

February 2014

BLU-V[®] Viability PMA Kit Handbook

For live-dead cell differentiation of microbiological organisms
in water, food, or environmental samples using real-time PCR



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Kit Contents

BLU-V Viability PMA Kit	
Catalogue no.	296015
PMA Reagent	2 x 0.7 mg
Buffer EB	55 ml
RNase-Free Water	1.9 ml
Quick-Start Protocol	1

Storage

PMA Reagent is shipped at room temperature. It should be stored at 2–8°C, upon delivery. The PMA reagent can be stored under these conditions until the expiration date on the tube without showing any reduction in performance. For information on the correct reconstitution of the PMA Reagent, see page 10. Buffer EB can be stored at room temperature.

Intended Use

The BLU-V Viability PMA Kit is intended for molecular biology applications. The product is not intended for the diagnosis, prevention, or treatment of a disease. The BLU-V Viability PMA Kit permits the discrimination between live and dead cell microorganisms to be determined quickly, sensitively, and reliably.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

24-hour emergency information

Chemical emergency or accident assistance is available 24 hours a day from:
CHEMTREC

USA & Canada ■ Tel: 1-800-424-9300

Outside USA & Canada ■ Tel: +1-703-527-3887 (collect calls accepted)

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of BLU-V PMA Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

Viability PCR is an innovative technology that allows differentiation between live and dead microorganisms, based on the loss of cell membrane integrity in dead cells. QIAGEN's viability PCR system uses the DNA-intercalating agent propidium monoazide (PMA). PMA is a chemical molecule that is applied in a constantly growing range of molecular biology applications for the differentiation of live and dead microorganisms at the DNA level. After photoactivation with a defined wavelength, PMA intercalates and binds covalently to DNA. Subsequent amplification of the modified DNA is inhibited, resulting in a reduction in the amplification signal.

PMA is unable to pass through intact biological membranes. This means that DNA from living microorganisms, which have intact membranes, is protected from PMA modifications and is therefore detectable by PCR. Membranes of dead microorganisms lose their protective functionality, and PMA can enter these cells and modify their DNA. The modified DNA of dead microorganisms is inhibited from detection by PCR, resulting in a reduced amplification signal.

PMA carries a strong positive charge that ensures it is unable to pass through intact live cell membranes. It also has an anchor group that binds irreversibly to DNA upon activation with light of a defined wavelength. These special features make PMA superior to other DNA intercalating dyes and the basis for QIAGEN's BLU-V Viability Portfolio.

For an overview of the PMA Kit components and the workflow, please refer to Appendix A, page 19.

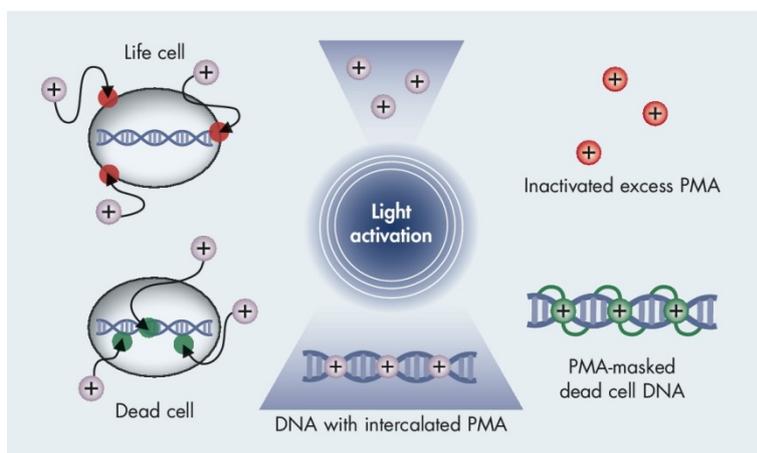


Figure 1. Schematic representation of viability PCR.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

General equipment and reagents

- Pipets (adjustable)*
- Sterile pipet tips with filters
- Tube racks

PMA treatment

- SafeSeal Micro Tubes 2 ml (Sarstedt cat. no. 72.695)
- Vortexer*
- Microcentrifuge with rotor for 2 ml tubes*
- Thermomixer, or shaking water bath for heating 2 ml tubes at 70°C*

DNA purification kit

QIAGEN offers multiple different DNA sample preparation kits. The PMA workflow is designed to function optimally in combination with all QIAGEN extraction chemistries. The choice of kit depends on the sample material and sample throughput. The following table gives an overview of the DNA purification kits, which are well suited for extraction of microbial DNA from various sources.

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Table 1. DNA purification kits.

Sample type	Low-throughput (manual or automated)	Medium to high- throughput (automated)
Bacterial DNA from culture, blood and tissue	DNeasy® Blood and Tissue (cat. no. 69504 or 69506)	EZ1® DNA Tissue Kit (cat. no. 953034)
Microbial DNA from whole blood, swabs, cultures, and body fluids	QIAamp® UCP Pathogen Mini (cat. no. 50214)	
Microbial DNA from whole blood, plasma, serum, buffy coat, lymphocytes, dried blood spots, body fluids, cultured cells, swabs, and tissue	QIAamp DNA Mini Kit (cat. no. 51304 or 51306)	QIAsymphony DNA Mini Kit (192)(cat. no. 931236)
Bacterial DNA from animal whole blood, serum, plasma, other body fluids, swabs and washes, and tissue	QIAamp <i>cador</i> ® Pathogen Mini (cat. no. 54104, or 54106)	
Bacterial DNA from food and environmental samples		QIAsymphony® <i>mericon</i> Bacteria Kit (cat. no. 93115)
High-molecular-weight DNA from blood, tissue, and bacteria using a simple, magnetic bead-based protocol	MagAttract® HMW DNA Kit (cat. no. 67563)	

For difficult-to-lyse pathogens, such as fungi, and also spores, a mechanical lysis is recommended to carry out an additional mechanical (pre-) lysis step prior to the mainly enzymatic lysis procedures of the extraction kit protocols. During mechanical lysis, the cells are burst open by bead-beating with glass beads. The force of the mechanical lysis increases the yield of DNA and thereby the success

rate of real-time PCR. In order to perform standardized mechanical lysis, the following equipment is required:

- Pathogen Lysis Tubes L (cat. no. 19092)
- A vortexer adapter plate for horizontal or vertical fixation of 2 ml screw cap tubes (e.g., Shaking Plate [P509.1] with vortex adapter [P510.1] available from Carl Roth may be used)

Real-time PCR equipment and reagents

- Real-time PCR Buffer, DNA polymerase, nucleotides or a ready-to-use real-time PCR kit, for example, QuantiFast® Pathogen PCR +IC Kit (QIAGEN GmbH, cat. no. 211352) or QuantiNova Probe PCR Kit (QIAGEN GmbH, cat. no. 208252 or 208254)
- *mericon* Kits for Pathogen detection in food or animal feed samples (e.g., *mericon* Salmonella spp. Kit (see ordering information, page 25)
- Primers and TaqMan® probes for detection of the microbiological target from an established oligonucleotide manufacturer. Primers should be of standard quality or high performance liquid chromatography (HPLC) pure, and probes should be highly purified, by for example, HPLC. Lyophilized primers and probes should be dissolved in TE buffer to provide a stock solution of 100 µM. Primer and probe stock solutions should be stored in aliquots at –20°C. For additional information, see “Recommendations for the real-time PCR assay”, page 22)
- Probe stock solutions should be protected from exposure to light. Typically, the target specific probe should be labeled with FAM™ and a non-fluorescent quencher.
- DNase-free consumables. Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive detection of nucleic acids.
- Rotor-Gene® Q or other real-time PCR instrument*
- PCR plastics for the thermal cycler to be used
- For Rotor-Gene Q: Strip Tubes and Caps, 0.1 ml, for use with 72-well rotor (cat. no. 981103 or 981106)
- For Rotor-Gene Q: Loading Block 72 x 0.1 ml Tubes (cat. no. 9018901) or Loading Block 96 x 0.2 ml Tubes (cat. no. 9018905)

* Ensure that instruments have been checked and calibrated according to the manufacturer’s recommendations.

Important Notes

The user should always pay attention to the following:

- Use gloves, as well as sterile pipet tips with filters.
- All materials and media possibly containing the tested target organism should be autoclaved for 20 min at 120°C.
- Store and extract positive materials (specimens, positive controls, and amplicons) separately from all other reagents, and add them to the reaction mix in a spatially separated facility.
- Equilibrate all cooled or frozen components thoroughly to room temperature (15–25°C) before starting the workflow.
- For PCR chemicals: When thawed, mix the components (by pipetting repeatedly up and down or by pulse vortexing) and centrifuge briefly.

Preparation of PMA reagent

PMA reagent

Add 550 µl RNase-Free water to each vial of PMA reagent, to obtain a 2.5 mM PMA solution. Mix by pipetting up and down 5 times or vortexing the tube for 4–6 sec and centrifuge briefly. PMA is a photo-reactive reagent. Store the reconstituted reagent only in light non-transparent tubes to avoid light exposure.

Relevant assay controls

No PMA control sample

The No PMA control sample is implemented into each experiment (for each target pathogen and/or each matrix) to assess the DNA masking effect of PMA on the test samples. When test samples are prepared from the original sample solution, containing a concentrated mixture of live and dead cells of the target organism, one sample is retained to serve as the No PMA control sample. It is set up as a normal test sample and runs through the entire workflow with the only difference being that it is not treated with the PMA reagent.

Dead cell control samples

In order to verify and visualize the maximum PMA masking effect for the respective sample type, two separate samples are set up as dead cell control samples. Pathogens contained in these samples are heat-killed for 10 minutes at 70°C and exposed to permanent shaking on a thermoshaker. One heat-treated

sample runs through the entire workflow with PMA being added. The other sample runs through the entire workflow without PMA being added. The latter representing the heat-treated analogue of the regular No PMA Control.

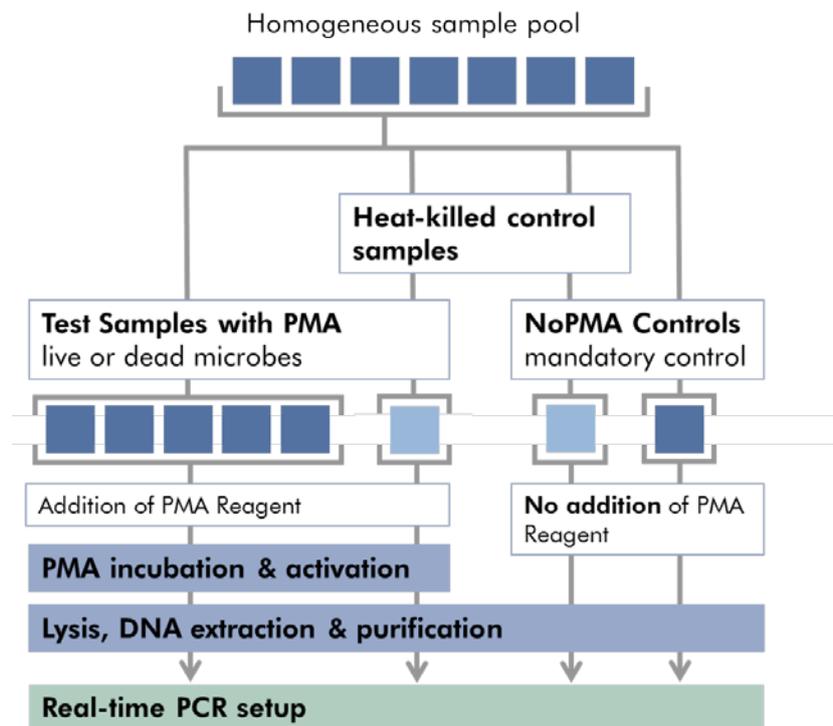


Figure 2. Schematic of required control samples.

Successful purification and amplification of the No PMA control should result in a C_T difference of >6 between the amplification of the heat-killed and PMA-treated sample and the heat-killed No PMA control sample (when Buffer EB is used). The real-time PCR signal of the No PMA control samples therefore serves as the necessary point of comparison to observe the PMA-induced signal shift.

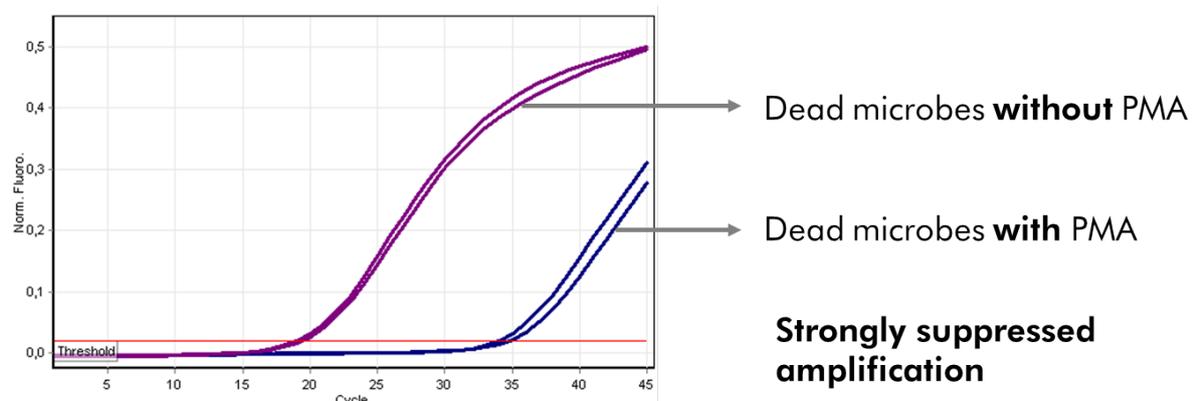


Figure 3. Example amplification plot of maximum PMA masking efficiency.

Note: The above described C_T specifications only apply for a PMA reaction using Buffer EB. Application of a different buffer may lead to a different C_T value shift in the subsequent PCR reaction.

Handling different sample types

The BLU-V Viability PMA Kit is designed as a versatile and flexible tool. The described workflow is suitable for the specific demands of a broad range of different microbiological target organisms such as bacteria, fungi, and yeasts. The kit contains all necessary consumables to carry out the PMA treatment and can easily be combined with a QIAGEN sample preparation kit and QIAGEN real-time PCR Probe assay consumables.

Nevertheless, different target organisms and background matrices have different influences on the performance of the BLU-V Viability PMA Kit.

Small adjustments can be easily conducted by the user in order to optimize the PMA reaction with respect to a specific sample type or target pathogen. The following chapters offer advice and support for specific workflow steps which are likely parameters for individual adjustments.

Sample pre-treatment

Depending on the background matrix, the sample solution used as a starting material might have characteristics, which can impair the efficiency of the initial sample handling and/or PMA treatment procedures. Although most background components will be removed by the first centrifugation step of the protocol, matrix particles can remain in the pellet. Depending on the matrix, this can occur to a greater or lesser extent. Very viscous and/or turbid samples represent highly demanding sample types. While sample viscosity mainly influences the initial sample handling, sample turbidity influences the efficiency of the PMA treatment.

Sample turbidity strongly interferes with the PMA light activation step as solid matrix components, distributed in the sample solution, hinder homogeneous sample illumination. In the worst case scenario, only sample layers exposed at the outer surface of the sample tube are efficiently illuminated while the sample solution in the middle of the tube remains only insufficiently illuminated. Optimal light permeation of the sample is crucial for an overall activation of the PMA reagent. Sample mixing by vortexing the sample tubes every 2 minutes (as recommended in the protocol) supports a homogeneous sample illumination, although this is heavily influenced by the degree of turbidity.

In case of strongly turbid samples, filtration or dilution of the starting material is recommended. If the sample has already been centrifuged and re-suspended in

Buffer EB (protocol step 8), further dilution of the sample in Buffer EB is recommended. The dilution factor depends on the respective sample. However, for the PMA light activation step the sample solution needs to be fairly transparent although not completely clear.

For viscous samples the same approach applies as for turbid samples. Dilution of the starting material up to a suitable degree of viscosity is recommended.

PMA reaction buffers

The overall efficiency of the PMA reagent can be modulated on the basis of the buffer, in which the PMA masking reaction is carried out. Depending on the reaction buffer, permeation of the PMA reagent into dead cells can be enhanced, resulting in stronger signal reduction in the subsequent PCR reaction.

To choose a suitable PMA reaction buffer, its ability to improve PMA accumulation in dead cells, but also its compatibility with live cells need to be balanced. In the BLU-V Viability PMA Kit, Buffer EB is provided as an optimal PMA reaction buffer. It significantly enhances PMA efficiency while exerting only minimal effects on a range of tested live organisms. However, in case Buffer EB proves to be incompatible with a specific target organism, a different PMA reaction buffer needs to be applied. Recommended alternatives to Buffer EB include well established buffer solutions:

- Pathogen specific growth media
- PBS buffer
- Physiological NaCl-solution

Please note that although these buffers have proven to be suitable reaction buffers for the PMA masking reaction, they might not exhibit the same PMA efficiency increase as achieved with Buffer EB. Pathogen specific growth media have shown to have a stable but sometimes only minor performance in supporting the PMA masking efficiency.

PMA concentration

The effect of PMA partially depends on the respective target organisms. The default PMA concentration of 50 μM was found to be optimal for *Salmonella enterica*, *Listeria monocytogenes*, and *Campylobacter jejuni*. Experiences with other microorganisms showed that small alterations of the PMA concentration can in some cases increase the PMA masking efficiency without suffering cytotoxic side effects of the reagent. Therefore, PMA concentrations of 10–100 μM /reaction should be assessed when adjusting the PMA workflow to specific target organisms.

Table 2. PMA concentrations and respective volumes for 500 μ l sample volume

Final PMA conc. (μM)	Volume PMA stock solution (μl)	Volume H₂O (μl)	Applied volume of mixture (μl/rxn)
10	10	40	10
20	20	30	10
30	30	20	10
40	40	10	10
50	10	–	10
60	12	–	12
70	14	–	14
80	16	–	16
90	18	–	18
100	20	–	20

Protocol: Selective Masking of DNA from Dead Organisms with PMA Reagent

Important points before starting

- If working with infectious pathogens, the protocol and subsequent sample analysis via PCR must be carried out in a suitable laboratory area, designated for working with infectious pathogens.

Things to do before starting

- Reconstitute the PMA reagent according to the instructions on page 10.
- Keep the reconstituted PMA reagent protected from light until its application in protocol step 9.

Procedure 1 — preparation of workflow controls

1. Prepare two 2 ml SafeSeal Micro Tubes with 1 ml of sample solution for each homogenous sample pool for the dead cell control samples.
2. Close the tubes, label one tube as “Dead + PMA” and the other tube as “Dead No PMA”.
3. Heat-kill the bacteria for 10 min at 70°C in a thermomixer, or shaking water bath, and let them cool to room temperature.
4. Treat the tube “Dead + PMA” according to the normal workflow; treat the tube “Dead No PMA” according to the descriptions for the “No PMA control sample”. This dead cell control will show the maximum capacity of PMA masking.

Procedure 2 — processing of test samples

5. Add 1 ml of sample solution containing a concentrated mixture of live and dead target organism into a 2 ml SafeSeal Micro Tube.
6. Centrifuge the tube for 5 min at 13,000 x g.

Note: For each experiment, implement at least one additional sample as a No PMA control sample. The No PMA control sample runs through the entire workflow but is not treated with the PMA reagent to serve as an unmasked C_T control in the subsequent real-time PCR reaction (see page 11). The C_T value of the No PMA control sample provides a point of comparison to determine the PMA induced signal shift indicating the presence of dead microorganisms.

7. Remove and discard the supernatant.

Note: For fast and efficient disposal of the supernatant, carefully decant the supernatant into a suitable waste container and softly tap the empty tube onto an absorbent tissue afterwards to remove any drops hanging at the rim of the tube.

8. Add 500 µl of Buffer EB to the pellet of the target organism, close the cap of the tube, and resuspend the pellet by brief, vigorous vortexing.

Note: Buffer EB enhances the sensitivity of dead organisms to the PMA reagent, thereby efficiently supporting the overall PMA masking reaction. It was shown to be compatible with a range of different microbiological organisms, exerting minimal effects on live cells. In case Buffer EB proves to be incompatible with a specific target organism, a different PMA reaction buffer has to be applied. For information on the application of alternative PMA reaction buffers, see page 13.

9. Add 10 µl of the reconstituted PMA reagent to the samples. Do not add PMA reagent to the No PMA control sample (see step 1) and the heat-killed sample labeled "Dead No PMA". Tightly cap the tubes, and mix by vortexing or inverting the tubes several times.

Note: To ensure exposition of all samples to the PMA reagent for the same amount of time, it is recommended to pipet the reconstituted PMA reagent into the caps of the 2 ml SafeSeal Micro Tube. Even when the cap is closed, the 10 µl drop will remain in the cap. To mix the reaction solution, invert all sample tube simultaneously 3–4 times and vortex. This ensures contact of the PMA reagent with all samples at the same time.

10. Place the tubes into the sample positions of the Incubation Box, close the lid, and incubate the mixtures for 10 min at room temperature in the dark.

Note: PMA is a photo-reactive reagent which is already partially activated at the exposure to normal day light or laboratory lamp light. To avoid nonspecific reactions of the PMA reagent with random sample components, ensure a fast transfer of the PMA containing sample solution to the dark incubation box and allow only minimal light exposure.

11. After incubation, transfer the sample tubes to the sample positions of the BLU-V System and illuminate the sample-PMA solution for 10 min. Vortex the sample tubes within the BLU-V System approximately every 2 min to allow a homogeneous sample illumination.

Note: Light intensity, as well as illumination time, can be manually set for the PMA light activation step using the buttons of BLU-V System display. For standard illumination settings, keep the light intensity at 100%. The illumination will stop automatically after the set time. During illumination the

time is displayed as a minute count-up to simplify sample mixing at regular 2 min intervals. For safety reasons, illumination and time count will stop whenever the lid is lifted and will switch on again when the lid is closed.

Homogeneous light penetration is crucial for a quantitative PMA masking reaction. For strongly turbid samples, start the overall protocol from a diluted sample to improve the overall sample light permeability during the described illumination step (see page 12).

Mixing samples more often during the illumination also supports the overall PMA masking efficiency in turbid samples.

12. Following light treatment, centrifuge at 13,000 x g for 5 min and discard the supernatant.

Note: For fast and efficient disposal of the supernatant, carefully decant the supernatant into a suitable waste container and softly tap the empty tube onto an absorbent tissue afterwards to remove any drops hanging at the rim of the tube.

13. If you want to continue with the DNA purification using the QIAamp UCP Pathogen Mini Kit, QIAamp *cador* Pathogen Mini Kit, or the QIASymphony *mericon* Bacteria Kit, continue with step 14. If you want to continue with the DNA purification using the DNeasy Blood & Tissue Kit, the QIAamp DNA Mini Kit, QIASymphony DNA Mini Kit or EZ1 DNA Tissue Kit, continue with step 16.

14. Add 500 µl of Buffer EB to the pellet of the target organism, tightly cap the tube, and resuspend the pellet by brief, vigorous vortexing.

15. Continue according to the *QIAamp UCP Pathogen Mini Handbook*, page 26 (Pretreatment of Microbial DNA from Biological Fluids or Cultures), *QIAamp *cador* Pathogen Mini Handbook*, page 24 (Pretreatment B2 — for Difficult-to-Lyse Bacteria in Cell-Free Fluids), or the *QIASymphony mericon Bacteria Handbook* (*mericon Automated Pathogen Detection Workflow Handbook*, page 20).

16. Continue with the respective protocol for the DNeasy Blood & Tissue Kit, page 44 (Gram-negative bacteria) or page 45 (Gram-positive bacteria), QIAamp DNA Mini Kit, page 55 (Appendix D: Protocols for Bacteria), MagAttract HMW DNA Kit, page 22 (Gram-negative bacteria) or page 25 (Gram-positive bacteria), QIASymphony DNA Mini Kit (QIASymphony SP Protocol Sheet: *Tissue_LC_200_V7_DSP* and *Tissue_HC_200_V7_DSP*, page 11 for Gram-negative bacteria or page 12 for Gram-positive bacteria), or EZ1 DNA Tissue Kit, page 39 (Purification of DNA from Bacterial Culture Samples).

Troubleshooting Guide

The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Appendix A: BLU-V Viability PMA Kit Components and Workflow Overview

PMA Reagent

Propidium monoazide (PMA) is a photo activated chemical compound that is implemented extensively in molecular biology applications as a technology for live-dead cell differentiation in microbiological organisms.

In the BLU-V Viability PMA Kit, the PMA Reagent is conveniently provided as a lyophilized solid. For its application in the live-dead cell differentiation workflow, it is reconstituted in water to yield a ready-to-use working solution. PMA is activated upon illumination with light of a suitable wavelength. Standardized illumination of PMA treated sample solutions is performed using the BLU-V System. A small table top illumination device providing an efficient instrument to subject samples to a defined dose of PMA activating light.

Buffer EB

Buffer EB is supplied within the BLU-V Viability PMA Kit as a standard PMA reaction buffer. It notably enhances PMA efficiency for a range of different sample types, while exerting only minimal effects on live organisms.

Overview of the PMA live-dead cell differentiation workflow

The general setup of the QIAGEN PMA live-dead cell differentiation workflow is illustrated in Figure 1, page 6. The workflow consists of 4 main segments, which include sample pre-treatment (steps 1– 3), PMA treatment (steps 4– 6), DNA extraction (step 7), and real-time PCR (step 8).

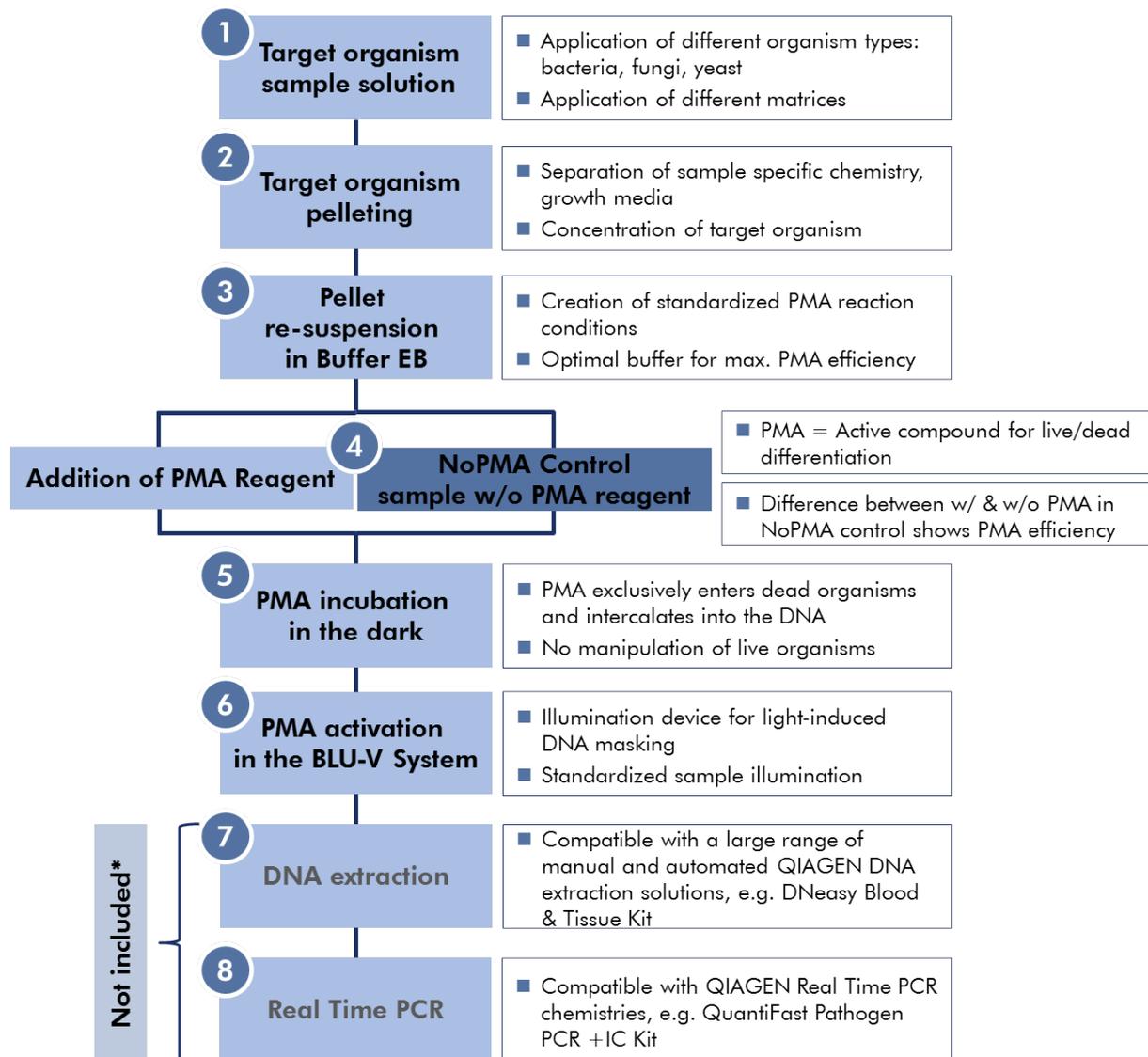


Figure 4. Overview of the steps in the recommended PMA live-dead cell differentiation workflow.

* Please choose from a range of workflow optimized manual and automated QIAGEN DNA extraction and real-time PCR solutions.

The following section provides a short description of each step and explains the different workflow segments.

- Step 1: As starting material, a solution containing a mixture of live and dead cells of the microbiological target organism is applied. The starting material can, for example, be an environmental swab sample or a contaminated water sample. The workflow was optimized to be applicable for a diverse range of starting materials.
- Step 2: To separate most of the background matrix and free DNA from the target organism, and to concentrate the target organisms, the starting solution is centrifuged.
- Step 3: The pelleted target organisms are resuspended in Buffer EB. Buffer EB is shown to enhance the sensitivity of dead cells towards the PMA reagent, supporting the overall DNA masking reaction.
- Step 4: PMA is added to all samples. At least one sample per matrix is retained, which serves as the No PMA control sample. The real-time PCR signal of this sample is needed to verify the above mentioned signal shift expected to result from the PMA treated workflow control.
- Step 5: To allow the PMA reagent to efficiently enter dead cells, incubation at room temperature of PMA treated samples for 10 min is carried out. To avoid premature PMA activation, following exposure of the samples to day light, the incubation has to be carried out in the dark.
- Step 6: PMA activation to initiate the DNA masking reaction is achieved by standardized light treatment in the BLU-V System illumination device. A defined light dose of a suitable wavelength is applied to all samples for 10 min to ensure complete PMA masking.

Continue with reagents not included in the kit:

- Step 7: Following PMA treatment, the masked or unmasked DNA of live and dead target organisms is extracted. The PMA protocol was designed to work optimally with QIAGEN DNA extractions chemistries. For the most suitable DNA extraction protocol, please choose from a wide range of manual or automated DNA sample preparation kits by QIAGEN.
- Step 8: The purified DNA is amplified with a probe-based real-time PCR assay, specific for the target organism. Usage of QIAGEN's real-time PCR chemistries, for example, the QuantiFast Pathogen PCR +IC Kit completes the harmonized PMA detection workflow.

Appendix B: Recommendations for the Real-Time PCR Assay

Assay design

Guidelines for the optimal design of primers and probes are given below. It is particularly important to minimize non-specific annealing of primers and probes. This can be achieved through careful assay design.

- Use specialized design software (e.g., Primer Express® Software) to design primers and probes
- All assays should be designed using the same settings to ensure that they will work optimally under the same cycling conditions (60°C annealing/extension)
- T_m of all primers should be 58–62°C and within 2°C of each other
- T_m of probes should be 8–10°C higher than the T_m of the primers
- Avoid a guanidine at the 5' end of probes, next to the reporter, since this causes quenching
- Avoid runs of 4 or more of the same nucleotide, especially of guanidine
- Choose the binding strand in a way that the probe has more C than G bases
- Primer length: 18–30 nucleotides
- GC content of primers: 30–70%.
- Check that primers and probes are not complementary to each other
- Try to avoid highly repetitive sequences
- Avoid complementarity of 2 or 3 bases at the 3' ends of primer pairs to minimize primer dimer formation
- Avoid mismatches between the 3' end of primers and the template sequence
- Avoid runs of 3 or more Gs and/or Cs at the 3' end
- Avoid complementary sequences within a primer sequence and between the primer pair
- We recommend an amplicon length of 250–1600 base pairs, PMA masking efficiency increases with amplicon length, because the likelihood of PMA binding is higher

- The probe should be labeled at the 5'-end with a fluorescent dye like 6-FAM and quenched at the 3'-end with a non-fluorescent quencher, for example, BHQ[®]1
- Always check the specificity of primers by performing a BLAST search (www.ncbi.nlm.nih.gov/blast). Ensure that primer sequences are unique for your template sequence

Table 3. Standard PCR Cycling for long amplicon sizes

Step	Time	Temperature	Additional comments
Initial PCR activation	5 min	95°C	HotStarTaq [®] Plus DNA Polymerase is activated
3-step cycling:			
Denaturation	15 s	95°C	45 cycles
Annealing*†	30 s	60°C	
Extension	75 s	72°C	

* Fluorescence data collection.

† On the Rotor-Gene Q, perform “gain optimization before first acquisition” in the target channel to adjust sensitivity for your pathogen assay.

Appendix C: Interpretation of Results

The PMA masking effect might vary depending on the microorganisms and the matrix applied. Therefore, it should be evaluated according to the following example.

In this example a pure *Salmonella enterica* overnight culture was prepared. Dead cell control samples were prepared and live and dead samples were treated with and without PMA, according to the protocol. Subsequently, DNA was extracted with the DNeasy Blood & Tissue Kit. The DNA was analyzed with real-time PCR using the *mericon* Salmonella spp. Kit.

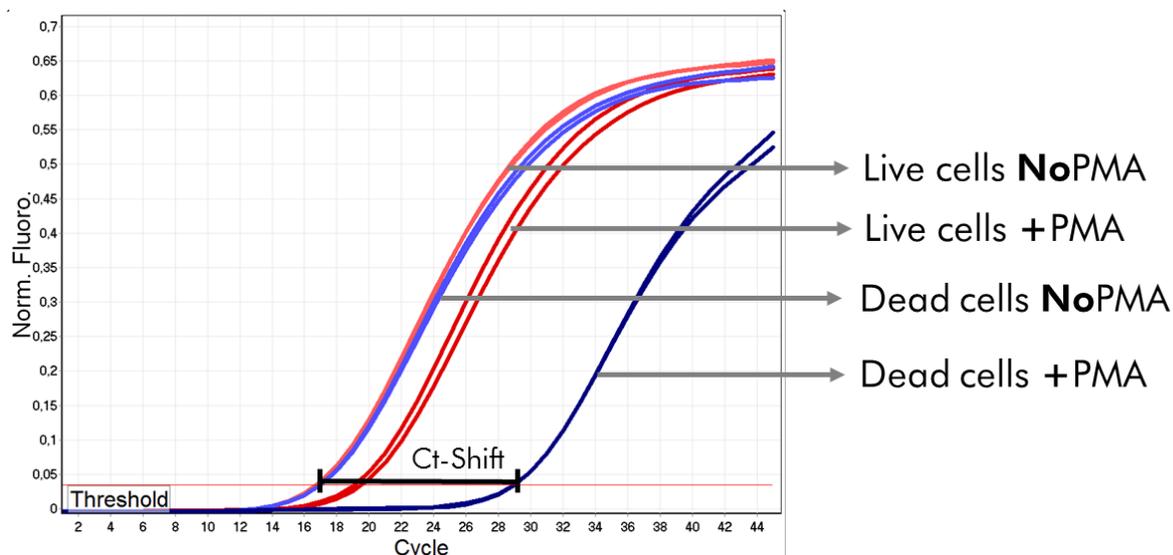


Figure 5. Anticipated amplification curves for the four different sample groups.

The figure shows the anticipated amplification curves for the four different sample groups. The C_T values of live cells treated with PMA might be slightly higher compared to the No PMA control of live cells. This is due to a naturally occurring portion of dead cells in a microbial culture. This C_T -shift usually ranges between 1 and 3 C_T values. The dead cells control sample treated with PMA should exhibit a prominent C_T -shift compared with the dead cells No PMA control. This C_T -shift demonstrates the PMA masking effect for the respective sample type. In the example, the C_T -shift of 12 makes it very clear to distinguish between cells that are dead or alive.

Table 4. C_T values

Experimental condition	C_T	C_T -shift
Live cells no PMA	16.9	2.5
Live cells plus PMA	19.4	
Dead cells no PMA	17.0	12
Dead cells plus PMA	29.0	

Ordering Information

Product	Contents	Cat. no.
BLU-V System	Instrument for photo-activation of dyes, includes one BLU-V Sample Holder.	9002300
BLU-V Incubation Box	Box for up to 12 tubes (tube volumes up to 2 ml) for light protection during incubation	9022908
Fuse T2A, 250V	Set of fuses	9240628
Power cord	Power cord for use in Australia	9232548
Power cord	Power cord for use in Switzerland	9232546
BLU-V Sample Holder	Sample holder for up to 12 tubes (tube volumes up to 2 ml)	9022909
<i>mericon</i> Salmonella spp Kit (24)*	For 24 reactions: PCR Assay Salmonella spp, Positive Control, Multiplex PCR Master Mix, QuantiTect® Nucleic Acid Dilution Buffer, RNase-Free Water, 50x ROX Dye Solution	290013
<i>mericon</i> Listeria spp Kit (24)*	For 24 reactions: PCR Assay Listeria spp, Internal Control, Positive Control, Multiplex PCR Master Mix, QuantiTect Nucleic Acid Dilution Buffer, RNase-free water	290123
<i>mericon</i> Campylobacter spp Kit (24)*	For 24 reactions: PCR Assay Campylobacter spp, Positive Control, Multiplex PCR Master Mix, QuantiTect Nucleic Acid Dilution Buffer, RNase-Free Water, 50x ROX Dye Solution	290033

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* Other kit sizes are available; please see www.qiagen.com.

Notes

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