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Protocol status: Working
 We use this protocol and it's working

Live/Dead qPCR of *B. pertussis* IS481

PLOS One

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ABSTRACT

This protocol describes the use of qPCR for the quantitative determination of live *B. pertussis* bacteria in a sample. The method uses propidium monoazide, a compound that can cross the cell wall of dead bacteria and binds DNA following photo activation to inhibit amplification of DNA from dead bacteria. This method uses a TaqMan probe specific to IS481 to ensure maximum sensitivity due to the high copy number of the target. Although IS481 is not specific to *B. pertussis* for experimental purposes it provides acceptable specificity.

ATTACHMENTS

[SOP_001.3_live_dead_qPCR.docx](#)

MATERIALS

MATERIALS

- ☒ Water Contributed by users
- ☒ Centrifuge Contributed by users
- ☒ Microcentrifuge Contributed by users
- ☒ Microcentrifuge tubes Denville Scientific Inc. Catalog #C2170
- ☒ StepOnePlus™ Real-Time PCR System Thermo Fisher Scientific Catalog #4376600
- ☒ MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL Thermo Fisher Catalog #4346907
- ☒ TaqMan™ Gene Expression Master Mix Thermo Fisher Catalog #4369016

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PROTOCOL integer ID:
33678

Keywords: enumeration,
live, dead, qPCR, propidium
monoazide, PMA

Adhesive PCR Plate Seals Thermo Fisher Catalog #AB0558
PMA-Lite™ LED Photolysis Device Biotium Catalog #E90002
1.5 ml Crystal Clear Microcentrifuge Tube StarLab Catalog #E1415-1500
QIAamp DNA Mini Kit Qiagen Catalog #51304
RNase A Merck Millipore Sigma Catalog #R4642
PMA Dye 20 mM in H2O Biotium Catalog #40019
1000 µl Filter Tip (Sterile) Racked StarLab Catalog #S1126-7810
200 µl Graduated Filter Tip (Sterile) Racked StarLab Catalog #S1120-8810
20 µl Bevelled Filter Tip (Sterile) Racked StarLab Catalog #S1120-1810
10 µl Graduated Filter Tip (Sterile) Racked StarLab Catalog #S1121-3810
Primers (IS481F/R) Contributed by users
Probe (IS481) Contributed by users
Control DNA for standard curve (Purified DNA from B1917 or strain being tested) Contributed by users

		Seque nce (5'-3')
IS481 Forwa rd Prime r	ATCA AGCA CCGC TTTA CCC	
IS481 Rever se Prime r	TTGG GAGT TCTG GTAG GTGT G	
IS481 Probe	FAM- AATG GCAA GGCC GAAC GCTT CA- BHQ1	

SAFETY WARNINGS

Good Laboratory Practice must be followed. Wear laboratory coats and gloves.

BEFORE START INSTRUCTIONS

Principle

To use propidium monoazide (PMA) to inhibit the PCR amplification signal of DNA from dead bacteria. Purified DNA will be used as a template for the quantification of *IS481* using a TaqMan probe. A standard curve consisting of DNA from pure B1917 will be used to determine absolute quantity in unknowns.

PMA treatment

- 1** Pellet fresh samples by centrifuging at 2,000 x g for 10 minutes and resuspend in 1.2 ml of PBS. 10m
- 2** Transfer 200µl of resuspended sample into two clear microfuge tubes (continue with 1 sample to Step #3 and 1 sample skip to Step #4).
- 3** Add 0.5µl of PMA to one of the 200µl of samples from Step #2.
- 4** Incubate microfuge tubes in the dark for 10 min at room temperature. Cover samples with aluminium foil and incubate on a rocker. 10m
- 5** Expose samples to light using the PMA-Lite™ LED Photolysis Device for 5 min. 5m

DNA purification

- 6 Add 20µl of QIAGEN Protease and 4 µl of RNase A.
- 7 Add 200µl of Buffer AL and vortex for 15 s. 15s
- 8 Incubate at 56°C for 10 min. 10m
- 9 Briefly spin tube to recover condensation.
- 10 Add 200µl of ethanol (96-100%), vortex and spin briefly.
- 11 Add to spin column and centrifuge for 1 min at 6000xg. 1m
- 12 Transfer to a new collection tube.
- 13 Add 500µl AW1 buffer and spin for 1 min at 6000xg. 1m

- 14 Transfer to a new collection tube.
- 15 Add 500µl AW2 buffer and spin for 3 min at 17000xg 3m
- 16 Transfer to a 2ml microfuge tube.
- 17 Spin at max speed for 1 min. 1m
- 18 Transfer to a 1.5ml microfuge tube.
- 19 Add 200µl of Buffer AE and incubate at room temperature for 1 min. 1m
- 20 Elute by centrifuging for 1 min at 6000xg. 1m

qPCR

21 Dilute stock primers (50 μ M) 9 in 50 μ l, to give a reaction concentration of 900nM.

22 Dilute stock probe (50 μ M) 3 in 100 μ l, to give a reaction concentration of 150nM.

23

Prepare Mastermix as follows:

Taqman MM	10 μ l	1x
Forward primer	2 μ l	900nM
Reverse primer	2 μ l	900nM
Probe	2 μ l	150nM

Mastermix per reaction

24 Add 16 μ l of master mix per well.

25 Serial dilute 9 times, 5:50 positive control DNA (25ng/ μ l).

26 Use dilutions 3-9 for 1000, 100, 10, 1, 0.1, 0.01, 0.001pg.

27 Load in triplicate 4 μ l of DNA for each control dilution, unknown and water samples.

PCR and analysis

- 28 Cover plate with film and spin for 1 min at 1000xg

1m

- 29 Set up qPCR program as follows:

		50°C	2min
		95°C	10min
40 cycles :		95°C	15sec
		60°C	1min

qPCR Cycle

Results

- 30 Run analysis on data using StepOnePlus™ Software v2.3.
- 31 Outliers of triplicate samples should be ignored (omit).
- 32 Confirm standard curve has an $r^2 > 0.95$.
- 33 Convert pg of DNA to copy number: $B. pertussis/\text{sample} = (50 \times (\text{mass of template in pg}) \times 6.022 \times 10^{23}) / (\text{length of genome in bp} \times 1 \times 10^{12} \times 650)$.

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- 34** Untreated sample (no PMA), represents total *B. pertussis* in the sample.
- 35** PMA treated sample gives number of live *B. pertussis* in the sample.
- 36** The number of dead can be determined from the difference between untreated and treated.