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GenIUL, experts on viability PCR

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ABSTRACT

The standard PCR technique allows the rapid detection of microorganisms, difficult to detect by traditional culture methods. However, the control of samples through PCR has the drawback of false positives due to amplification of DNA/RNA of non-viable cells. This can be overcome by adding intercalating agents (EMA or PMA) to the sample, which selectively penetrates nonviable cells and blocks their DNA/RNA after a photo-activation process, preventing its subsequent amplification by PCR. This process is known as viability PCR (v-PCR). v-PCR has been used as an accurate, reliable and simple method to detect and quantify viable microorganism involved in environmental samples, food production process and clinical samples.

GenIUL provides the reagents (EMA and PMA) and the PhAST Blue photo-activation system needed to carry out v-PCR, ensuring optimum and uniform photochemical reaction of intercalating agents prior to PCR.

Keywords: PhAST Blue photo-activation system, GenIUL, Ethidium monoazide, Propidium monoazide, Viable cell detection, Viability PCR.

INTRODUCTION

Even though the advent of quantitative polymerase chain reaction (PCR) has improved the detection of pathogen microorganisms in most of areas of microbiology, a serious limitation of this method may arise from the inability to discriminate between viable and nonviable pathogens (Wang et al. 2006) and the overestimation of potentially viable (or infectious) biomass. Therefore, the inhibition of the nonviable cells detection by molecular methods based on nucleic acid amplification is one of the main interests in applied microbiology. After the first publication of a real-time PCR procedure using ethidium monoazide (EMA), this premise began to change (Nogva et al. 2003).

The utilization of selective nucleic acid intercalating dyes, like EMA and propidium monoazide (PMA), has been suggested as a means to reduce PCR signals from DNA originating from dead cells (Cawthorn et al. 2008, Nocker et al. 2006, Nogva et al. 2003, Rudi et al. 2005). Therefore, it is one of the most successful approaches to detect viable cells by PCR (herein called v-PCR).

V-PCR method uses membrane integrity as the criterion to differentiate between live and dead cells (Fig. 1). Theoretically, selective nucleic acid intercalating dyes should only penetrate into membrane compromised cells or dead cells. The presence of an azide group is believed to permit crosslinking of the dye to the DNA after exposure to strong visible light. Photolysis of EMA and PMA converts the azide group into a highly reactive nitrene radical, which can react with any organic molecule in its proximity, including DNA. In this bound state, DNA cannot be amplified by PCR (Nocker and Camper 2009; Rudi et al. 2005).

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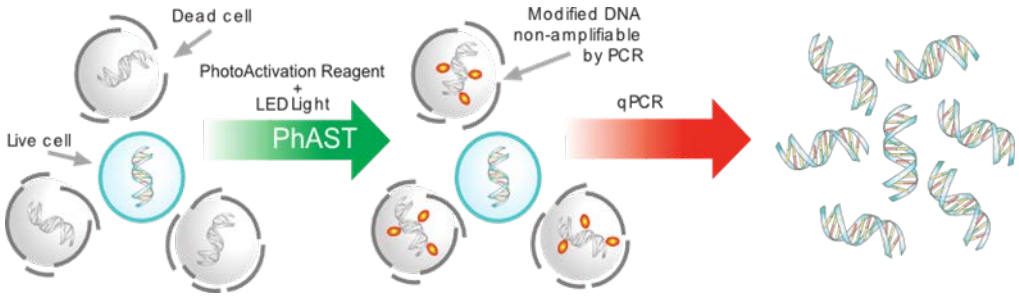
Figure 1. Outline of viability PCR process



Viability PCR is a promising technique because it makes use of the speed and sensitivity of the molecular detection while at the same time providing viability information. EMA and PMA have since their invention been applied to a wide variety of microorganisms including bacteria, fungi, protozoan, and viruses.

Moreover, in the detection and enumeration of microorganisms in clinical samples, environmental or food control, by molecular biology techniques it is crucial to differentiate live from dead cells. The assessment and understanding of microbial reality may have large biases and alter our conclusions. V-PCR technology will turn the applicability of genetic techniques and their applications in many fields (Table 1).

Table 1. Fields of application and strengths of viability-qPCR.

Application field	**Examples**	**Strengths**
Pathogen detection	• *Escherichia coli* • *Helicobacter pylori* • Acanthamoeba • *Staphylococcus aureus*	• Rapid detection method • Precise procedures adapted to each sample or microorganism • Assessment and understanding of microbial reality
Infectious disease evolution and susceptibility studies	• *Mycobacterium tuberculosis* • *Candida albicans*	• Feasible technique • Rapid diagnostic • Effective evaluation of disease evolution • Susceptibility testing method
Quality control and health risk assessment of environmental and food samples	• *Legionella pneumophila* • *Salmonella enterica* • *Clostridium perfringens* • *Listeria monocytogenes* • *Escherichia coli* O157:H7 • *Mycobacterium avium subsp. paratuberculosis*	• Rapid and inexpensive analytical tool • Effective evaluations of disinfection treatments • Screening of the efficacy of disinfectants • Adequate estimation of health risk in food and environmental samples
Infective Virus Detection	• Hepatitis A virus • Enteric virus • Bacteriophage T4 virus • RNA viruses	• Real significance of virus levels detection • Discrimination of infectious and non-infectious viruses

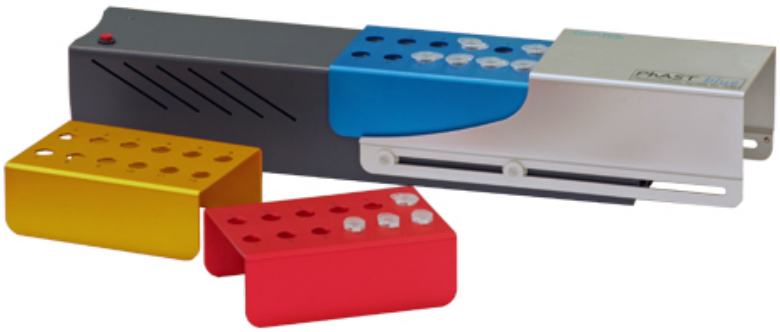
Microbial ecology	• *Escherichia coli* • *Enterococcus faecalis*	• Rapid and sensitive technique • Precise procedures adapted to each sample or microorganism • Improve the data on environmental exposures to several microorganisms • Enhances the validity of human risk assessment

PHAST BLUE THE SOLUTION FOR PRECISE PHOTO-ACTIVATION

Initial research with viability dyes employed photo-activation of viability dyes using high-power halogen lamps (500–750 W) developed for stage lighting. These lamps are currently still used and are functional for research purposes although different lamps differ in their efficiency for light activation, probably due to different emission spectra. A lamp (or more precisely the bulb) can be considered suitable if the spectrum has a high proportion of the wavelengths necessary for photo-activation. Absorption maxima are 456 nm for EMA and 464 nm for PMA. Samples are typically placed horizontally on ice with the most transparent side facing up at a distance of 20–30 cm from the light source. During exposure it is advised to mix samples by tilting the ice container to guarantee homogeneous exposure of the sample both to the dye and to the light. Ice can be seen to be advantageous both by preventing excessive heating of the samples (halogen lamps emit a substantial amount of heat) as well as by maximizing light exposure due to its reflective properties. A disadvantage of the procedure using halogen lamps can be seen, apart from the intense emission of heat, in experimental variation caused by the manual procedure. Differences in exposure or mixing efficiency can be expected to result in experimental variation and lower reproducibility between assays. Indeed it is difficult to cross-compare results from different studies given the wide variety of lamps and differences in exposure procedures.

An alternative to halogen lamps is the use of light-emitting diodes (LEDs), which were first introduced for this application by Vesper et al. (2008), using an array of LEDs to expose PMA-treated cells collected on a filter. Following the same concept, GenIUL company designed the PhAST Blue equipment, a LED-based instrument for light exposing cell suspensions (Fig. 2).

Figure 2. PhAST Blue equipment to GenIUL company



PhAST Blue instrument combines high power LED with the proper optical alignment of the reaction tube to ensure the maximum efficiency in the binding of the reagent to DNA.

Furthermore, PhAST Blue improves reproducibility and avoids variations due to manual photoactivation. The PhAST equipment is thermally stable with a constant and uniform light dose, and allows simultaneous photo-activation of 12 samples in a simple and efficient manner (Fig. 2).
Additionally, PhAST Blue allows optimizing the viable PCR method by programming different parameters such as light intensities and photo-activation times.
Its functionality was successfully demonstrated in studies by Agusti et al. (2010), Fittipaldi et al. (2010, 2011a), Elizaquivel et al. (2012), Miotto et al. (2012), Nkuipou-Kenfacka (2013), Rogers et al. (2013), Sanchez et al. (2012), Schnetzinger et al. (2013) and Vendrame et al. (2013). In comparison with halogen lamps, LEDs have a decisive advantage in emitting light of a defined specific wavelength (in this case in the blue range of the visible spectrum) allowing for optimal dye activation and avoiding the generation of heat.
The implementation of LED technology can thus be seen as an important step in the standardization of photoactivation.

FINAL REMARKS

- Viability PCR is a promising technique. It makes use of the speed and sensitivity of the molecular detection while at the same time providing viability information.
- GenIUL has developed PhAST Blue equipment able to optimize the viability PCR method.
- Results obtained with PhAST Blue equipment are comparable to results obtained using halogen lamps.
- PhAST Blue equipment offers greater convenience in the sample processing since the samples are not heated, it is not necessary to use ice, and avoids variations due to manual photo-activation.
- GenIUL is the unique company providing high-pure reagents for v-PCR, in the market

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