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Detection of Bordetella holmesii and Bordetella bronchiseptica Using the ABI 7500 Real-time PCR System

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We use this protocol and it's working

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Abstract

This procedure provides instructions for how to perform qualitative PCR (qPCR) for the detection of the hIS1001 insertion sequence of *Bordetella holmesii* and the bfrZ gene of *Bordetella bronchiseptica* from appropriate direct specimens. This test is intended to be used as a secondary assay to differentiate *B. pertussis* (IS481 positive) from *B. holmesii*.

Guidelines

Perform all manipulations of samples and DNA in a genomic level PCR laboratory. Prepare all PCR master mixes in a Reagent Preparation Clean Room.

Materials

Samples

IS481 Positive lysates from the primary detection assay for *Bordetella pertussis* and *Bordetella parapertussis* on the ABI 7500 FAST.

Reagents, Materials, and Equipment

	Reagents	Materials	Equipment
	Life Technolgies Fast Advanced master mix (#4444557)	Microcentrifuge tubes	Pipettes (various volumes)
	PCR grade water	pipette tips (various volumes)	Biological Safety Cabinet Class 2 Type A/B3
	IDTE 1x TE Buffer pH 8.0	ABI 96-well optical plate	Fully equipped reagent and genomic preparation clean rooms
	hIS1001 primers and probe (B. holmesii)	optical plate adhesive film	Applied Biosystems 7500 Real-Time Analyzer
	bfrZ primers and probe (B. bronchiseptica)	adhesive film applicator	
	Instagene Matrix (BioRad)	tube racks	
	bfrZ-hIS1001 gBlock		

Primers

	Primer ID	Sequence	Product Size (bp)	Final Concentration (nM)	Target	Reference
	bfrZ-Qr (Bronch- R)	CCACCAAACGCA ATGACCTG	99	100	bfrZ	Modified Jinnerot et al., 2015
	bfrZ-Qf-mod (Bronch-F)	CGAATTGCGCCC ATCCCATG		100		
	BHIS41U20 (Holm-F)	GGCGACAGCGAG ACAGAATC	67	300	hIS1001	Tatti et al., 2011
	BHIS91L17 (Holm-R)	GCCGCCTTGGCT CACTT		300		

Probes

	Probe ID	Sequence	Target	Dye/Quencher	Final Concentration (nM)	Reference
	bfrZ-Qp (Bronch-P)	TCGGGAAGGTGCAGCATGTCCTGGAAATA	bfrZ	FAM/ZEN	100	Jinnerot et al., 2015
	BHIS62U28P (Holm-P)	CGTGCAGATAGGCTTTTAGCTTGAGCGC	hIS1001	CY5/TAO	150	Tatti et al., 2011

Controls

	PCR Controls	Control Organism/Reagent	Comment
	bfrZ-hIS1001 gBlock	frozen aliquots of bfrZ and hIS1001 gblock diluted to 4.2 × 10^2 copies/ul	See below for sequence
	NTC: No Template Control	PCR grade water	Use the same lot of water as was used in the preparation of the master mix. Tests for the sterility of the master mix reagents.

Note: Extraction controls are not required as sample lysate has already been run with these controls on the primary Pertussis PCR.

bfrZ-hIS1001 gBlock sequence (PCR gBlock)

CCGGTGGCGACAGCGAGACAGAATCCCGTGCAGATAGGCTTTTAGCTTGAGCGCGAAGTGAGCCAAGGCGGCGATGCCGC
TGCCCTGAGCCTGGGAAGACCCACTGGCCGCCGCCACCAAACGCAATGACCTGAACCTGTATTTCCAGGACATGCTGCAC
CTTCCCGACGAGAAGACGCGCCTGCTGCTGGCCATGGGATGGGCGCAATTCGACAGCCGCCCGCCCGACAA

Reconstitute the gBlock as per the manufacturers instructions, and then dilute to desired working concentration (copies/ul) using 1 part Carrier RNA to 5 parts IDTE buffer.

Note: dilutions of gBlock are best kept stored in 0.5 mL lo-bind PCR tubes at -20 °C

Troubleshooting

Safety warnings

Method Limitations:

1. Coinfections of *B. pertussis* with *B. holmesii* will be called *B. holmesii* positive with the use of this assay in combination with the IS481 assay.

2. High Ct IS481 positive and negative for hIS1001 may be false negative for *B. holmesii.*

Procedure A: Preparing 20X PCR Master Mix

1 All primer and probe sequences are listed in the materials section of this protocol.

Order all necessary primers and probes; if received lyophilized reconstitute as per the manufacturers instructions before making the desired stock dilutions.

2 Turn on the Biological Safety Cabinet (BSC) in the Reagent Preparation Clean Room. Allow the BSC to stabilize, ensure it is functioning as expected, and decontaminate prior to using.

Inside the BSC, prepare **20X HOLM mix** as per the following table. Prepare 1 mL total volume for each batch.

	Primer/Probe	Stock Concentration (uM)	Final PCR Concentration (nM)	Volume (uL) for 1000 reactions
	bfrZ-Qp (FAM) (Bronch-P)	100	100	20
	bfrZ-Qf-mod (Bronch-F)	100	100	20
	bfrZ-Qr (Bronch-R)	100	100	20
	BHIS62U28P (CY5) (Holm-P)	100	150	30
	BHIS41U20 (Holm-F)	100	300	60
	BHIS91L17 (Holm-R)	100	300	60
	IDTE Volume			790
	Final Volume			1000

3 Pipette the 20X Holm mix into 100 µL aliquots. Label each aliquot with **20X Holm Mix.**

4 Store the 20X Holm aliquots in 4 °C fridge for short-term storage and a -20 °C freezer in side a Reagent Preparation Clean Room for long-term storage.

Procedure B: Setting up the Real-Time PCR Reactions

5 Turn on the BSC in the Reagent Preparation Clean Room. Allow the BSC to stabilize, and decontaminate before use.

6 Remove an aliquot of the 20X Holm Mix from its place of storage.

If frozen let thaw completely before using.

7 When the 20X mix is completely thawed vortex briefly and spin down.

8 In a 1.7 mL microcentrifuge tube prepare the master mix cocktail as follows:

	Reagent	1x reaction (uL)
	PCR Grade Water	4
	Fast Advanced Master Mix	10
	20X Holm Mix	1

Make enough master mix to account for the total number of reactions on your PCR plate + 15% to account for pipetting errors.

9 Vortex and briefly spin down the master mix before pipetting

10 Aliquot 15 µL of master mix into the required number of wells of a 96-well optical plate

11 Seal the 96-well optical plate with adhesive film using a seal applicator and place in a clear plastic bag for transport to a Genomic PCR Room.

12 Turn on the BSC in a Genomic PCR Room. Allow the BSC to stabilize, ensure it is functioning as expected and decontaminate before use.

13 Pipette samples from the sample extract plate and controls in the following order:

1. NTC: 5 µL of the same lot PCR grade water that was used to prepare the master mix.
2. Patient Samples: 5 µL of each sample extract from the extract plate
3. HOLM/BRONCH (bfrZ-hIS1001) gBlock: 5 µL

14 Apply an optical adhesive film to the plate using a plate seal applicator, taking care to seal the plate edges and avoid touching the top of the film.

Note: any fingerprints or residue on the film will alter the optical readings

	If	Then
	Plate will not be run immediately	Store plate in a 4C fridge until ready to perform PCR
	Plate will be run immediately	Proceed to load and run the plate on the ABI 7500

15 On the ABI 7500 instrument create a new experiment for the current run as per the ABI 7500 User Manual

16 Check that the following reaction conditions are correctly programmed:

	Temperature (°C)	Time	Number of Cycles
	50	2 min	Hold
	95	20 sec	Hold
	95	3 sec	40
	60	30 sec	

17 Either input your samples and controls into the current run file manually or import the data from a saved run file on a memory stick.

Ensure all wells are assigned correctly on the run file as per the pipetting of the sample plate.

18 Check that each sample well has the correct target, dye, and quencher assigned to it.

	Target	Dye	Quencher
	bfrZ (Bronch)	FAM	None
	hIS1001 (Holm)	CY5	None

19 Load the plate onto the ABI 7500 instrument.

20 Save the run on the instrument and start the run as per the ABI 7500 user manual.

Procedure C: 7500 FAST Run Analysis

21 Ensure the run has completed successfully. Click OK.

22 Select the Analysis tab after the run has completed, and then select the "Amplification Plot" tab.

23 To view all samples, click on the small square at the top left of the plate map to select all the wells on the plate at once. Curves should appear on the graph.

24 Under Options (at the bottom of the screen), select "hIS1001" (*B. holmesii*)

25 Unclick "Auto Threshold" and enter a manual value of **0.1** in the box. Click Auto Baseline.

26 Repeat steps 25 and 26 but for the target "bfrZ" (*B. bronchiseptica*)

27 Click on the "Analyze" button.

28 Ensure that under "Plot Settings" the "Delta Rn vs. Cycle" option is selected and plot colour is set to "target".

29 View the results of all run controls and ensure they have produced acceptable values before proceeding with clinical sample analysis.

30 Examine the results for hIS1001 and bfrZ targets for each clinical sample.

Note: All Ct values must be confirmed by viewing the amplification curve and multicomponent plot for appropriate graphing of positive result.

See the Applied Biosystems 7500 Fast Real-Time PCR System Presence/Absence Experiments manual at:

http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_050341.pdf

Interpretation Table
POS: Ct < or = 35
NEG: Ct = Undetermined
If Ct >35 sample is indeterminate and may require retesting or recollection.

	IS481	pIS1001	hIS1001	bfrZ	Interpretation
	POS	NEG	NEG	NEG	B. pertussis Positive
	POS/NEG	NEG	POS	NEG	B. holmesii Positive
	POS/NEG	POS/NEG	NEG	POS	B. bronchiseptica Positive
	NEG	POS	NEG	NEG	B. parapertussis Positive

Protocol references

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