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Live/Dead qPCR of *B. pertussis* IS481

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

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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_...

30KB

Materials

MATERIALS

- Water
- Centrifuge
- Microcentrifuge
- 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
- 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 MilliporeSigma (Sigma-Aldrich)** **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)
- Probe (IS481)
- Control DNA for standard curve (Purified DNA from B1917 or strain being tested)

		Sequence (5′-3′)
	IS481 Forward Primer	ATCAAGCACCGCTTTACCC
	IS481 Reverse Primer	TTGGGAGTTCTGGTAGGTGTG

	IS481 Probe	FAM-AATGGCAAGGCCGAACGCTTCA-BHQ1

Primers (IS481F/R) and Probe (IS481)

Safety warnings

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

Before start

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 56ºC 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 (50µM) 9 in 50µl, to give a reaction concentration of 900nm.

22 Dilute stock probe (50µM) 3 in 100µl, to give a reaction concentration of 150nm.

23 Prepare Mastermix as follows:

	Taqman MM	10µl	1x
	Forward primer	2µl	900nM

	Reverse primer	2µl	900nM
	Probe	2µl	150nM

Mastermix per reaction

24 Add 16µl of master mix per well.

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

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

27 Load in triplicate 4µl 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:

		50ºC	2min
		95ºC	10min
	40 cycles:	95ºC	15sec
		60ºC	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*/sample=(50x(mass of template in pg)x6.022×10^{23})/length of genome in bp*1×10^{12}x650).

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.