dark irreversibly binds to cellular DNA; as a result,

this DNA will be excluded from PCR. Quantitative assessment of the live/dead bacteria ratio can be performed by real-time PCR after dividing the specimen concerned in two parts and treating one of them with a phenanthridine dye, while leaving the other one untreated. Next, DNA is isolated from both specimens using an appropriate technique and amplified, and the proportion of live and dead bacteria in the initial specimen is determined as the ratio of the obtained product amounts. Importantly, EMA that has undergone degradation by photolysis, cannot interact in the solution with native DNA isolated from live cells.

PCR Combined with Ethidium Monoazide Treatment (EMA–PCR)

The first experimental article that described successful application of EMA–PCR for differentiation of live and dead bacteria was published in 2003 (Nogva et al., 2003). This work was performed using the following microorganisms: several strains of toxicogenic *E. coli* variant O157:H7, *L. monocytogenes,* and *Salmonella* sp., which were killed by exposure to high temperature and chemical disinfectants. The authors tested different EMA concentrations, as well as treatment conditions, in particular, the duration and intensity of illumination. The results of viability evaluation were verified using conventional microscopy with a commercial LIVE/DEAD *Bac*Light kit (Molecular Probes, Inc., currently ThermoFisher Scientific) including two dyes: SYTO 9 and propidium iodide; this test confirmed validity of the quantitative EMA–PCR microbial viability assay. A study using *Campylobacter jejuni* isolated from different foodstuffs showed that EMA–PCR was superior to microscopic analysis, in particular, due to the possibility to discriminate concominant microorganisms (Rudi et al., 2005). The EMA–PCR technique was adapted to differentiate live and dead bacteria in soil (Pisz et al., 2007) and water samples (Inoue et al., 2008). A considerable discussion (Hein et al., 2006) was provoked by the finding that exposure to EMA caused selective DNA elimination from dead cells in mixed bacterial samples (Nocker and Camper, 2006). It turned out that there occurred DNA fragmentation rather than irreversible EMA binding to DNA (Soejima et al., 2007). The authors noticed that the amount of DNA isolated from dead bacteria after EMA treatment decreased significantly and undertook a special study to find out what actually happened to this DNA, taking into account that similar observations have been reported previously (Nocker and Camper, 2006). The products obtained in vitro were analyzed using several techniques of electrophoresis, as well as by electron microscopy, and it was found that exposure to EMA and light caused single-strand breaks in double-stranded DNA; at higher EMA concentrations in the reaction mixture, they became so numerous that actually resulted in double-strand breaks (Soejima et al., 2007). These data invited some hypotheses concerning the mechanism of DNA disintegration and suggested new considerations on using EMA–PCR to differentiate live and dead bacteria. Thus, assuming that the permeation of EMA into damaged bacterial cells and its interaction with intracellular DNA before and after exposure to light compromised the integrity of bacterial DNA, the researchers evaluated the likelihood of obtaining amplicons of different sizes in *L. monocytogenes* (Soejima et al., 2008). It was found that a 894-bp-long target fragment was not amplified, whereas a shorter one (113 bp) still could be detected, which further confirmed the fact of DNA fragmentation caused by double-strand DNA breaks.

It should be noted that a study performed with two bacterial species, *C. jejuni* and *L. monocytogenes*, reported that real-time EMA–PCR did not ensure the required level of differentiation between live and dead cells (Flekna et al., 2007). Nevertheless, even after the more appropriate assay with propidium monoazide (PMA) was introduced, EMA has not fallen out of use (Fukuzawa et al., 2019), in particular, due to the lower cost of this reagent. To improve the reliability of the EMA assay, Minami et al. (2010) proposed to perform repeated treatments of specimens with low EMA concentrations, using *Enterobacter sakazakii* as a control.

PCR Combined with Propidium Monoazide Treatment

Taking into account that propidium iodide, which permeates only the membranes of those bacterial cells that have lost viability, has long been used for differentiation of live and dead bacteria by means of microscopy and flow cytometry (Nebe-von Caron et al., 1998), it was proposed to perform viability PCR (vPCR) using its analog, propidium monoazide (3-amino-8-azido-5-{3-[diethyl(methyl)ammonio]pro-pyl}-6-phenylphenanthridinium dichloride; Fig. 2), which was first utilized by Nocker et al. in 2006. Subsequently, a series of experimental studies that analyzed a broad range of microbial species using quantitative PCR, DNA microchip technology, and pyrosequencing showed that PMA could be efficiently employed for differentiation of live and dead bacteria (Nocker et al., 2007, 2009, 2010; Contreras et al., 2011; Yáñez et al., 2011; Schnetzinger et al., 2013).

A considerable number of articles have addressed the issues of quantifying live and dead bacteria using real-time PCR for the purposes of monitoring bacterial abundance in the samples, in particular, after different types of disinfecting treatment (Bae and Wuertz, 2009; Wahman et al., 2009; Yokomachi and Yaguchi, 2012; Kaushik and Balasubramanian, 2013; Xing-Long et al., 2013; Lee et al., 2015; Kibbee and Örmeci, 2017; Zhou et al., 2017). For instance, the abundance of live bacteria in a wastewater microbial community before and after disinfection, as well as in specimens of feces was assessed using digital droplet

PCR in combination with PMA treatment (Gobert et al., 2018).

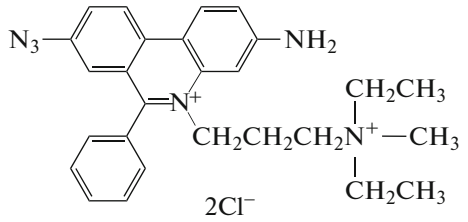
The study by Janssen et al. (2016), who applied real-time quantitative PMA–PCR to detect live and dead *Chlamydia trachomatis* cells, was the first to employ vPCR to control the microbial abundance in sexually transmitted diseases. Along with analysis of clinical specimens, the study included model experiments performed to validate the technique, which confirmed its appropriateness for monitoring antibiotic therapy of chlamydiosis. It was also shown that PMA treatment of microbial samples followed by PCR is a promising approach to treatment monitoring in patients with tuberculosis (Nikolayevskyy et al., 2015). A recent work found that, along with PMA–PCR, the effect of antibiotics on *P. aeroginosa* can also be assessed using PCR-based detection of pre-rRNA (Lee and Bae, 2018a).

Li et al. (2015) reported using multiplex real-time vPCR after PMA treatment of water samples for simultaneous detection of viable bacteria of three species: *Legionella pneumophila*, *Salmonella typhimurium,* and *S. aureus*. Previously, multiplex vPCR in combination with immunomagnetic separation and PMA treatment was utilized for simultaneous detection of viable *Listeria monocytogenes*, *S. typhimurium,* and *E. coli* O157:H7 in food products (Yang et al., 2013).

It should be noted, however, that, according to certain publications, PMA–vPCR overestimated the abundance of live bacteria in the specimens tested (Lovdal et al., 2011; Ditommaso et al., 2014). To achieve better discrimination between live and dead bacteria by increasing the permeability of damaged bacterial membranes for PMA, some studies utilized various detergents, such as sodium deoxycholate, sarkosyl, sodium dodecyl sulfate, or Triton X-100, as well as EDTA, a chelating agent (Wang et al., 2014a, 2014b; Dong et al., 2018; Zi et al., 2018; Kontchou et al., 2019). Dimethyl sulfoxide could also be used for the same purpose (Seidel et al., 2017).

A model system study where PMA–PCR was used for relative quantification of live and dead *L. pneumophila* cells after their experimental introduction in wastewater and disinfection treatments showed that there was a risk of both false positive and false negative results (Fittipaldi et al., 2011). Several works report that PMA treatment of microorganisms, including exposure to light, could be successfully performed directly on the membrane filters that were used to concentrate bacterial cells from water samples (Slimani et al., 2012; Villarreal et al., 2013). In addition to evaluating bacterial viability state, quantitative PMA–PCR was efficiently utilized to determine viability of *Bacillus subtilis* spores (Rawsthorne et al., 2009).

For a rather long time, vPCR assays with phenanthridine dyes involved exposure to visible light using high-power halogen lamps, which heated the specimens and therefore implied the need for some cooling



Propidium Monoazide (PMA)

Fig. 2. Structural formula of propidium monoazide.

systems. The problem was resolved after it was proposed to use blue LED lamps with emission wavelengths from 445 to 485 nm (Vesper et al., 2008). Although halogen lamps are still sometimes used in vPCR assays, LED-based light sources are significantly more common and they are manufactured by several companies. For instance, two devices by Biotium, Inc., PMA-Lite and Glo-Plate, are designed for blue-wave illumination of PMA bound to bacterial DNA in test tubes or plates. The same company also produces a broad range of reagents and kits for differentiation of live and dead bacteria, in particular, PMAxx, a novel dye advertised as an improved PMA version. In addition, the well-known company Qiagen produces the BLU-V System, a heating-free illuminating device.

At this point, we feel it necessary to discuss two recent studies on differentiation of live and dead bacteria performed by two different research groups (Willers et al., 2017; Huang et al., 2018). Huang et al. (2018) compared the efficiency of culturing, vPCR, and flow cytometry using the already mentioned LIVE/DEAD *Bac*Light kit with SYTO 9 (which stains all cells green) and propidium iodide (which stains only dead cells red) for the assessment of *E. coli* viability state. This work evaluated a number of parameters, such as the duration of both preliminary steps and the experiment as such, the waiting period until final results, the cost of the analysis, and its throughput (parallelism). On the whole, in contrast to flow cytometry, which is a rapid assay but does not discriminate different species of bacteria, vPCR enables selective amplification of the desired target and should be considered a promising approach. The study by Willers et al. (2017) compared the efficiency of PMA–PCR and flow cytometry that utilized only PMA instead on the standard LIVE/DEAD *Bac*Light kit. Although the latter assay had an advantage of rapidity, it should be noted the described experiments were performed with pure cultures of *Helicobacter pylori*. At the same time, it cannot compete with vPCR if applied to real specimens that might contain numerous bacterial species, including unknown ones, mainly because of nonspecific detection of viable bacteria.

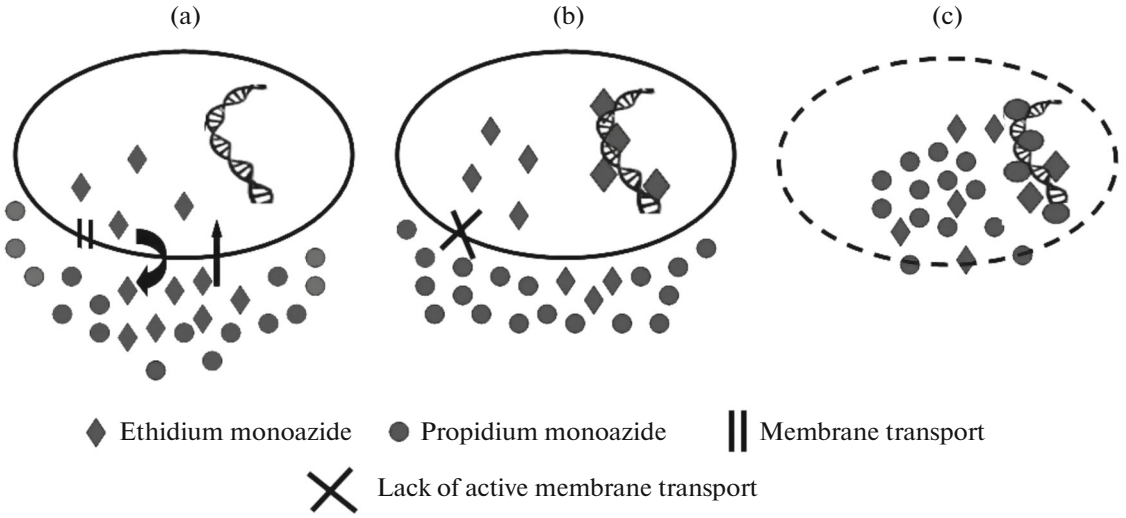


Fig. 3. Interaction of ethidium monoazide and propidium monoazide with DNA of live (a), dormant (b), and dead (c) bacteria.

Combined Use of EMA and PMA in PCR Assays

As soon as PMA was proposed as a phenanthridine dye for differentiation between live and dead bacteria, experiments were performed to compare its efficiency to that of the already known EMA (Nocker et al., 2006). The principal difference between EMA and PMA is that the latter dye cannot permeate into a living bacterial cell, because it carries two positive charges, whereas EMA with a single positive charge still can permeate an undamaged membrane and then be exported, which results in an equilibrium shown schematically on Fig. 3. Moreover, this process may either occur by passive diffusion or be mediated by a cell pump that, in particular, ensures antibiotic resistance in bacterial cells (Willers et al., 2017).

A further advantage of PMA is its lower toxicity for microorganisms. For instance, it was shown that PMA exhibits no antibacterial activity against *L. monocytogenes* (Pan and Breidt, 2007). Another study that analyzed viability of *E. sakazakii* using vPCR found that both PMA and EMA prevented amplification of DNA from dead bacteria, but EMA treatment also had an inhibiting effect on PCR with DNA isolated from live cells (Cawthorn and Witthuhn, 2008).

A study on the viability of *Clostridium perfringens*, *L. monocytogenes*, and *Salmonella enterica* showed that neither EMA nor PMA treatment could completely exclude amplification of DNA from dead bacteria (Wagner et al., 2008). Some authors found that treatment of mixed bacterial communities with phenanthridine dyes EMA and PMA produced an elevated rate of false results for the latter (Lee and Levin, 2009). Another study determined that the PMA concentration required to inhibit vPCR with DNA from *L. pneumophila* was 4 times higher than the necessary EMA concentration, which suggested that EMA was a more appropriate reagent (Chang et al., 2010). Apparently, the already mentioned lower cost of EMA is also of substantial importance. On the other hand, it was shown that PMA was superior to EMA for differentiation of live and dead *H. pylori*, because the latter dye also permeated living cells and prevented amplification of their DNA (Nam et al., 2011). A similar conclusion was proposed by other authors who analyzed live and dead pathogenic bacteria in the mouth cavity: *Streptococcus mutans*, *Prevotella intermedia,* and *Aggregatibacter actinomycetemcomitans* (Loozen et al., 2011). Relatively recently, a fairly large-scale study was performed in five different model objects: gram-negative bacteria *L. pneumophila*, *P. aeruginosa*, and *S. enterica*, as well as gram-positive bacteria *S. aureus* and *Enterococcus faecalis*, to determine the optimal EMA and PMA concentrations for differentiation of live and dead bacteria of these species (6 and 50 μM, respectively), as well as to evaluate the efficiency of using DNase for the same purpose (Reyneke et al., 2017). This work showed that the efficiency of EMA for the viability detection in the analyzed species of gram-negative and gram-positive microorganisms was basically the same. Previously, other authors reported that EMA had similar effects on gram-negative *C. jejuni* and gram-positive *L. monocytogenes* (Flekna et al., 2007). Nevertheless, it should be noted that there exist certain differences in PMA penetration through the cell walls of gram-positive and gram-negative microorganisms; for the latter group, the company Biotium produces an enhancer of PMA penetration to ensure better differentiation of live and dead bacteria.

We should certainly mention an alternative approach to specific detection of live bacteria using DNase I that was proposed by Villarreal et al. (2013). DNase I treatment degrades extracellular DNA and DNA of dead bacteria with a damaged cell wall, while

DNA subsequently isolated from the remaining (live) bacterial cells can be analyzed by conventional PCR. To obtain accurate results, DNase had to be degraded by proteinase K treatment after the DNA degradation step and prior to DNA isolation. The efficiency of elimination of undesirable DNA was controlled using PMA–vPCR, which showed similar results in the analysis of autochthonous microbial communities of biofilms developing in the reservoirs for drinking water production.

PCR Assay with PEMAX

The study by Fittipaldi et al. (2011) cited above probably served as a starting point for vPCR improvement research by this group. In their review published in 2012, the authors provided a detailed analysis of various advantages and shortcomings of phenanthridine dyes used for vPCR (Fittipaldi et al., 2012), which became the basis for subsequent development of a novel bacterial viability assay, followed by a series of similar studies. For instance, an analysis performed in a model system of *Salmonella enteritidis* showed that combined treatment with 10 μM EMA and 50 μM PMA with subsequent vPCR produced more accurate results and allowed detection of not only live and dead bacteria, but also specifically of VBNC cells (Codony et al., 2015). The Spanish company GenIUL manufactures reagents and equipment required for these vPCR assays. To standardize the critical procedure of exposure to light, the company proposes several types of LED devices: PAUL (Photo Activation Universal Light system), PhotoActivation System for Tubes (PhAST), and Phast Blue, saving the researchers the trouble of selecting the light source, as well as the necessity of cooling the specimens to prevent their overheating by halogen lamps. The product worth special mentioning is the PEMAX reagent: its exact composition is kept secret, but it is described as a blend of two dyes (probably, EMA, PMA, or their analogs) supposed to ensure improved differentiation of live and dead bacteria. Several recently published methodical articles describe using the PEMAX reagent to determine the viability state in different bacterial species and suggest, in particular, that the issue with false positive results has been basically resolved (Agusti et al., 2017; Lizana et al., 2017). For instance, combined double photo-activation treatment of *S. enterica* cells, including those artificially added to food products, prevented amplification of the PCR product that could have represented over 10^7 dead bacteria, while the use of PEMAX completely excluded their DNA from the reaction as potential templates (Dinh Thanh et al., 2017). Moreover, changing the reaction tubes and continuing the experiment in new ones helped decrease the rate of false positive results, since it is long known that DNA can be adsorbed on polypropylene (Belotserkovskii et al., 1996).

Amplicon Size for Detection of Viable Microorganisms

Irrespectively of the technique employed, the size of amplified DNA or RNA fragments is critically important for the assessment of bacterial viability state. For instance, a special experiment performed as long ago as in 1993 showed that *L. pneumophila* treated with different chlorine concentrations for different periods of time lost their ability to grow on solid media, but a 168 bp-long DNA fragment sometimes could still be detected by conventional PCR, in contrast to a longer amplicon of 650 bp, which was not amplified (McCarty and Atlas, 1993). This suggested that cell death was associated with progressive DNA degradation and with disappearance of large DNA fragments. Thus, the size of amplified fragments is an indirect indicator of the bacterial cells viability state. It is therefore no wonder that the amplicon size issue attracts considerable attention in vPCR protocols, considering that it provides a further sign of viable or unviable state in microorganisms. As mentioned above, the results of an EMA assay for detection of live *L. monocytogenes* cells were more accurate when longer DNA fragments were amplified (Soejima et al., 2008). Subsequently, the same group applied this approach to other bacteria. For instance, an EMA-based assay for differentiation of live and dead cells of five species of the family *Enterobacteriaceae* revealed an evident inverse relationship between the size of amplicons (110, 340, 670, 1490, and 2840 bp) and the efficiency of their accumulation in vPCR (Soejima et al., 2011b). Another study on differentiation of live and dead *E. sakazakii* bacteria was performed using EMA treatment with nested vPCR, where stage 1 involved amplification of a larger 1514-bp-long fragment, and a shorter fragment of 560 bp was amplified at the second stage to improve the sensitivity of the assay (Soejima et al., 2012). Moreover, a 2451-bp-long DNA fragment present in various coliform bacteria was detected directly in EMA-treated milk, without the DNA isolation step (Soejima et al., 2012). Taking into account the presence of numerous PCR inhibitors in milk, the authors used proteinase K treatment to prevent false negative results. A further difficulty was related to the fact that pasteurized milk contains many dead bacteria, which also could affect the final result.

A number of studies have evaluated the efficiency of nested vPCR with PMA treatment (Contreras et al., 2011; Banihashemi et al., 2012; Martin et al., 2013; Schnetzinger et al., 2013; Ditommaso et al., 2014; Banihashemi et al., 2017). It was shown that the assay for the presence of live salmonella in ham was more reliable when the amplified DNA fragment was 417 bp long, in comparison to shorter fragments of 95 and 285 bp (Martin et al., 2013). The death rate of pathogenic enterobacteria *Yersinia enterocolitica*, *S. enterica*, *C. jejuni*, and *Arcobacter butzleri* in river water was assessed by PMA–PCR amplifying even longer frag-

ments: 1213, 1614, 1512, and 1415 bp, respectively (Banihashemi et al., 2017). Previously, model experiments of the same research group showed that the best results in detection of live *S. enterica* and *C. jejuni* after PMA treatment were obtained by amplification of 1614- and 1512-bp-long DNA fragments, respectively, whereas amplicons of 119, 174, and even 899 bp could not ensure the necessary level of discrimination (Banihashemi et al., 2012).

Differentiation of Live and Dead Bacteria by Isothermal Amplification of DNA or RNA Fragments Involving Phenanthridine Dyes

Alternatively to PCR, differentiation of live and dead microorganisms can utilize other methods of nucleic acid amplification, in particular, those performed under isothermal conditions, which provides certain advantages. For instance, in 2009, the EMA–LAMP technique (Ethidium MonoAzide–Loop-mediated isothermal AMPlification) was proposed for differentiation between the live and dead *S. enterica* cells (Lu et al., 2009). Subsequently, the more appropriate PMA replaced EMA in the LAMP assay employed to detect both salmonella and other bacteria, e.g., *E. coli*, *Vibrio parahaemolyticus*, or *S. aureus* (Chen et al., 2011; Youn et al., 2017; Li et al., 2017; Yan et al., 2017; Fang et al., 2018). However, the group that developed an EMA-based Rti-LAMP technique with real-time detection of amplification results also reported that the use of PMA for differentiation of live and dead *S. enterica* cells was less efficient (Wu et al., 2015). It was proposed that, after eliminating DNA of dead bacteria from the reaction using PMA treatment, the results of LAMP assay with DNA of live bacteria could be detected using other technical variations, e.g., on a microfluidic platform or in combination with biosensor techniques or lateral chromatography (Ahmad et al., 2017; Zhao et al., 2017; Zhang et al., 2017). Another method of nucleic acid amplification, microarray-based recombinase polymerase amplification using PMA was applied to enable rapid (in approximately 1 h) differentiation of live and dead *Legionella* spp. cells (Kober et al., 2018). The current standard for detection of these dangerous bacteria, causative agents of legionellosis, are culture-based methods, which, however, require at least 10 days, and moreover, cannot detect VBNC bacteria. In another study, differentiation of live and dead streptococci using the same technique could be performed as rapidly as in 20 min (Chen et al., 2018).

CONCLUSIONS

There can be no doubt about the importance of detecting viable bacteria in diverse environments, including food products, drinking water, human and animal tissues and excretions, and environmental media (water, soil, or air), especially in isolated compartments, such as space stations. Essential information concerns not only the presence of microorganisms or their abundance, but also their ability to proliferate and thus to represent a danger for human health or for other biological objects. PCR-based approaches to differentiation of live and dead bacteria have been developing for nearly three decades, and during this time, some techniques of this group have advanced considerably, while other turned out to be less efficacious (Table 1).

Most commonly, viable bacteria are detected using phenanthridine dyes, and there has been considerable progress in this field; nevertheless, this approach might still require adaptation to particular bacterial species or genera. Our review does not discuss the technical issues of using ethidium and propidium monoazides for differentiation of live and dead bacteria, such as the range of their concentrations, temperatures, or duration of treatment, which can vary strongly depending on bacterial species in question, but the necessary information can be found in the original articles listed in References. Another promising approach is detection of viable microorganisms by means of RT–PCR amplification of intact pre-rRNA, which is not preserved in nonviable bacterial cells. Ongoing research aims to find or synthesize some novel compounds that could help differentiate live and dead bacteria, in particular, using nucleic acid amplification. For instance, in a recent study by Lee and Bae (2018b), viability state of both gram-negative (*P. aeruginosa*) and gram-positive (*E. faecalis*) bacteria was successfully determined using a derivative of the known SYTO 13 dye, which was termed DyeTox13 Green C-2 Azide and shown to be superior to both EMA and PMA. Furthermore, it was proposed to replace phenanthridine dyes with certain platinum compounds, which also penetrate only compromised membranes of nonviable bacteria and form chelate complexes with purine bases of their DNA, preventing further DNA amplification (Soejima et al., 2016a). The authors underline that the entire assay could be performed at light without any risk that these platinum compounds would enter the reaction prematurely; moreover, they are hundreds of times cheaper than phenanthridine dyes. The same research group achieved further cost reduction in differentiation of live and dead bacteria by using a number of palladium compounds that also penetrate only damaged bacterial membranes; it was possible to perform this assay in pasteurized milk containing numerous dead microorganisms (Soejima and Iwatsuki, 2016).

For the most accurate assessment of the bacterial viability state in various media, it seems optimal to divide the initial sample in three aliquots and to perform three assays: one of them will amplify all bacterial DNA, the second will detect only viable bacteria that maintain their cell wall and/or plasma membrane impermeable for monoazide phenanthridine dyes (or their analogs), and the third one should involve an

Table 1. Methods of differentiating live and dead bacteria based on DNA or RNA amplification in combination with additional treatments (in the chronological order)

Year	Method	Target and features	Reference
1988	PCR	The first use of PCR to detect bacterial presence in a specimen; no differentiation of live and dead bacteria	Oliver et al., 1988
1991	RT–PCR	The first use of PCR with an mRNA target to differentiate live and dead bacteria	Bej et al., 1991
1993	PCR	The first study to differentiate live and dead bacteria by detecting amplicons of different sizes	Jamil et al., 1993
1994	NASBA	Isothermal RNA amplification of live and dead bacteria that did not involve DNA	van der Vliet et al., 1994
1996	RT–PCR	The first use of the pre-rRNA and rRNA boundary region as a target	Candelosi et al., 1996
1999	RT–SDA	Isothermal RNA (cDNA) amplification with preliminary DNA elimination	Hellyer et al., 1999
2003	PCR	The first use of PCR with phenathridine dye (EMA) treatment allowing fairly reliable differentiation of live and dead bacteria	Nogva et al., 2003
2006	PCR	PMA is proposed as a more specific phenathridine dye enabling better differentiation of live and dead bacteria	Nocker et al., 2006
2008	PCR	Treatment with a mixture of two phenathridine dye (EMA and PMA) is proposed for more reliable differentiation of live and dead bacteria	Cawthorn and Witthuhn, 2008
2009	EMA–LAMP	A technique of highly sensitive isothermal amplification with phenathridine dye (EMA) treatment	Lu et al., 2009
2010	PCR (MVT)	Revised technique using pre-rRNA and rRNA boundary region as a target	Candelosi et al., 2010
2011	PMA–LAMP	A technique of highly sensitive isothermal amplification with PMA treatment	Chen et al., 2011
2016	PCR	PCR-based differentiation of live and dead bacteria using certain platinum and palladium compounds	Soejima et al., 2016; Soejima and Iwatsuki, 2016
2018	PMA-RPA	A rapid and highly sensitive technique of isothermal amplification with phenathridine dye PMA treatment proposed for differentiation of live and dead bacteria	Kober et al., 2018
2018	DyeTox-PCR	PCR-based differentiation of live and dead bacteria using a novel promising dye, DyeTox13 Green C-2 Azide	Lee and Bae, 2018b

appropriate technique of highly sensitive pre-rRNA amplification without DNase treatment under isothermal conditions. This could prevent both the potential degradation of RNA molecules and DNA amplification, so that amplicons would be produced only from RNA templates. A further important aspect of this triple assay is the fact that bacteria exposed to UV radiation can retain PMA-impermeable cell walls, and even though they lose their viability, the corresponding information cannot be obtained. In spite of the necessity to conduct three independent reactions, this expenditure of resources may be justified in a number of cases requiring differentiation of live and dead bacteria.

FUNDING

An.Kh. Baymiev, Al.Kh. Baymiev, B.R. Kuluev, K.Yu. Shvets, R.S. Yamidanov, Ya.A. Ivanenkov, and A.V. Chemeris were supported by the Russian Science Foundation, project no. 17-74-30012.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. This article does not contain any studies involving animals or human subjects performed by any of the authors.

REFERENCES

Agustí, G., Fittipaldi, M., and Codony, F., False-positive viability PCR results: an association with microtubes, *Curr. Microbiol.*, 2017, vol. 74, pp. 377–380. https://doi.org/10.1007/s00284-016-1189-3

Ahmad, F., Stedtfeld, R.D., Waseem, H., Williams, M.R., Cupples, A.M., Tiedje, J.M., and Hashsham, S.A., Most probable number–loop mediated isothermal amplification (MPN-LAMP) for quantifying waterborne pathogens in less than 25 minutes, *J. Microbiol. Methods*, 2017, vol. 132, pp. 27–33. https://doi.org/10.1016/j.mimet.2016.11.010

Bae, S. and Wuertz S., Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide, *Appl. Environ. Microbiol.*, 2009, vol. 75, pp. 2940–2944. https://doi.org/10.1128/AEM.01333-08

Banihashemi, A., Van Dyke, M., and Huck, P.M., Long-amplicon propidium monoazide-PCR enumeration assay to detect viable *Campylobacter* and *Salmonella, J. Appl. Microbiol.*, 2012, vol. 113, pp. 863–873. https://doi.org/10.1111/j.1365-2672.2012.05382.x

Banihashemi, A., Van Dyke, M.I., and Huck, P.M., Application of long amplicon propidium monoazide-PCR to assess the effects of temperature and background microbiota on pathogens in river water, *J. Water Health*, 2017, vol. 15, pp. 418–428. https://doi.org/10.2166/wh.2017.161

Barer, M.R. and Harwood, C.R., Bacterial viability and culturability, *Adv. Microb. Physiol.*, 1999, vol. 41, pp. 93–137. https://doi.org/10.1016/S0065-2911(08)60166-6

Bej, A.K., Mahbubani, M.H., and Atlas, R.M., Detection of viable *Legionella pneumophila* in water by polymerase chain reaction and gene probe methods, *Appl. Environ. Microbiol.*, 1991, vol. 57, pp. 597–600.

Bej, A.K., Ng, W.Y., Morgan, S., Jones, D.D., and Mahbubani, M.H., Detection of viable *Vibrio cholerae* by reverse-transcriptase polymerase chain reaction (RT-PCR), *Mol. Biotechnol.*, 1996, vol. 5, pp. 1–10. https://doi.org/10.1007/BF02762407

Belotserkovskii, B.P. and Johnston, B.H., Polypropylene tube surfaces may induce denaturation and multimerization of DNA, *Science*, 1996, vol. 271, no. 5246, pp. 222–223. https://doi.org/10.1126/science.271.5246.222

Bernet, C., Garret, M., de Barbeyrac, B., Bebear, C., and Bonnet J., Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction, *J. Clin. Microbiol.*, 1989, vol. 27, pp. 2492–2496.

Birch, L., Dawson, C.E., Cornett, J.H., and Keer, J.T., A comparison of nucleic acid amplification techniques for the assessment of bacterial viability, *Lett. Appl. Microbiol.*, 2001, vol. 33, pp. 296–301. https://doi.org/10.1046/j.1472-765X.2001.00999.x

Cangelosi, G.A. and Meschke, J.S., Dead or alive: molecular assessment of microbial viability, *Appl. Environ. Microbiol.*, 2014, vol. 80, pp. 5884–5891. https://doi.org/10.1128/AEM.01763-14

Cangelosi, G.A., Brabant, W.H., Britschgi, T.B., and Wallis, C.K., Detection of rifampin- and ciprofloxacin-resistant *Mycobacterium tuberculosis* by using species-specific assays for precursor rRNA, *Antimicrob. Agents Chemother.*, 1996, vol. 40, pp. 1790–1795. https://doi.org/10.1128/AAC.40.8.1790

Cangelosi, G.A., Weigel, K.M., Lefthand-Begay, C., and Meschke, J.S., Molecular detection of viable bacterial pathogens in water by ratiometric pre-rRNA analysis, *Appl. Environ. Microbiol.*, 2010, vol. 76, pp. 960–962. https://doi.org/10.1128/AEM.01810-09

Cawthorn, D.M. and Witthuhn, R.C., Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide, *J. Appl. Microbiol.*, 2008, vol. 105, pp. 1178–1185. https://doi.org/10.1111/j.1365-2672.2008.03851.x

Chang, B., Taguri, T., Sugiyama, K., Amemura-Maekawa, J., Kura, F., and Watanabe, H., Comparison of ethidium monoazide and propidium monoazide for the selective detection of viable *Legionella* cells, *Jpn. J. Infect. Dis.*, 2010, vol. 63, pp. 119–123. https://doi.org/10.4018/978-1-60566-840-6.ch008

Chemeris, A.V., Magdanov, E.G., Garafutdinov, R.R., and Vakhitov, V.A., How to exclude false positive results in polymerase chain reaction, *Vestm. Biotekhnol. Fiz.-Khim. Biol.*, 2012a, vol. 8, no. 3, pp. 34–45.

Chemeris, A.V., Chemeris, D.A., Magdanov, E.U., Garafutdinov, R.R., Nagaev, N.R., and Vakhitov, V.A., Causes of false negative PCR and preventing some of them, *Biomika*, 2012b, vol. 4, no. 1, pp. 31–47.

Chemeris, D.A., Magdanov, E.U., Mashkov, O.I., Garafutdinov, R.R., and Chemeris, A.V., PCR with delayed (hot) start, *Biomika*, 2011, vol. 2, no. 1, pp. 1–8.

Chen, J., Wang, Y., Liu, X., Chen, G., Chen, X., Chen, J., Liu, Z., Gong, J., Yang, G., and Lan, Q., Development of propidium monoazide-recombinase polymerase amplification (PMA-RPA) assay for rapid detection of *Streptococcus pyogenes* and *Streptococcus agalactiae, Mol. Cell. Probes*, 2018, vol. 41, pp. 32–38. https://doi.org/10.1016/j.mcp.2018.08.007

Chen, S., Wang, F., Beaulieu, J.C., Stein, R.E., and Ge, B., Rapid detection of viable salmonellae in produce by coupling propidium monoazide with Loop-Mediated Isothermal Amplification, *Appl. Environ. Microbiol.*, 2011, vol. 77, pp. 4008–4016. https://doi.org/10.1128/AEM.00354-11

Codony, F., Agustí, G., and Allué-Guardia, A., Cell membrane integrity and distinguishing between metabolically active and inactive cells as a means of improving viability PCR, *Mol. Cell. Probes*, 2015, vol. 29, pp. 190–192. https://doi.org/10.1016/j.mcp.2015.03.003

Contreras, P.J., Urrutia, H., Sossa, K., and Nocker, A., Effect of PCR amplicon length on suppressing signals from membrane-compromised cells by propidium monoazide treatment, *J. Microbiol. Methods*, 2011, vol. 7, pp. 89–95. https://doi.org/10.1016/j.mimet.2011.07.016

Davey, H.M., Life, death, and in-between: meanings and methods in microbiology, *Appl. Environ. Microbiol.*, 2011, vol. 77, pp. 5571–5576. https://doi.org/10.1128/AEM.00744-11

Dinh Thanh, M., Agustí, G., Mader, A., Appel, B., and Codony, F., Improved sample treatment protocol for accurate detection of live *Salmonella* spp. in food samples by viability PCR, *PLoS One*, 2017, vol. 12. e0189302. https://doi.org/10.1371/journal.pone.0189302

Ditommaso, S., Ricciardi, E., Giacomuzzi, M., Arauco Rivera, S.R., Ceccarelli, A., and Zotti, C.M., Overestimation of the *Legionella* spp. load in environmental samples by quantitative real-time PCR: pretreatment with propidium monoazide as a tool for the assessment of an association between *Legionella* concentration and sanitary risk, *Diagn. Microbiol. Infect. Dis.*, 2014, vol. 80, pp. 260–266. https://doi.org/10.1016/j.diagmicrobio.2014.09.010

Dong, L., Liu, H., Meng, L., Xing, M., Wang, J., Wang, C., Chen, H., and Zheng, N., Quantitative PCR coupled with sodium dodecyl sulfate and propidium monoazide for detection of viable *Staphylococcus aureus* in milk, *J. Dairy Sci.*, 2018, vol. 101, pp. 4936–4943. https://doi.org/10.3168/jds.2017-14087

Dutilh, B., Bébéar, C., Rodriguez, P., Vekris, A., Bonnet, J., and Garret, M., Specific amplification of a DNA sequence common to all *Chlamydia trachomatis* serovars using the polymerase chain reaction, *Res. Microbiol.*, 1989, vol. 140, pp. 7–16. https://doi.org/10.1016/0923-2508(89)90053-3

Fang, J., Wu, Y., Qu, D., Ma, B., Yu, X., Zhang, M., and Han, J., Propidium monoazide real-time loop-mediated isothermal amplification for specific visualization of viable *Salmonella* in food, *Lett. Appl. Microbiol.*, 2018, vol. 67, pp. 79–88. https://doi.org/10.1111/lam.12992

Fittipaldi, M., Codony, F., Adrados, B., Camper, A.K., and Morató, J., Viable real-time PCR in environmental samples: can all data be interpreted directly?, *Microb. Ecol.*, 2011, vol. 61, pp. 7–12. https://doi.org/10.1007/s00248-010-9719-1

Fittipaldi, M., Nocker, A., and Codony, F., Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification, *J. Microbiol. Methods*, 2012, vol. 91, pp. 276–289. https://doi.org/10.1016/j.mimet.2012.08.007

Flekna, G., Stefanic, P., Wagner, M., Smulders, F.J., Mozina, S.S., and Hein, I., Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR, *Res. Microbiol.*, 2007, vol. 158, pp. 405–412. https://doi.org/10.1016/j.resmic.2007.02.008

Fukuzawa, S., Shiho, H., and Fujita, T., Selective detection of DNA from viable *Mycobacterium tuberculosis* complex strains using the EMA-PCR method, *Jpn. J. Infect. Dis.*, 2019, vol. 72, pp. 19–22. https://doi.org/10.7883/yoken.JJID.2018.111

Fykse, E.M., Skogan, G., Davies, W., Olsen, J.S., and Blatny J.M., Detection of *Vibrio cholerae* by real-time nucleic acid sequence-based amplification, *Appl. Environ. Microbiol.*, 2007, vol. 73, pp. 1457–1466. https://doi.org/10.1128/AEM.01635-06

Gobert, G., Cotillard, A., Fourmestraux, C., Pruvost, L., Miguet, J., and Boyer, M., Droplet digital PCR improves absolute quantification of viable lactic acid bacteria in faecal samples, *J. Microbiol. Methods*, 2018, vol. 148, pp. 64–73. https://doi.org/10.1016/j.mimet.2018.03.004

Graves, D.E., Watkins, C.L., and Yielding, L.W., Ethidium bromide and its photoreactive analogues: spectroscopic analysis of deoxyribonucleic acid binding properties, *Biochemistry*, 1981, vol. 20, pp. 1887–1892. https://doi.org/10.1021/bi00510a026

Hartskeerl, R.A., de Wit, M.Y., and Klatser, P.R., Polymerase chain reaction for the detection of *Mycobacterium leprae, J. Gen. Microbiol.*, 1989, vol. 135, pp. 2357–2364. https://doi.org/10.1099/00221287-135-9-2357

Hein, I., Flekna, G., and Wagner, M., Possible errors in the interpretation of ethidium bromide and PicoGreen DNA staining results from ethidium monoazide-treated DNA, *Appl. Environ. Microbiol.*, 2006, vol. 72, pp. 6860–6861. https://doi.org/10.1128/AEM.01243-06

Hellyer, T.J., DesJardin, L.E., Teixeira, L., Perkins, M.D., Cave, M.D., and Eisenach, K.D., Detection of viable *Mycobacterium tuberculosis* by reverse transcriptase-strand displacement amplification of mRNA, *J. Clin. Microbiol.*, 1999, vol. 37, pp. 518–523.

Huang, Z., Zheng, J., Shi, C., and Chen, Q., Flow cytometry-based method facilitates optimization of PMA treatment condition for PMA-qPCR method, *Mol. Cell. Probes*, 2018, vol. 40, pp. 37–39. https://doi.org/10.1016/j.mcp.2018.05.002

Inoue, D., Tsutsui, H., Yamazaki, Y., Sei, K., Soda, S., Fujita, M., and Ike, M., Application of real-time polymerase chain reaction (PCR) coupled with ethidium monoazide treatment for selective quantification of viable bacteria in aquatic environment, *Water Sci. Technol.*, 2008, vol. 58, pp. 1107–1112. https://doi.org/10.2166/wst.2008.474

Jamil, S., Keer, J.T., Lucas, S.B., Dockrell, H.M., Chiang, T.J., Hussain, R., and Stoker, N.G., Use of polymerase chain reaction to assess efficacy of leprosy chemotherapy, *Lancet*, 1993, vol. 342, no. 8866, pp. 264–268. https://doi.org/10.1016/0140-6736(93)91816-5

Janssen, K.J., Hoebe, C.J., Dukers-Muijrers, N.H., Eppings, L., Lucchesi, M., and Wolffs, P.F., Viability-PCR shows that NAAT detects a high proportion of DNA from non-viable *Chlamydia trachomatis, PLoS One*, 2016, vol. 11. e0165920. https://doi.org/10.1371/journal.pone.0165920

Kaushik, R. and Balasubramanian, R., Discrimination of viable from non-viable gram-negative bacterial pathogens in airborne particles using propidium monoazide-assisted qPCR, *Sci. Total Environ.*, 2013, vol. 449, pp. 237–243. https://doi.org/10.1016/j.scitotenv.2013.01.065

Kell, D.B., Kaprelyants, A.S., Weichart, D.H., Harwood, C.R., and Barer, M.R., Viability and activity in readily culturable bacteria: a review and discussion of the practical issues, *Antonie van Leeuwenhoek*, 1998, vol. 73, pp. 169–187. https://doi.org/10.1023/A:1000664013047

Kibbee, R.J. and Örmeci, B., Development of a sensitive and false-positive free PMA-qPCR viability assay to quantify VBNC *Escherichia coli* and evaluate disinfection performance in wastewater effluent, *J. Microbiol. Methods*, 2017, vol. 132, pp. 139–147. https://doi.org/10.1016/j.mimet.2016.12.004

Klein, P.G. and Juneja, V.K., Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR, *Appl. Environ. Microbiol.*, 1997, vol. 63, pp. 4441–4448.

Knowles, J.R., Photogenerated reagents for biological receptor-site labeling, *Acc. Chem. Res.* 1972, vol. 5, pp. 155–160.
https://doi.org/10.1021/ar50052a006

Kobayashi, H., Oethinger, M., Tuohy, M.J., Procop, G.W., Hall, G.S., and Bauer, T.W., Limiting false-positive polymerase chain reaction results: detection of DNA and mRNA to differentiate viable from dead bacteria, *Diagn. Microbiol. Infect. Dis.*, 2009, vol. 64, no. 4, pp. 445–447.
https://doi.org/10.1016/j.diagmicrobio.2009.04.004

Kober, C., Niessner, R., and Seidel, M., Quantification of viable and non-viable *Legionella* spp. by heterogeneous asymmetric recombinase polymerase amplification (haRPA) on a flow-based chemiluminescence microarray, *Biosens. Bioelectron.*, 2018, vol. 100, pp. 49–55.
https://doi.org/10.1016/j.bios.2017.08.053

Kontchou, J.A. and Nocker, A., Optimization of viability qPCR for selective detection of membrane-intact *Legionella pneumophila, J. Microbiol. Methods*, 2019, vol. 156, pp. 68–76.
https://doi.org/10.1016/j.mimet.2018.12.003

Lee, E.S., Lee, M.H., and Kim, B.S., Evaluation of propidium monoazide-quantitative PCR to detect viable *Mycobacterium fortuitum* after chlorine, ozone, and ultraviolet disinfection, *Int. J. Food Microbiol.*, 2015, vol. 210, pp. 143–148.
https://doi.org/10.1016/j.ijfoodmicro.2015.06.019

Lee, J.L. and Levin, R.E., A comparative study of the ability of EMA and PMA to distinguish viable from heat killed mixed bacterial flora from fish fillets, *J. Microbiol. Methods*, 2009, vol. 76, pp. 93–96.
https://doi.org/10.1016/j.mimet.2008.08.008

Lee, S. and Bae, S., Evaluating the newly developed dye, DyeTox13 Green C-2 Azide, and comparing it with existing EMA and PMA for the differentiation of viable and nonviable bacteria, *J. Microbiol Methods*, 2018b, vol. 148, pp. 33–39.
https://doi.org/10.1016/j.mimet.2018.03.018

Lee, S. and Bae, S., Molecular viability testing of viable but non-culturable bacteria induced by antibiotic exposure, *Microb. Biotechnol.*, 2018a, vol. 11, pp. 1008–1016.
https://doi.org/10.1111/1751-7915.13039

Li, H., Xin, H., and Li, S.F., Multiplex PMA-qPCR assay with internal amplification control for simultaneous detection of viable *Legionella pneumophila, Salmonella typhimurium*, and *Staphylococcus aureus* in environmental waters, *Environ. Sci. Technol.*, 2015, vol. 49, pp. 14249–14256.
https://doi.org/10.1021/acs.est.5b03583

Li, Y., Yang, L., Fu, J., Yan, M., Chen, D., and Zhang, L., The novel loop-mediated isothermal amplification based confirmation methodology on the bacteria in Viable but Non-Culturable (VBNC) state, *Microb. Pathog.*, 2017, vol. 111, pp. 280–284.
https://doi.org/10.1016/j.micpath.2017.09.007

Lizana, X., López, A., Benito, S., Agustí, G., Ríos, M., Piqué, N., Marqués, A.M., and Codony, F., Viability qPdCR, a new tool for *Legionella* risk management, *Int. J. Hyg. Environ. Health*, 2017, vol. 220, pp. 1318–1324.
https://doi.org/10.1016/j.ijheh.2017.08.007

Lleò, M.M., Pierobon, S., Tafi, M.C., Signoretto, C., and Canepari, P., mRNA detection by reverse transcription-PCR for monitoring viability over time in an *Enterococcus faecalis* viable but nonculturable population maintained in a laboratory microcosm, *Appl. Environ. Microbiol.*, 2000, vol. 66, pp. 4564–4567.
https://doi.org/10.1128/AEM.66.10.4564-4567.2000

Loozen, G., Boon, N., Pauwels, M., Quirynen, M., and Teughels, W., Live/dead real-time polymerase chain reaction to assess new therapies against dental plaque-related pathologies, *Mol. Oral. Microbiol.*, 2011, vol. 26, pp. 253–261.
https://doi.org/10.1111/j.2041-1014.2011.00615.x

Lovdal, T., Hovda, M.B., Björkblom, B., and Møller, S.G., Propidium monoazide combined with real-time quantitative PCR underestimates heat-killed *Listeria innocua, J. Microbiol. Methods*, 2011, vol. 85, pp. 164–169.
https://doi.org/10.1016/j.mimet.2011.01.027

Lu, Y., Yang, W., Shi, L., Li, L., Alam, M.J., Guo, S., and Miyoshi, S.-I., Specific detection of viable *Salmonella* cells by an ethidium monoazide-Loop Mediated Isothermal Amplification (EMA-LAMP) method, *J. Health Sci.*, 2009, vol. 55, pp. 820–824.
https://doi.org/10.1248/jhs.55.820

Martin, B., Raurich, S., Garriga, M., and Aymerich, T., Effect of amplicon length in propidium monoazide quantitative PCR for the enumeration of viable cells of *Salmonella* in cooked ham, *Food Anal. Methods*, 2013, vol. 6, pp. 683–690.
https://doi.org/10.1007/s12161-012-9460-0

McCarty, S.C. and Atlas, R.M., Effect of amplicon size on PCR detection of bacteria exposed to chlorine, *PCR Methods Appl.*, 1993, vol. 3, pp. 181–185.
https://doi.org/10.1101/gr.3.3.181

McKillip, J.L., Jaykus, L.A., and Drake, M., rRNA stability in heat-killed and UV-irradiated enterotoxigenic *Staphylococcus aureus* and *Escherichia coli* O157:H7, *Appl. Environ. Microbiol.*, 1998, vol. 64, pp. 4264–4268.

Minami, J., Yoshida, K., Soejima, T., Yaeshima, T., and Iwatsuki, K., New approach to use ethidium bromide monoazide as an analytical tool, *J. Appl. Microbiol.*, 2010, vol. 109, pp. 900–909.
https://doi.org/10.1111/j.1365-2672.2010.04716.x

Nam, S., Kwon, S., Kim, M.J., Chae, J.C., Jae Maeng, P., Park, J.G., and Lee, G.C., Selective detection of viable *Helicobacter pylori* using ethidium monoazide or propidium monoazide in combination with real-time polymerase chain reaction, *Microbiol. Immunol.*, 2011, vol. 55, pp. 841–846.
https://doi.org/10.1111/j.1348-0421.2011.00388.x

Nebe-von Caron, G., Stephens, P., and Badley, R.A., Assessment of bacterial viability status by flow cytometry and single cell sorting, *J. Appl. Microbiol.*, 1998, vol. 84, pp. 988–998.
https://doi.org/10.1046/j.1365-2672.1998.00436.x

Nikolayevskyy, V., Miotto, P., Pimkina, E., Balabanova, Y., Kontsevaya, I., Ignatyeva, O., Ambrosi, A., Skenders, G., Ambrozaitis, A., Kovalyov, A., Sadykhova, A., Simak, T., Kritsky, A., Mironova, S., Tikhonova, O., et al., Utility of propidium monoazide viability assay as a biomarker for a

tuberculosis disease, *Tuberculosis* (Edinb.), 2015, vol. 95, pp. 179–185.
https://doi.org/10.1016/j.tube.2014.11.005

Nocker, A. and Camper, A.K., Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide, *Appl. Environ. Microbiol.*, 2006, vol. 72, pp. 1997–2004.
https://doi.org/10.1128/AEM.72.3.1997-2004.2006

Nocker, A., Cheung, C.Y., and Camper, A.K., Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells, *J. Microbiol. Methods.*, 2006, vol. 67, pp. 310–320.
https://doi.org/10.1016/j.mimet.2006.04.015

Nocker, A., Mazza, A., Masson, L., Camper, A.K., and Brousseau, R., Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology, *J. Microbiol. Methods*, 2009, vol. 76, pp. 253–261.
https://doi.org/10.1016/j.mimet.2008.11.004

Nocker, A., Richter-Heitmann, T., Montijn, R., Schuren, F., and Kort, R., Discrimination between live and dead cells in bacterial communities from environmental water samples analyzed by 454 pyrosequencing, *Int. Microbiol.*, 2010, vol. 13, pp. 59–65.
https://doi.org/10.2436/20.1501.01.111

Nocker, A., Sossa, K.E., and Camper, A.K., Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR, *J. Microbiol. Methods*, 2007, vol. 70, pp. 252–260.
https://doi.org/10.1016/j.mimet.2007.04.014

Nogva, H.K., Drømtorp, S.M., Nissen, H., and Rudi, K., Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR, *Biotechniques*, 2003, vol. 34, pp. 804–813.
https://doi.org/10.2144/03344rr02

Olive, D.M., Atta, A.I., and Setti, S.K., Detection of toxigenic Escherichia coli using biotin-labelled DNA probes following enzymatic amplification of the heat labile toxin gene, *Mol. Cell. Probes*, 1988, vol. 2, pp. 47–57.
https://doi.org/10.1016/0890-8508(88)90043-6

Olive, D.M., Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase, *J. Clin. Microbiol.*, 1989, vol. 27, no. 2, pp. 261–265.
https://doi.org/10.1016/S0934-8840(11)80610-9

Oliver, J.D., Recent findings on the viable but nonculturable state in pathogenic bacteria, *FEMS Microbiol. Rev.*, 2010, vol. 34, pp. 415–425.
https://doi.org/10.1111/j.1574-6976.2009.00200.x

Pan, Y. and Breidt, F., Jr., Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells, *Appl. Environ. Microbiol.*, 2007, vol. 73, pp. 8028–8031.
https://doi.org/10.1128/AEM.01198-07

Pinto, D., Santos, M.A., and Chambel, L. Thirty years of viable but nonculturable state research: unsolved molecular mechanisms, *Crit. Rev. Microbiol.*, 2015, vol. 41, pp. 61–76.
https://doi.org/10.3109/1040841X.2013.794127

Pisz, J.M., Lawrence, J.R., Schafer, A.N., and Siciliano, S.D., Differentiation of genes extracted from non-viable versus viable microorganisms in environmental samples using ethidium monoazide bromide, *J. Microbiol. Methods*, 2007, vol. 71, pp. 312–318.
https://doi.org/10.1016/j.mimet.2007.09.015

Rawsthorne, H., Dock, C.N., and Jaykus, L.A., PCR-based method using propidium monoazide to distinguish viable from nonviable *Bacillus subtilis* spores, *Appl. Environ. Microbiol.*, 2009, vol. 75, no. 9, pp. 2936–2939.
https://doi.org/10.1128/AEM.02524-08

Reyneke, B., Ndlovu, T., Khan, S., and Khan, W., Comparison of EMA-, PMA- and DNase qPCR for the determination of microbial cell viability, *Appl. Microbiol. Biotechnol.*, 2017, vol. 101, no. 19, pp. 7371–7383.
https://doi.org/10.1007/s00253-017-8471-6

Riedy, M.C., Muirhead, K.A., Jensen, C.P., and Stewart, C.C., Use of a photolabeling technique to identify nonviable cells in fixed homologous or heterologous cell populations, *Cytometry*, 1991, vol. 12, pp. 133–139.
https://doi.org/10.1002/cyto.990120206

Rosa, P.A. and Schwan, T.G., A specific and sensitive assay for the Lyme disease spirochete *Borrelia burgdorferi* using the polymerase chain reaction, *J. Infect. Dis.*, 1989, vol. 160, no. 6, pp. 1018–1029.
https://doi.org/10.1093/infdis/160.6.1018

Roussel, C., Galia, W., Leriche, F., Chalancon, S., Denis, S., Van de Wiele, T., and Blanquet-Diot, S., Comparison of conventional plating, PMA-qPCR, and flow cytometry for the determination of viable enterotoxigenic *Escherichia coli* along a gastrointestinal in vitro model, *Appl. Microbiol. Biotechnol.*, 2018, vol. 102, pp. 9793–9802.
https://doi.org/10.1007/s00253-018-9380-z

Rudi, K., Moen, B., Drømtorp, S.M., and Holck, A.L., Polymerase chain reaction for the detection of *Mycobacterium leprae, Appl. Environ. Microbiol.*, 2005, vol. 71, pp. 1018–1024.
https://doi.org/10.1128/AEM.71.2.1018-1024.2005

Schnetzinger, F., Pan, Y., and Nocker, A., Use of propidium monoazide and increased amplicon length reduce false-positive signals in quantitative PCR for bioburden analysis, *Appl. Microbiol. Biotechnol.*, 2013, vol. 97, pp. 2153–2162.
https://doi.org/10.1007/s00253-013-4711-6

Seidel, L., Strathmann, M., and Nocker, A., The feasibility of improved live-dead distinction in qPCR-based microbial source tracking, *J. Microbiol. Methods*, 2017, vol. 140, pp. 23–31.
https://doi.org/10.1016/j.mimet.2017.06.013

Sheridan, G.E., Masters, C.I., Shallcross, J.A., and MacKey, B.M., Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells, *Appl. Environ. Microbiol.*, 1998, vol. 64, pp. 1313–1318.

Simpkins, S.A., Chan, A.B., Hays, J., Pöpping, B., and Cook, N., An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica, Lett. Appl. Microbiol.*, 2000, vol. 30, pp. 75–79.
https://doi.org/10.1046/j.1472-765x.2000.00670.x

Slimani, S., Robyns, A., Jarraud, S., Molmeret, M., Dusserre, E., Mazure, C., Facon, J.P, Lina, G., Etienne, J., and Ginevra, C., Evaluation of propidium monoazide (PMA) treatment directly on membrane filter for the enumeration of viable but non cultivable *Legionella* by qPCR, *J. Microbiol. Methods*, 2012, vol. 88, pp. 319–321.
https://doi.org/10.1016/j.mimet.2011.12.010

Soejima, T. and Iwatsuki, K.J., Innovative use of palladium compounds to selectively detect live *Enterobacteriaceae* cells in milk by polymerase chain reaction, *Appl. Environ. Microbiol.*, 2016, vol. 82, pp. 6930–6941.
https://doi.org/10.1128/AEM.01613-16

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., and Yoshida, S., Method to detect only live bacteria during PCR amplification, *J. Clin. Microbiol.*, 2008, vol. 46, pp. 2305–2313.
https://doi.org/10.1128/JCM.02171-07

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Takade, A., and Yoshida, S., Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria, *Microbiol. Immunol.*, 2007, vol. 51, pp. 763–775.
https://doi.org/10.1111/j.1348-0421.2007.tb03966.x

Soejima, T., Minami, J., Xiao, J.Z., and Abe, F., Innovative use of platinum compounds to selectively detect live microorganisms by polymerase chain reaction, *Biotechnol. Bioeng.*, 2016, vol. 113, pp. 301–310.
https://doi.org/10.1002/bit.25711

Soejima, T., Minami, J., Yaeshima, T., and Iwatsuki, K., An advanced PCR method for the specific detection of viable total coliform bacteria in pasteurized milk, *Appl. Microbiol. Biotechnol.*, 2012, vol. 95, pp. 485–497.
https://doi.org/10.1007/s00253-012-4086-0

Soejima, T., Schlitt-Dittrich, F., and Yoshida, S., Polymerase chain reaction amplification length-dependent ethidium, *Anal. Biochem.*, 2011b, vol. 418, pp. 37–43.
https://doi.org/10.1016/j.ab.2011.06.027

Soejima, T., Schlitt-Dittrich, F., and Yoshida, S., Rapid detection of viable bacteria by nested polymerase chain reaction via long DNA amplification after ethidium monoazide treatment, *Anal. Biochem.*, 2011a, vol. 418, pp. 286–289.
https://doi.org/10.1016/j.ab.2011.06.033

van der Vliet, G.M., Schepers, P., Schukkink, R.A., van Gemen, B., and Klatser, P.R., Assessment of mycobacterial viability by RNA amplification, *Antimicrob. Agents Chemother.*, 1994, vol. 38, pp. 1959–1965.
https://doi.org/10.1128/AAC.38.9.1959

Vesper, S., McKinstry, C., Hartmann, C., Neace, M., Yoder, S., and Vesper, A., Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA), *J. Microbiol. Methods*, 2008, vol. 72, pp. 180–184.
https://doi.org/10.1016/j.mimet.2007.11.017

Villarreal, J.V., Jungfer, C., Obst, U., and Schwartz, T., DNase I and Proteinase K eliminate DNA from injured or dead bacteria but not from living bacteria in microbial reference systems and natural drinking water biofilms for subsequent molecular biology analyses, *J. Microbiol. Methods*, 2013, vol. 94, pp. 161–169.
https://doi.org/10.1016/j.mimet.2013.06.009

Wagner, A.O., Malin, C., Knapp, B.A., and Illmer, P., Removal of free extracellular DNA from environmental samples by ethidium monoazide and propidium monoazide, *Appl. Environ. Microbiol.*, 2008, vol. 74, pp. 2537–2539.
https://doi.org/10.1128/AEM.02288-07

Wahman, D.G., Wulfeck-Kleier, K.A., and Pressman, J.G., Monochloramine disinfection kinetics of *Nitrosomonas europaea* by propidium monoazide quantitative PCR and Live/dead BacLight methods, *Appl. Environ. Microbiol.*, 2009, vol. 75, pp. 5555–5562.
https://doi.org/10.1128/AEM.00407-09

Wang, H., Gill, C.O., and Yang, X., Use of sodium lauroyl sarcosinate (sarkosyl) in viable real-time PCR for enumeration of *Escherichia coli, J. Microbiol. Methods*, 2014a, vol. 98, pp. 89–93.
https://doi.org/10.1016/j.mimet.2014.01.004

Wang, L., Li, P., Zhang, Z., Chen, Q., Aguilar, Z.P., Xu, H., Yang, L., Xu, F., Lai, W., Xiong, Y., and Wei, H., Rapid and accurate detection of viable *Escherichia coli* O157:H7 in milk using a combined IMS, sodium deoxycholate, PMA and real-time quantitative PCR process, *Food Control*, 2014b, vol. 36, pp. 119–125.
https://doi.org/10.1016/j.foodcont.2013.08.011

Weigel, K.M., Nguyen, F.K., Kearney, M.R., Meschke, J.S., and Cangelosi, G.A., Molecular viability testing of UV-inactivated bacteria, *Appl. Environ. Microbiol.*, 2017, vol. 83. pii: e00331-17.
https://doi.org/10.1128/AEM.00331-17

Willers, C., Wentzel, J.F., du Plessis, L.H., Gouws, C., and Hamman, J.H., Efflux as a mechanism of antimicrobial drug resistance in clinical relevant microorganisms: the role of efflux inhibitors, *Expert Opin. Ther. Targets*, 2017, vol. 21, pp. 23–36.
https://doi.org/10.1080/14728222.2017.1265105

Wu, G.P., Chen, S.H., and Levin, R.E., Application of ethidium bromide monoazide for quantification of viable and dead cells of *Salmonella enterica* by real-time loop-mediated isothermal amplification, *J. Microbiol. Methods*, 2015, vol. 117, pp. 41–48.
https://doi.org/10.1016/j.mimet.2015.07.012

Xing-Long, X., Cong-Cong, L., Yang, Q., Yi-Gang, Y., and Hui, W., Molecular monitoring of *Escherichia coli* O157:H7 sterilization rate using qPCR and propidium monoazide treatment, *Lett. Appl. Microbiol.*, 2013, vol. 56, pp. 333–339.
https://doi.org/10.1111/lam.12052

Yáñez, M.A., Nocker, A., Soria-Soria, E., Múrtula, R., Martínez, L., and Catalán, V., Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR, *J. Microbiol. Methods*, 2011, vol. 85, pp. 124–130.
https://doi.org/10.1016/j.mimet.2011.02.004

Yang, Y., Xu, F., Xu, H., Aguilar, Z.P., Niu, R., Yuan, Y., Sun, J., You, X., Lai, W., Xiong, Y., Wan, C., and Wei, H., Magnetic nano-beads based separation combined with propidium monoazide treatment and multiplex PCR assay for simultaneous detection of viable *Salmonella typhimurium, Escherichia coli* O157:H7 and *Listeria monocytogenes* in food products, *Food Microbiol.*, 2013, vol. 34, pp. 418–424.
https://doi.org/10.1016/j.fm.2013.01.004

Yan, M., Xu, L., Jiang, H., Zhou, Z., Zhou, S., and Zhang, L., PMA-LAMP for rapid detection of *Escherichia coli* and shiga toxins from viable but non-culturable state, *Microb. Pathog.*, 2017, vol. 105, pp. 245–250.
https://doi.org/10.1016/j.micpath.2017.02.001

Yokomachi, N. and Yaguchi, J., Enumeration of viable *Escherichia coli* by real-time PCR with propidium monoazide, *Water Sci. Technol.*, 2012, vol. 66, pp. 2065–2073.
https://doi.org/10.2166/wst.2012.370

Youn, S.Y., Jeong, O.M., Choi, B.K., Jung, S.C., and Kang, M.S., Application of loop-mediated isothermal am-

plification with propidium monoazide treatment to detect live *Salmonella* in chicken carcasses, *Poult. Sci.*, 2017, vol. 96, pp. 458–464.
https://doi.org/10.3382/ps/pew341

Zhang, L., Chen, Y., Cheng, N., Xu, Y., Huang, K., Luo, Y., Wang, P., Duan, D., and Xu, W., Ultrasensitive detection of viable *Enterobacter sakazakii* by a continual cascade nanozyme biosensor, *Anal. Chem.*, 2017, vol. 89, pp. 10194–10200.
https://doi.org/10.1021/acs.analchem.7b01266

Zhao, Y., Jiang, X., Qu, Y., Pan, R., Pang, X., Jiang, Y., and Man, C., *Salmonella* detection in powdered dairy products using a novel molecular tool, *J. Dairy Sci.*, 2017, vol. 100, pp. 3480–3496.
https://doi.org/10.3168/jds.2016-12535

Zhou, B., Liang, T., Zhan, Z., Liu, R., Li, F., and Xu, H., Rapid and simultaneous quantification of viable *Escherichia coli* O157:H7 and *Salmonella* spp. in milk through multiplex real-time PCR, *J. Dairy Sci.*, 2017, vol. 100, pp. 8804–8813.
https://doi.org/10.3168/jds.2017-13362

Zi, C., Zeng, D., Ling, N., Dai, J., Xue, F., Jiang, Y., and Li, B., An improved assay for rapid detection of viable *Staphylococcus aureus* cells by incorporating surfactant and PMA treatments in qPCR, *BMC Microbiol.*, 2018, vol. 18, p. 132.
https://doi.org/10.1186/s12866-018-1273-x

Translated by D. Timchenko