Frequently Asked Questions about GenIUL´s vPCR Portfolio

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1. What does the PhAST Blue or PAUL do?

The PhotoActivation System for Tubes - PhAST - and PhotoActivation Universal Light - PAUL System - can photo label the DNA/RNA of dead organisms through the photoactivation of a mix of photoactivable intercalating dyes with intact and non-intact membrane cells by using blue light.

Our PhAST Blue system allows simultaneous photoactivation of 12 samples in a simple and efficient manner.

Our PAUL system has been designed for working with other sample containers formats as filter membranes, 96 well plates or culture bottles.

2. What the PhAST Blue or PAUL doesn't do?

It is not a detection system by itself. Once the nucleic acid from dead microorganisms has reacted with EMA, PMA and PEMAX by photoactivation, a molecular analysis for the target detection needs to be performed e.g. PCR, qPCR, DGGE, pyro-sequencing, flow cytometry or fluorescence microscopy.

3. Why GenIUL's systems are the best option for reagents photoactivation?

Devices based on high power halogen lamps, have been successfully used despite of some serious limitation, e.g. sample overheat risk and non-homogeneous light dose.

Nowadays our systems offer the optimum light spectrum emission for the current vPCR reagents (464 to 476 nm). Additionally they allow better control of light dose without sample overheating. Moreover, by the means of its

specific software it is possible to have a total control of the photoactivation process parameters.

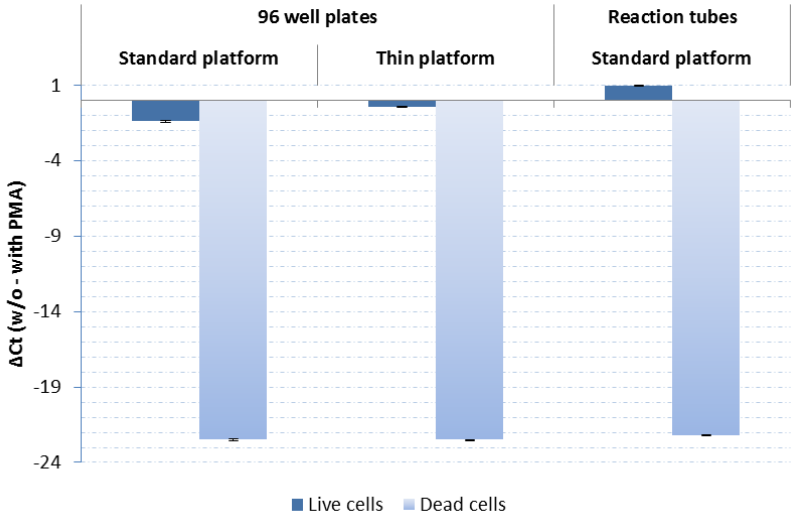
The use of our systems results in increases the workflow simplicity and reduces experimental variation.

4. Who has validated our photoactivation systems?

At this moment, at lot scientific papers have been published in indexed journals, where our systems were used. In our web page (News section), or in our Linkedin group (Viability PCR), you can find links to relevant scientific papers where our system was used.

5. Can PAUL system be used with microtubes like the PhAST Blue system?

Our PAUL system has been designed for working with sample containers formats such as filter membranes, 96 well plates or culture bottles, although the use of microtubes is also possible.



vPCR workflow with PAUL system: heat-killed and live P. aeruginosa cells (5x106 CFU/ml) with or without PMA (50 µM) treatment, exposed 15 min of photo-activation (100% light intensity) using GenIUL Reaction tubes or 96 well microplates (using standard or thin platform). Samples were incubated in the dark, at room temperature, during 10 min. Results are depicted as the ΔCt differences (without PMA-with PMA). Error bars represent standard deviations obtained from experiments performed in duplicate.

6. Is the PhAST Blue or PAUL light spectrum adequate for PEMAX/ PMA/ EMA photoactivation?

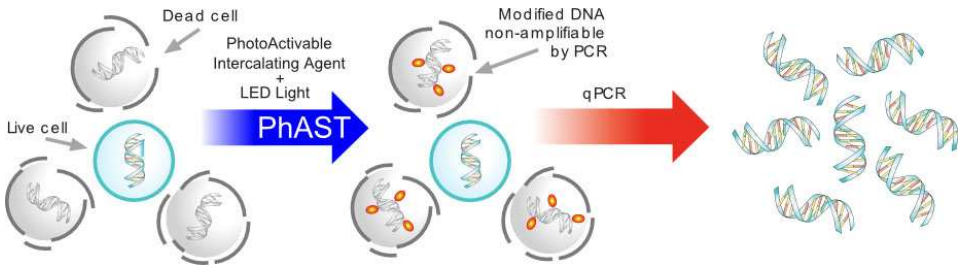
The dominant wavelength of the system is 464-476 nm. It is important to note that the maximum absorbance for EMA is 460 nm and for the photo-lysed EMA is 475 nm. In the same way, 470 nm is the optimum wavelength for the PEMAX and PMA photoactivation. So, our systems have a correct range of wavelength in order to reach good EMA/PEMAX/PMA-nucleic acids cross-linkage.

http://nar.oxfordjournals.org/content/5/12/4891.abstract

7. What is Viability PCR?

Viability PCR combines the use of photo-reactive reagents with a high affinity for DNA/RNA with a photo-chemical reaction. The nature of the reagents precludes it to pass through cell membranes. For this reason the DNA from cells with undamaged membrane will be free of photo blockage. After the treatment of microbial aqueous suspension with our reagents combined with a photoactivation step, only DNA from live microorganisms

will be detected by molecular procedures: PCR, qPCR, DGGE, Pyro-Sequencing…



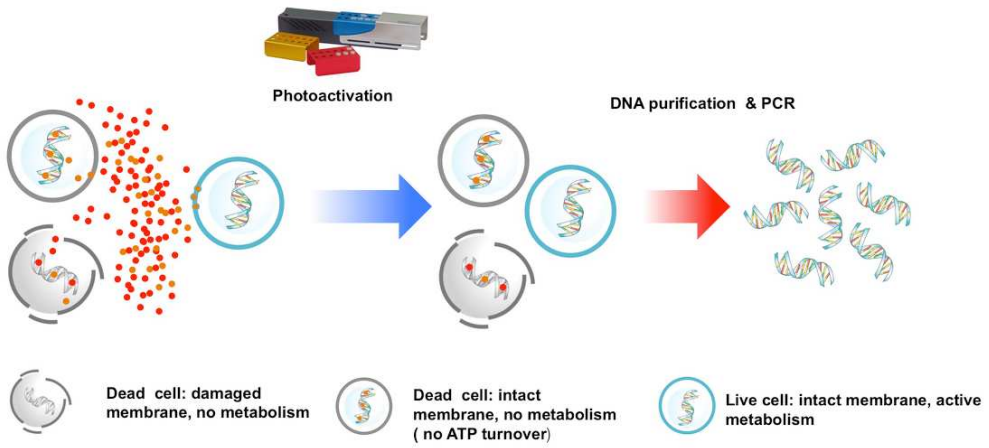
8. How can I prevent the amplification of DNA or RNA from dead microorganisms by using culture-independent methods?

A promising strategy is the use of nucleic acid intercalating dyes which can penetrate membrane damaged cells and are nearly completely cell membrane-impermeable, such as EMA, PMA and PEMAX in conjunction with molecular detection methods.

9. What is the scientific concept of the PEMAX Reagent in the viability PCR?

This new reagent, combined with the appropriate reaction buffer, extends the concept of viability PCR to cells with intact cell membrane structure but also with capability to actively maintain bacterial homeostasis, as a result of active metabolism.

Viability PCR uses cell membrane integrity to differentiate live cells from dead. This new approach improves viability PCR by enabling it to also discriminate between cells with an intact cell membrane and the ability to actively maintain bacterial homeostasis and cells that have an intact membrane but are metabolically inactive.



The PEMAX Reagent is a double dye technology patented and developed by GenIUL, S.L. in order to overcome the current limitations of viability PCR procedures.
Furthermore, the combination of PEMAX Reagent and vPCR buffers is a technology developed by GenIUL.

http://www.sciencedirect.com/science/article/pii/S0890850815000304

See our Doc. Code 450000101 for more information.

10. What does EMA, PMA and PEMAX stands for?

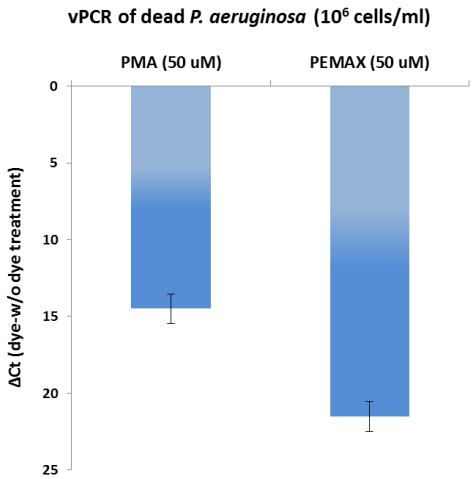
PEMAX is a GenIUL's trademark. PMA is the acronym for Propidium Monoazide and EMA is the acronym for Ethidium Monoazide.

11. What are HighPure - EMA?

This is our brand name for Ethidium Monoazide with a high purity (>95%). The performance of our reagents is specifically tested for viable PCR.

12. What is the performance of PEMAX Reagent in the viability PCR?

PEMAX dye is more effective than PMA to dim fluorescence PCR signals of dead cells, allowing better discrimination between live and dead cells, and thus improving the limitations of vPCR technology.



Representation of ΔCt values (Dye treated cells-untreated cells) in thermally inactivated (70 ºC, 30 min.) *P. aeruginosa* cells untreated or subjected to 50 ・M PMA or PEMAX dye and photo-activated for 15 min. using PhAST Blue equipment.

13. How many reactions can I do with 1 mg of PEMAX/PMA/EMA reagent?

Calculations of the number of assays will vary with the final concentration of dye in the sample and final reaction volumes used.

Usually, 0.5 mg of PEMAX or PMA reagent is dissolved in 500 ・l of water (molecular grade) to obtain a stock solution of 2 mM. Then, samples are treated with 25, 50 or 100 ・M of dye, depending on the sample. Treating samples (500 ・l) with 50 ・M of PEMAX or PMA dye (a commonly used procedure), with 1 mg of PEMAX/PMA dye can be treated up to 80 samples. For EMA reagent, 0.5 mg of dye is dissolved in 1064 ・l of DMSO 20% solution to obtain a stock solution of 1.25 mM. If 10 µl of the stock solution (1.25 mM) are added to 490 µl of the sample, the final concentration in the reaction will be 25 µM (a commonly used concentration). Taking into account this, 210 assays can be performed with 1 mg of EMA reagent.

14. What do means “best before” in our reagents?

Most of our reagents are quite stable if are correctly stored, up to now we have experimental data demonstrating their fully reactivity during the specified period. Probably they will be functional after this period however we don’t have complete evidences to support it.

15. Can the viability PCR concept be applied as molecular Biomarker?

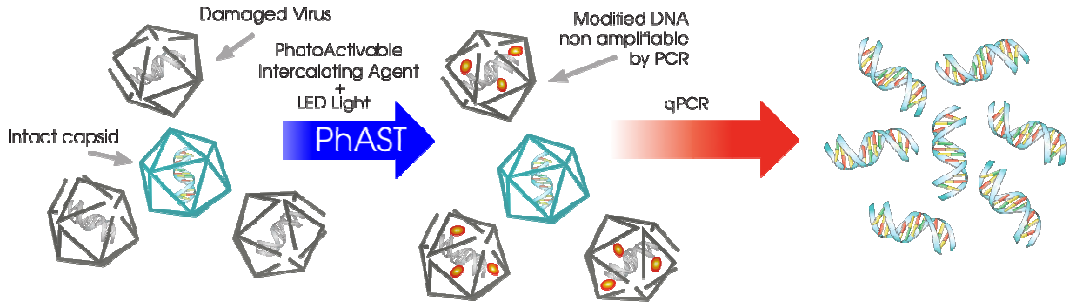
Yes, it can be applied; viability PCR has potential in monitoring bacterial load in sputum specimens and it has also a role as a biomarker of cure in TB treatment.
There is a scientific publication about this subject and in which the authors use our technology:

http://www.ncbi.nlm.nih.gov/pubmed/25534168

For this purpose, GenIUL has developed PEMAX Reagent monodoses (TBC-Biomarker kit) (Cat. No. 4900013025) suitable for use in *Mycobacterium tuberculosis* biomarker studies.

16. Can the viability PCR concept be applied for virus detection?

Yes, it can be applied, now by the means of viable PCR we will detect virus with undamaged capsid. After the treatment of microbial aqueous suspension with our reagents combined with a photoactivation step, only DNA/RNA from intact viral capsid will be detected by molecular procedures.

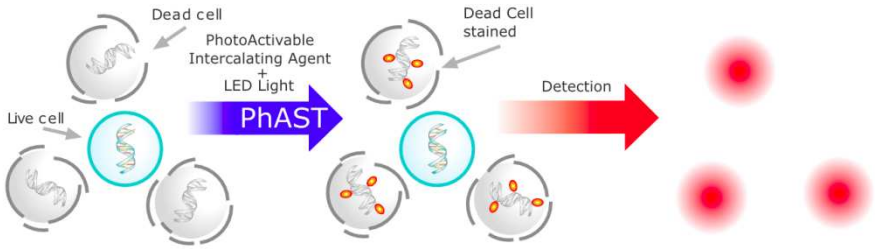


There exist different scientific publications about this subject. Some of them use our system:

http://www.ncbi.nlm.nih.gov/pubmed/20599560
http://www.ncbi.nlm.nih.gov/pubmed/20472736
http://www.ncbi.nlm.nih.gov/pubmed/21278473

17. Nucleic acids photo labeling can be used for purposes other than viability PCR?

Yes, this approach has been successfully used for decades for flow cytometry and fluorescence microscopy. In our web page you can find specific data sheets.



18. Is the EMA cytotoxic for live cells?

Although EMA shows higher cytotoxicity than PMA in viable cells, most of the times cytotoxic effects could be reduced or eliminated with bacterial specie dependence, if a right concentration is used. Because EMA shows more efficient penetration than PMA into membrane-compromised cells, lower EMA concentration should be used.

Moreover, the molecular weight of EMA is lower than PMA molecular weight; therefore if the same w/v concentration is used for both dyes, there will be more EMA present in the reaction.

Minimum amounts of EMA such as 2.5, 2.3, 1.5, and 1 µg/ml resulted be effective to suppress the DNA amplification from dead cells when Vibrio vulnificus (Wang and Levin, 2006), Legionella (Chen and Chang 2010), Bifidobacterium (Meng et al. 2010), and bacterial flora from fish fillet (Lee and Levin, 2009) were analyzed, respectively. For this reasons the recommended EMA concentration is ≤ 10 ・g/mL or ≤ 25 ・M.

http://www.ncbi.nlm.nih.gov/pubmed/15932774
http://www.ncbi.nlm.nih.gov/pubmed/20163500
http://www.ncbi.nlm.nih.gov/pubmed/20822571
http://www.ncbi.nlm.nih.gov/pubmed/18817818

On the other hand there exist different papers that demonstrate that EMA is not cytotoxic for yeast.
http://www.ncbi.nlm.nih.gov/pubmed/23132341
http://www.ncbi.nlm.nih.gov/pubmed/21036413

19. Are the PhAST Blue from GenIUL and the LED Active from IB-AS equivalent systems?

Yes, both systems are equivalent. PhAST Blue from GenIUL is the first evolution and new brand name of LED Active blue (From IB-applied Science, www.ib-as.com). PhAST Blue has some user friendliness and software improvements, but internally they share the same hardware design concept.

20. How does EMA/PMA/PEMAX combined with PhAST Blue or PAUL suppress nucleic acid amplification signal?

A simple sample pre-treatment using EMA, PMA and PEMAX previous to the molecular detection method, is necessary to only detect DNA from live or infectious microorganism. The pre-treatment comprises two steps: **Step one,** mixing of the EMA/PMA/PEMAX reagent with the sample and incubation in dark conditions, and **Step two,** sample photoactivation using our PhAST **Blue** instrument.

The nucleic acid intercalating dyes should only penetrate into membrane compromised cells or dead cells. The photolysis of EMA, PMA and PEMAX permits cross-linking of the dye to the DNA after exposure to strong visible light, and in this bound state, the DNA cannot be amplified by molecular procedures.

http://www.ncbi.nlm.nih.gov/pubmed/15691961
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1393219/

21. What is the scientific basis of this technique and our products?

The photo labeling properties of Azide Phenantridium derivatives were published several decades ago. From the 80's to today different applications have been published.

Flow Cytometry	http://www.ncbi.nlm.nih.gov/pubmed/2049970
Viability PCR	http://www.ncbi.nlm.nih.gov/pubmed/12703305
Fluorescence microscopy	http://www.ncbi.nlm.nih.gov/pubmed/11226693

22. What is the advisable amount of cells to work with viable PCR?

The optimum maximum amount of cells to work with viable PCR is around 10^6 cell/ml. However, an optimization step is always advisable.

http://www.ncbi.nlm.nih.gov/pubmed/22940102

23. What is the optimum incubation time?

The incubation of the sample with the reagent on darkness conditions may be critical for some applications. It is necessary to allow the reagent to entry in all the damaged membrane cells, including spores and protozoan cysts. For most purposes from 5 to 15 minutes may be satisfactory. In some cases longer incubation times are needed, for example, 30 minutes for protozoan cyst.

24. Is the incubation temperature important?

Yes, incubation temperature is important for several reasons:

- Cellular membrane fluidity is strongly influenced by temperature. The active ingredients of our reagents (Specially EMA) are quite soluble in hydrophobic solvents, so they have some ability to interact with cell membranes. At lower temperature, cell membranes are less fluid, so its specific interaction with the reagent and the subsequent diffusion to cytoplasm is greatly reduced.

- Constant temperature incubation minimizes result variability.

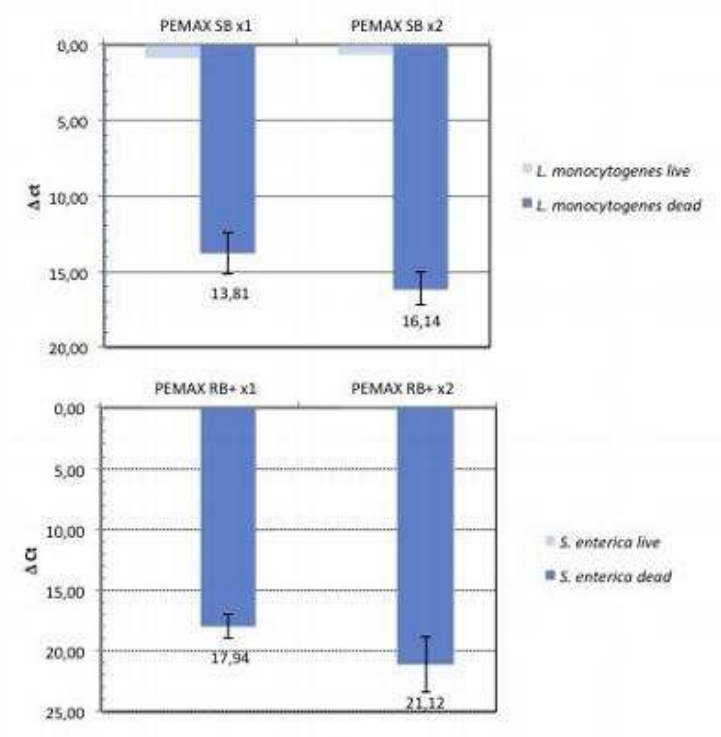
- Recent papers suggest that in some cases high temperatures incubation could improve PMA diffusion to dead cells and improve the treatment performance.

http://www.ncbi.nlm.nih.gov/pubmed/23389080

GenIUL has designed the **Dark Box system** (Cat. No. 90001200), in order to protect samples from light and to ensuring constant temperature incubation.

25. What is the optimum light exposure time?

The times required for optimal assay performance, reported in the literature, are in the range between 2 and 20 min in a unique exposure treatment (1x). Using our PhAST Blue and PAUL devices 15 min has been recommended to date. In addition, recent studies by our R+D team showed that the double photoactivation procedure (2x) (15 min light + 10 min dark+ 15 min light), at 100% intensity, can increase the signal reduction.



26. If I work with PhAST Blue, are the tube materials important?

The optimal performance of photochemistry reactions is highly related with the amount of light received by the sample.
For this reason we recommend our reaction tubes (Cat. No. 4900019000) which shows a very high light transmittance rate.

27. How can I handle liquid waste with viability dyes?

Our **D-Bag Holder** (Cat. No. 900099645) is a safe an easy system for managing these liquid wastes.

Visit our web page for more information.

28. If the treatment is not 100% effective, will I have false positive?

A treatment with a 99.9% effectiveness in a sample with $1{\cdot}10^5$ dead cells may still contain the DNA of 100 dead cells. Therefore, in some cases, for some techniques with high sensitivity such as nested PCR or real-time PCR, false positive results can be obtained.

For this reason, some authors recommend a result analysis based on relative rather than absolute values.
http://www.ncbi.nlm.nih.gov/pubmed/20632000

As stated below there exist different approaches for optimizing vPCR procedures, one of the more promising is to enhancing the reaction by the means of specific **reaction buffers.** In the current GenIUL portfolio you can find **reaction buffers** for specific microorganisms. For more information see our Doc. Code 450000062.

Despite many efforts, even using well-optimized viability PCR procedures and in spite of the expected full neutralization of nucleic acids of dead microorganisms, sometimes it is not unusual to obtain a partial signal reduction. In this sense, recent researches carried out by our R+D team demonstrated that a fraction of DNA remains inaccessible to viability PCR due to their interaction with the tube wall and in turns generates false positive results.

http://www.geniul.com/en/support/guides/62-understanding-the-tube-contribution-in-vpcr-results/file

In this direction, a change of the sample tube before the photoactivation step results in the largest signal reduction in dead cells and represents one of the best improvements in minimizing false-positive signal.

http://www.ncbi.nlm.nih.gov/pubmed/28175959

We recommend, as a rule of thumb, include the tube change approach and double light treatment in your vPCR workflow, in order to obtain a complete signal reduction.

29. There exist some practical guidelines for viability PCR procedures optimization?

Yes, you can find a complete review in the next link:

http://www.ncbi.nlm.nih.gov/pubmed/22940102

30. Does the viability PCR works well with complex samples?

The viability PCR technique has been used with complex samples such as fecal samples (1) and environmental samples with high turbidity levels (1, 2, 3).

(1) http://aem.asm.org/cgi/content/full/75/9/2940.
(2) http://www.ncbi.nlm.nih.gov/pubmed/19153730
(3) http://www.ncbi.nlm.nih.gov/pubmed/20024544

31. Can be used samples containing viability dyes in a direct PCR method?

We don't test the impact of PMA/EMA/PEMAX reagents for direct PCR methods from other brands. If you wish to do a simple DNA purification method and avoid PCR inhibition, you can use our vDNA Reagent (Cat. No. 4900014000) and vDNA Buffer (Cat. No. 4900014001) products.

vDNA Reagent provides a fast and easy genomic DNA extraction procedure, enabling very high DNA recovery rates from samples. This product is able to retain the viability PCR dyes providing a DNA suspension free of PCR inhibitors. It can be used from simple cell pellets up to complex samples as wastewater, soil and stool.

http://www.geniul.com/en/support/download-technical-guides/v-dna-reagent-documents/48-v-dna-reagent-note/file

32. And if I have more questions?

Please if you have any questions do not hesitate to contact us.

http://www.geniul.com/ca/contact-us/contact.html
http://www.linkedin.com/groups/Viability-PCR-4613707

33. Patent Rights

Our products and systems are not a detection technology. Our PhAST Blue instrument is covered by patent, which covers the optimized design and its use in bio molecules photo labeling. As stated in different points of this document, nucleic acid photo labeling can be used for multiple purposes in microbiology and cell biology, as starting point of different methods and test.

Some applications, in which Propidium Monoazide, Ethidium Monoazide can be used, may be covered by patents issued and applicable in the United States, Japan and certain other countries.

The use of PEMAX Reagent for vPCR, is covered by the patent pending request belonging to GenIUL. The customers that received this product can use it for research and evaluation purposes without infringing our intellectual property rights.