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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
99801: Forget-Me-Not™ qPCR Master Mix	2 X 1 mL
31042C: ROX Reference Dye	1 X 1 mL
31053A: mip primer mix, 5 uM each primer For: 5'-GCAATGTCAACAGCAA-3' Rev: 5'-CATAGCGTCTTGCATG-3'	1 X 400 uL

Storage and Handling

Store kit at -20 °C. After first thaw, PMA Enhancer should be stored at 4°C. Store PMA and Forget-Me-Not Master Mix protected from light. Protect PMA from light during use. Components are stable for at least 6 months when stored as recommended. Kit components are stable for several freeze/thaw cycles.

Spectral Properties

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

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

Product Description

Viability-PCR kits are designed for selective detection of viable bacteria by real-time PCR. Each kit contains a viability dye (PMA or PMAxx), Forget-Me-Not qPCR Master Mix, and PCR primers for detection of a specific strain of bacteria.

This kit contains primers for amplification within the *Legionella* mip gene, with reagents sufficient to treat 80 bacterial cultures and perform 200 PCR reactions. The number of samples that can be treated with PMA using the kit may vary depending on sample type.

PMA is a photoreactive DNA binding dye developed by Biotium. It is cell membrane-impermeable and so selectively binds to DNA from dead cells with compromised membrane integrity, while leaving DNA from viable cells intact. Upon photolysis, the dye forms a stable covalent bond, resulting in permanent DNA modification. 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). Thus the dye is useful in the selective detection of viable pathogenic cells by real-time qPCR (Figure 1).

PMA Enhancer for Gram Negative Bacteria is designed for use with PMA or PMAxx. 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. PMA Enhancer gives the most improvement when bacteria are dead but their membranes are not completely disrupted, as occurs after mild heat treatment.

Forget-Me-Not qPCR Master Mix is a hot-start EvaGreen® dye-based master mix for use in real time PCR applications and DNA melt curve analysis. Forget-Me-Not master mix contains a low concentration of blue dye which allows you to see at a glance whether you forgot to add master mix to any of your tubes, so you can catch pipetting mistakes and avoid wasting time, reagents, and your precious DNA samples. It is formulated for qPCR using a fast cycling protocol, but can also be used for qPCR using regular cycling protocols. Forget-Me-Not 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 Mixes (Figures 2-4).

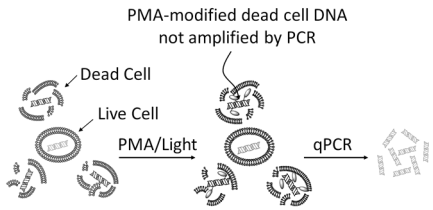

Figure 1. The cell membrane-impermeable PMA dye selectively and covalently modifies DNA from dead bacteria with compromised membranes. Subsequent PCR amplification of PMA-modified DNA templates is inhibited, allowing selective quantitation of viable bacteria.

Quick guide protocol

(Detailed protocol on following page)

1. Aliquot 400 uL cell culture or sample into tubes. If desired, prepare live and dead cell controls.

2. Add 100 uL 5X PMA Enhancer to a final concentration of 1X. See detailed protocol for more information.

3. Working in dim light, add 25-50 uM PMA or PMAxx viability dye to tubes. Include no-dye controls.

4. Incubate for 10 min, rocking, protected from light.

5. Expose samples to light to crosslink dye to DNA. We recommend 15 min in the PMA-Lite.

6. Isolate DNA using a commercial kit or other protocol.

7. Set up qPCR reactions, using 2 uL of each isolated DNA sample as templates. Do not normalize the DNA concentrations.

8. Compare the amount of total and live-cell-derived DNA in your sample by calculating the dCt (dCt = Ct with viability dye - Ct without viability dye). See detailed protocol for more information.

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).

Detailed 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 dye and light treatment. PMA Enhancer generally 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 or 95°C for 5 min. 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 PMA 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.

   a. For best results, we recommend that the photo-crosslinking be carried out on Biotium's PMA-Lite LED Photolysis Device see next page for more information). 15 min exposure should be sufficient for complete PMA or PMAxx 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 the mip primers included with this kit to detect *Legionella*. See reaction setup and fast cycling parameters below.
13. Data analysis: Compare the amount of total and live-cell-derived DNA in your sample by calculating the dCt (dCt = Ct with viability dye - Ct without viability dye). The dCt of a control sample of killed cells can be calculated to determine the maximum inhibition that can be achieved by PMA or PMAxx in your sample, and the dCt of control live cells can be calculated to control for false negatives that may arise from dye getting into live cells.

Note 1: 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, 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 Forget-Me-Not Master Mix	10 uL	1X
mip primer mix, 5 uM	2 uL	0.5 uM each
Template	x uL See Notes 1&2	See Note 1
ROX	Optional	See Table 1
dH_2O	Add to 20 uL	

Note 2: Template volume should not exceed 10% of final reaction volume.

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

Hold	
95 °C for 2-10 minutes (see Note 3)	
Cycling	
95 °C for 5 seconds	Cycle 40 times
52 °C for 30 seconds (acquire data)	
Melt	
57 °C to 99 °C	

Note 3 - 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.

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	Dilute ROX 1:100 with dH2O and add 3 uL diluted ROX per 20 uL reaction.
ABI: 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne, StepOne plus	High ROX	Dilute ROX 1:10 with dH2O and add 3 uL ROX Reference Dye per 20 uL reaction.

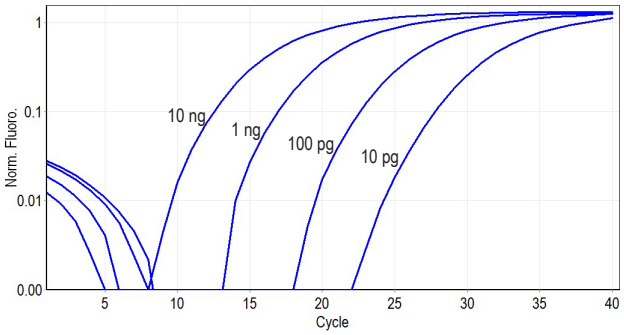

Figure 2. qPCR was performed to amplify a fragment of mip from 10 ng, 1 ng, 100 pg, or 10 pg of *Legionella pneumophila* gDNA. The real-time PCR was performed on a RotorGeneQ (Qiagen).

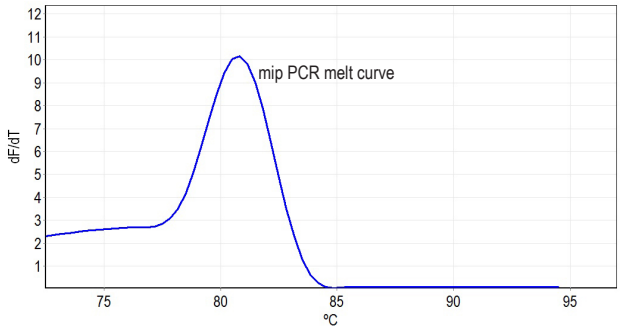

Figure 3. Melt curve analysis of the mip real-time PCR product generated in Figure 1, from 1 ng of *Legionella pneumophila* gDNA input.

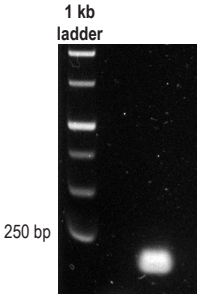

Figure 4. Reaction product from real-time PCR amplification of mip (159 bp fragment) from 1 ng *Legionella pneumophila* gDNA input. Biotium's 1 kb DNA ladder was run in the first lane. The 1% agarose 1X TBE gel was post-stained with 3X GelRed in water and imaged on a UVP GelDoc-iT using UV illumination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

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 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.

Related Products

Catalog number	Product
E90002	PMA-Lite™ LED Photolysis Device
40013	PMA dye, 1 mg
40019	PMA dye, 20 mM in dH2O, 100 uL
40069	PMAxx™ dye, 20 mM in dH2O, 100 uL
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)
31037	PMA Real-Time PCR Bacterial Viability Kit - E. coli O157:H7 (Z3276)
31050	PMA Real-Time PCR Bacterial Viability Kit - E. coli (uidA)
31051	PMA Real-Time PCR Bacterial Viability Kit - Listeria monocytogenes (hly)
31041-T	Forget-Me-Not™ qPCR Master Mix (100 rxn), 1 mL
31022	Ready-to-Use 1 kb DNA Ladder, 150 applications (1.5 mL)
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

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