Revised: September 25, 2015

Product Information

PMA™ Real-Time PCR Bacterial Viability Kit - *Legionella pneumophila* (mip)

Catalog Number: 31053

Unit Size: 1 kit (200 PCR reactions)

Kit Contents

Component	Size
40019: PMA™ dye, 20 mM in H_2O	1 X 100 uL
31038: PMA Enhancer for Gram Negative Bacteria, 5X Solution	1 X 16 mL
31003A: Fast EvaGreen® qPCR Master Mix	2 X 1 mL
99939: 10X ROX Reference Dye	1 X 1 mL
31053A: mip primer mix, 5 uM each primer For: 5'-GCAATGTCAACAGCAATGGCTG-3' Rev: 5'-CATAGCGTCTTGCATGCCTTTA-3'	1 X 400 uL

Storage and Handling

Store kit at -20 °C. PMA Enhancer can be stored at 4 °C. Protect PMA and Fast EvaGreen Master Mix from light. Components are stable for at least 6 months when stored as recommended. Before use, thaw at room temperature and mix well by gentle vortexing. Keep Fast EvaGreen qPCR Master Mix on ice before use. Kit components are stable for several freeze/thaw cycles.

Spectral Properties

PMA: λ_{abs} = 464 nm (before photolysis);
λ_{abs} /λ_{em} = ~510/~610 nm (following photolysis and reaction with DNA/RNA)

EvaGreen: λ_{abs} = 471 nm (without DNA)
λ_{abs}/λ_{em} = 500/530 nm (with DNA)

Product Description

PMA-PCR kits are designed for selective detection of viable bacteria from a specific strain using PMA dye and real-time PCR. The kits contain PMA™ dye, Fast EvaGreen® qPCR Master Mix, and PCR primers for detection of a specific strain of bacteria. Kits are available for detection of a selection of bacterial strains that are of widespread interest to food safety, public health, and antibacterial research.

This kit contains primers for amplification of *Legionella pneumophila* gene mip, with reagents sufficient to treat 80 bacterial cultures with DNA and perform 200 PCR reactions. The number of samples that can be treated with PMA using the kit may vary depending on sample type. See the product protocol under the downloads tab and references for more information.

PMA is a high affinity photoreactive DNA binding dye developed by Biotium. The dye is weakly fluorescent by itself but becomes highly fluorescent upon binding to nucleic acids. It preferentially binds to dsDNA with high affinity. Upon photolysis, the photoreactive azido group on the dye is converted to a highly reactive nitrene radical, which readily reacts with any hydrocarbon moiety at the binding site to form a stable covalent nitrogen-carbon bond, thus resulting in permanent DNA modification. The dye is cell membrane-impermeable and thus can be used to selectively modify DNA from dead cells with compromised membrane integrity, while leaving DNA from viable cells intact. PMA inhibits PCR amplification of modified DNA templates by a combination of removal of modified DNA during purification and inhibition of template amplification by DNA polymerases (1). Consequently the dye is useful in the selective detection of viable pathogenic cells by quantitative real-time PCR (Figure 1).

PMA Enhancer for Gram Negative Bacteria is designed for use with PMA to selectively detect viable gram-negative bacteria using real-time PCR. When Enhancer is added to gram negative bacteria before treatment with PMA, dead cell DNA levels are further decreased, and thus live-dead cell discrimination is improved. The amount of improvement varies depending on such factors as the bacterial strain and the way that the bacterial were killed. Enhancer gives the most improvement when bacteria are dead but their membranes are not completely disrupted, as occurs after mild heat treatment.

Fast EvaGreen Master Mix is a ready-to-use hot-start mix for qPCR and DNA melt curve analysis of PCR amplicons. It is formulated for qPCR using a fast cycling protocol, but also can be used for qPCR using regular cycling protocols. EvaGreen dye is a unique DNA-binding dye with features ideal for both qPCR and melt curve analysis. EvaGreen dye binds to dsDNA via a novel "release-on-demand" mechanism, which permits the use of a relatively high dye concentration in qPCR without PCR inhibition. Fast EvaGreen Master Mix contains Cheetah™ Taq, Biotium's fast-activating chemically-modified hot-start Taq polymerase, which is particularly suitable for fast PCR cycling protocols.

Legionella pneumophila is is a pathogenic species of gram-negative bacteria. It can infect the lungs and cause Legionnaire's Disease. *Legionella pneumophila* is sometimes detected in water sources such as cooling towers and swimming pools. PCR to amplify the gene mip has been published and shown to be highly specific for *Legionella pneumophila* (2). The primers provided in the kit have been validated at Biotium for real-time qPCR using EvaGreen Master Mix (Figures 2-4). An example of PMA-PCR using *E. coli* is shown in Figure 5.

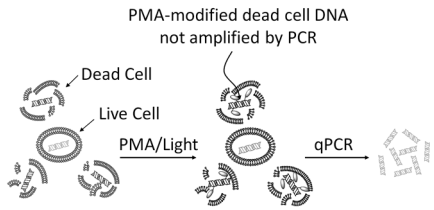


Figure 1. Principle of PMA modification for quantitation of viable bacteria by qPCR. The cell membrane-impermeable PMA dye selectively and covalently modifies DNA from dead bacteria with compromised membranes. Subsequent PCR amplification of PMA-modified DNA templates is inhibited, allowing selective quantitation of viable bacteria.

Protocol for treating gram-negative bacteria with PMA plus Enhancer for qPCR

The following is a protocol for treating cultured laboratory strains of gram-negative bacteria with PMA. Treatment of complex biological or environmental samples such as feces or soil may require optimization of sample dilution for PMA and light treatment. PMA Enhancer improves the activity of PMA on gram-negative bacteria, but has a detrimental effect on gram-positive bacteria. However, you may want to test whether it is beneficial in your assay of interest. If both gram-negative and gram-positive bacteria are to be treated in one sample, Enhancer should not be used.

1. Inoculate an appropriate media broth with bacteria (volume is dependent on size of experiment).
2. Shake cultures at 200 RPM at 37°C overnight.
3. Continuing culturing bacteria until the OD_{600} of the culture is approximately 1.
4. For dead cell control samples, heat inactivate bacteria at 58°C for 3 hours. To confirm killing of bacteria, plate 10 uL of heat inactivated bacteria on the appropriate media plate, and 10 uL of a 1:100 dilution of control bacteria on another plate. Place the plate at 37°C and check for colony growth after 24-48 hours.
5. Pipette 400 uL aliquots of bacterial culture into clear microcentrifuge tubes.
6. [Optional]: Add 100 uL of 5X Enhancer to each tube, for a 1X final Enhancer concentration.
7. Working quickly and in low light, thaw the 20 mM PMA stock and prepare a working stock by diluting to 5 mM in water. If using Enhancer, add 2.5 uL of working stock to each tube for a final concentration of 25 uM. If Enhancer is not used, we recommend adding 4 uL of working stock to 400 uL of sample for a final concentration of 50 uM.

8. Incubate tubes in the dark for 10 minutes at room temperature. Flick tubes occasionally to mix, or incubate on a rocker covered with aluminum foil.
9. Expose samples to light to cross-link PMA to DNA. See information on light sources below.

   a. For best results, we recommend that the photo-crosslinking be carried out on Biotium's PMA-Lite LED Photolysis Device (catalog # E90002). 15 min exposure should be sufficient for complete PMA activation.

   b. Commercial halogen lamps (>600 W) for home use have been employed for photoactivating PMA in some publications, though results have not been consistent due to inevitable variation in the set-up configurations. If you decide to use a halogen lamp, we recommend that you lay tubes on a block of ice set 20 cm from the light source. The ice block should be in a clear tray with a piece of aluminum foil under the clear tray to reflect the light upward. Set the lamp so that the light source is pointing directly downward onto the samples. Expose samples to light for 5-15 min.
10. Pellet cells by centrifuging at 5,000 x g for 10 minutes.
11. Extract genomic DNA using a standard protocol or commercially available kit. Use an appropriate protocol or kit for DNA extraction from complex biological or environmental samples (e.g., feces or soil).
12. Perform qPCR using primers against an appropriate genomic DNA target for your organism of interest. DNA templates modified with PMA will show delayed amplification by qPCR, and a further delay when Enhancer is used (Figure 5).

Note 1: Amplicons as short as 100 bp can be used, but longer target amplicons have been shown to decrease the signal from heat-killed PMA-treated cells.

Note 2: Part of the proposed mechanism of action of PMA is the removal of PMA-bound DNA from samples via precipitation; therefore the amount of input DNA in each sample should not be normalized between samples. Instead, PCR should be performed using equal volumes of gDNA eluate from each sample. For a positive control, 1 ng of live cell gDNA per reaction should be sufficient for achieving good signal. For gDNA extracted from bacterial cultures using a commercial extraction kit, 1-2 uL of eluted DNA can be used as a starting point for optimization.

PCR Reaction Setup

Add reaction components to each PCR tube or well according to the table below:

Reaction component	Amount per 20 uL reaction	Final concentration
2X Fast EvaGreen Master Mix	10 uL	1X
mip primer mix, 5 uM	2 uL	0.5 uM each
Template	x uL See Note 1	See Note 1
ROX	Optional	See Table 1
dH_2O	Add to 20 uL	

Note 3: Template volume should not exceed 10% of final reaction volume.

Fast-cycling parameters for mip real-time PCR on *Legionella pneumophila* gDNA

Hold
95 °C for 2-10 minutes (see Note 4)

Cycling	
95 °C for 5 seconds	Cycle 40 times
60 °C for 30 seconds (acquire data)	

Melt
57 °C to 99 °C

Note 4 - Activation of Cheetah™ Taq DNA Polymerase requires only 2 minutes at 95 °C, but genomic DNA can take longer to fully denature. If you observe high background fluorescence during intial amplification cycles, try increasing the hold time.

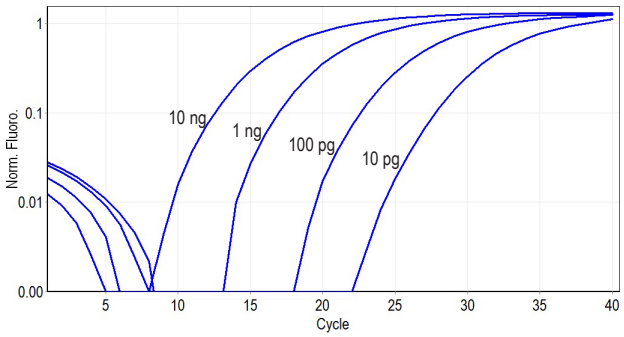


Figure 2. Fast EvaGreen® qPCR Master Mix was used to amplify a fragment of mip from 10 ng, 1 ng, 100 pg, or 10 pg of *Legionella pneumophila* gDNA. The real-time PCR was performed on a RotorGeneQ (Qiagen).

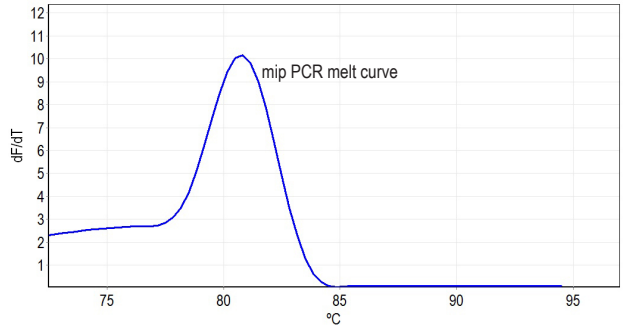


Figure 3. Melt curve analysis of the mip real-time PCR product generated in Figure 1, from 1 ng of *Legionella pneumophila* gDNA input.

Table 1. Recommended ROX Concentration for PCR Instruments

PCR Instrument	Recommended Rox Concentration	Amount of 10X ROX per 20 uL reaction
BioRad: iCycler, MyiQ, MiQ 2, iQ 5, CFX-96, CFX-384, MJ Opticon, Option2, Chromo4, MiniOpticon Qiagen: Rotor-Gene Q, Rotor-Gene3000, Rotor-Gene 6000 Eppendorf: Mastercycler realplex Illumina: Eco RealTime PCR System Cepheid: SmartCyler Roche: LightCycler 480, LightCycler 2.0	No ROX	None
ABI: 7500, 7500 Fast, ViiA 7 Stratagene: MX4000P, MX3000P, MX3005P	Low ROX 0.05-0.1X final	Dilute 10X ROX 1:10 with dH2O to obtain 1X ROX; add 1 to 2 uL of 1X ROX per 20 uL reaction
ABI: 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne, StepOne plus	High ROX 1X final	2 uL of 10X ROX per 20 uL reaction

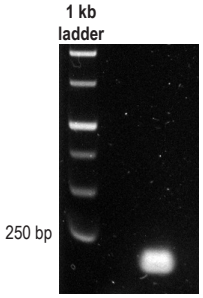
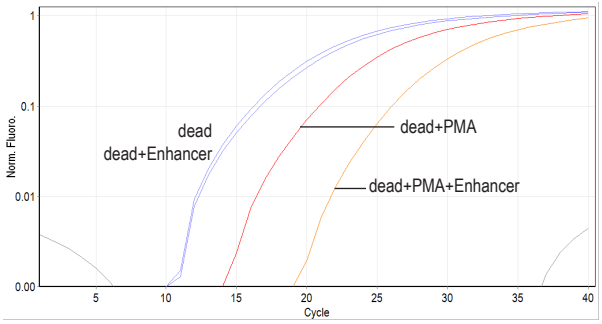


Figure 4. Reaction product from real-time PCR amplification of mip (159 bp fragment) from 1 ng *Legionella pneumophila* gDNA input. Biotium's 1 kb DNA ladder was run in the first lane. The 1% agarose 1X TBE gel was post-stained with 3X GelRed in water and imaged on a UVP GelDoc-iT using UV illumination and an ethidium bromide filter (1 second exposure).

A



B

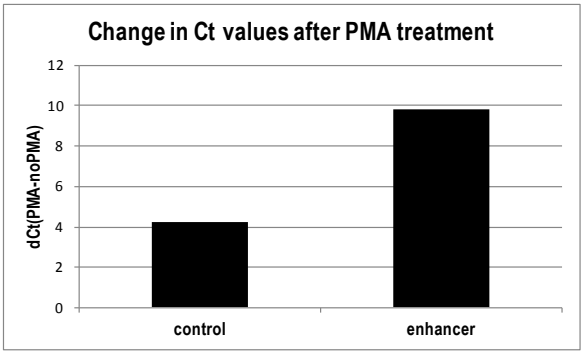


Figure 5. PMA plus Enhancer for quantitation of viable bacteria by Real-time PCR.
A. Mildly heat-killed *E. coli* were treated with PMA and/or Enhancer, followed by exposure with the PMA-Lite™ and DNA purification. Fast EvaGreen® qPCR Master Mix was used to amplify a 377-bp fragment of *E. coli* DNA. Dead cells treated with PMA+Enhancer showed a significant further delay in Ct compared to dead cells treated with PMA alone.
B. For either control- or enhancer-treated cells, dCt values were calculated by subtracting the Ct without PMA from the Ct with PMA. For dead cells, the use of Enhancer increased the dCt from 4 to 10, greatly increasing the specificity of viability PMA-PCR.

Light sources for photoactivation

Biotium offers the PMA-Lite™ LED Photolysis Device for light-induced cross-linking of PMA to dsDNA. The PMA-Lite™ LED Photolysis Device (catalog # E90002) is a thermally-stable blue LED light source that provides even illumination to all samples. It contains a cooling unit to prevent sample overheating as well as several timer settings to allow for precisely timed light treatment.

References

1. Nocker A., et al. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Meth. 67(2), 310-320 (2006).

2. Wilson DA, et al. Detection of Legionella pneumophila by real-time PCR for the mip gene. J. Clin. Microbiol. 41 (7) 3327-3330 (2003).

Related Products

Catalog number	Product
E90002	PMA-Lite™ LED Photolysis Device
40069	PMAxx™ dye, 20 mM in dH2O
40013	PMA™ dye, 1 mg
40019	PMA™ dye, 20 mM in dH2O
40015	Ethidium Monoazide (EMA), 5 mg
31038	PMA Enhancer for Gram Negative Bacteria, 5X Solution
31033	PMA Real-Time PCR Bacterial Viability Kit- Salmonella enterica (invA)
31034	PMA Real-Time PCR Bacterial Viability Kit - Mycobacterium tuberculosis (groEL2)
31035	PMA Real-Time PCR Bacterial Viability Kit - Staphylococcus aureus (nuc)
31036	PMA Real-Time PCR Bacterial Viability Kit - Staphylococcus aureus (mecA)
31050	PMA Real-Time PCR Bacterial Viability Kit - Escherichia coli (uidA)
31037	PMA Real-Time PCR Bacterial Viability Kit - Escherichia coli O157:H7 (Z3276)
31051	PMA Real-Time PCR Bacterial Viability Kit - Listeria monocytogenes (hly)
31003	Fast EvaGreen® qPCR Master Mix (200 rxn), 2 x 1 mL
31041-T	Forget-Me-Not qPCR Master Mix (100 rxn)
31021	1 kb DNA Ladder (100ng/uL), 300 ug/300 uL
31022	Ready-to-Use 1 kb DNA Ladder, 150 applications (1.5 mL)
31031	100 bp DNA Ladder, 30 ug/300 uL
31032	Ready-to-Use 100 bp DNA Ladder, 150 applications (1.5 mL)
41003	GelRed™ Nucleic Acid Gel Stain, 10,000X in water, 0.5 mL
32000-1	Live Bacterial Gram Stain Kit
32001	Bacterial Viability and Gram Stain Kit
30027	Viability/Cytotoxicity Assay Kit for Bacterial Live and Dead Cells

Please visit our website at www.biotium.com for information on our life science research products, including environmentally friendly EvaGreen® qPCR master mixes, fluorescent CF™dye antibody conjugates and reactive dyes, apoptosis reagents, fluorescent probes, and kits for cell biology research.