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Detection of Bordetella pertussis and Bordetella parapertussis 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 IS481 insertion sequence of *Bordetella pertussis* and the pIS1001 insertion sequence of the *Bordetella parapertussis* from appropriate direct specimens. The IS481 insertion has been found to be present in some strains of *B. bronchiseptica* and most strains of *B. holmesii*. Some strains of *B. bronchseptica* also carry the pIS1001insertion sequence. The *B. holmesii* and *B. bronchiseptica* multiplex qPCR should be performed when indicated.

Guidelines

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

Materials

Samples

Primary specimens:

I. Pernasal swab (2 is recommended and the specimen of choice)

II. nasal pharyngeal swab/wash/aspirate

III. post nasal swab

Referred Cultures:

Pure cultures of bacterial isolates on charcoal plates.

Reagents, Materials, and Equipment

Reagents	Materials	Equipment
Life Technologies Fast Advanced master mix (#4444557)	Microcentrifuge tubes	Biological Safety Cabinet Class 2 Type A/B3
PCR grade water	ABI 96-well optical plates	Fully equipped reagent and genomic preparation clean rooms
IDTE 1x TE Buffer pH 8.0	optical adhesive film	Heating Blocks
IS481 primers and probe	adhesive film	Pipettes (various volumes)
pIS1001 primers and probe	adhesive film applicator	Applied Biosystems 7500 Real-time Analyzer
Human Beta Globin (HBG) primers and probe	pipette tips (various volumes)	
Internal positive control gBlock and primers and probe	Lo-bind PCR tubes	
Instagene Matrix (BioRad)	tube racks	
HBG-IS481-pIS1001 gBlock	Discard pail and waste bags	
QC strain of Bordetella parapertussis organism	disposable gloves and gown	
Ambion Carrier RNA (thermofisher #4382878)	Clear plastic bags	

Primers

Primers	Sequence	Product Size	Final Concentration (nM)	Target	Reference
PPertM_mod (481-F)	CATCAAGCACCGCTTTACCC	117	300	IS481	Modified Kamachi et al., 2015
APPert_mod-R (481-R)	TGTTGGGAGTTCTGGTAGGTGTG				
135U17 (F) (1001-F)	TCGAACGCGTGGAATGG	65	150	pIS1001	Tatti et al., 2011
199L20 (R) (1001-R)	GGCCGTTGGCTTCAAATAGA				

	Primers	Sequence	Product Size	Final Concentration (nM)	Target	Reference
	HBG-F	ACCCAGAGGTTCTTTGAGTCCTTT	82	100	HBG	Mauritz et al.
	HBG-R	TGCCATGAGCCTTCACCTTAG				

Probes

	Probe	Sequence	Target	Dye/Quencher	Final Concentration (nM)	Reference
	871U22P_MGB (481-P)	TTGCGTGAGTGGGCT	IS481	FAM/MGB	150	Modified Tatti et al., 2011
	157U21P (1001-P)	AGACCCAGGGCGCACGCTGTC	pIS1001	VIC/QSY	150	Tatti et al., 2011
	HBG-P	CACTCCTGATGCTGTTATG	HBG	NED/MGB	100	Modified Mauritz et al.
	Pxd34_long (IPC-P)	AATGCCTGCGACAGCTACTGCAACTTCA	IPC	CY5/TAO	100	In-house

Controls

	Extraction Controls	Control Organism/Reagent	Comment
	NEC: Negative Extraction Control	PCR grade water	Negative extraction control is used to test the sterility of the extraction reagents.
	PEC: Positive Extraction Control	Material positive for Bordetella parapertussis	Positive extraction control is used to test the effectiveness of the extraction protocol with the organisms of interest

	PCR Controls	Control Organism/Reagent	Comment
	HBG-IS481-pIS1001 gBlock	Frozen aliquots of gBlock for HBG, B. pertussis, and B. parapertussis diluted to 2.46 x 10^2 copies/uL	See below for preparation and 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.

	Inhibition Control	Control Organism/Reagent	Comment
	IPC: Internal Positive Control	IPC gBlock diluted to 1000 copies/ul and 1 uL added to each reaction	IPC control is used to test for possible PCR inhibitors present in each patient sample. See below for preparation and sequence

gBlock Sequences

IPC gBlock (to be spiked into the master mix)
ATGAGCTGGGCATCAAGCACCGCTTTACCCGACCTTACCGCCCACAGACCAATGGGAGACAAGAATGGCGAGAAAATGCCTGC
GACAGCTACTGCAACTTCAAGTTCGGAAGGCGGCTGCCACACCTACCAGAACTCCCAACACCGAGCCGATGCCATGAAATC

PCR gBlock = HBG-IS481-pIS1001 gBlock (to be used as positive PCR control)
TGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGT
GAAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTCGCCGCGCTGTGCCATGAGCTGGGCATCAAGCACCGCTTTACCCGACCTTA
CCGCCCACAGACCAATGGCAAGGCCGAACGCTTCATCCAGTCGGCCTTGCGTGAGTGGGCTTACGCTCACACCTACCAGAACT
CCCAACACCGAGCCGATGCCATGAAATCCTGGGGGAGGCTGGCAGGGCTATGGCGTCGAACGCGTGGAATGGCCCGAAGACCC
AGGGCGCACGCTGTCGATCTATTTGAAGCCAACGGCCAAGGTGATGCTGTGCGAGCAGTGC

Reconstitute gBlocks as per the manufacturers instructions and dilute to desired 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

Safety warnings

Method Limitations:

1. The IS481 insertion element has been found to be present in some strains of *B. bronchiseptica* and most, if not all strains of *B. holmesii.*

2. Procedure for indications of when to run the PCR specific to *B. holmesii.*

3. The pIS1001 insertion sequence has been found in some strains of *B. bronchiseptica*.

4. The IPC uses the same primers as the IS481 amplicon, so a negative IPC signal when IS481 is positive is not indicative of inhibition.

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 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 PERT Mix** as per the following table. Prepare 1 mL total volume for each batch.

	Primer/Probe	Stock Concentration (uM)	Final PCR Concentration (nM)	(uL) for 1000 reactions
	481-F	100	300	60
	481-R	100	300	60
	481-P	100	150	30
	1001-F	100	150	30
	1001-R	100	150	30
	1001-P	100	150	30
	HBG-F	100	100	20
	HBG-R	100	100	20
	HBG-P	100	100	20
	IPC-P	100	100	20
	IDTE Volume			680
	Final Volume			1000

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

4 Store 20X PERT aliquots in 4 °C fridge for short-term storage and a -20 °C freezer inside 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 20X PERT Mix from the -20 °C freezer and allow to thaw.

Note: probes are light sensitive - store away from light.

7 Vortex the thawed 20X mix, then spin down quickly before use.

8 In a 1.7 mL microcentrifuge tube prepare the master mix cocktail as follows, ensuring all reagents are mixed thoroughly before pipetting into the 96-well plate.

Add the first 3 reagents in a Reagent Preparation Clean Room. The IPC gBlock is stored in the PCR Genomic Room and is added after transfer to that room.

	Reagents	1x reaction (uL)
	PCR grade water	3
	Fast Advanced Master Mix	10
	20X PERT Mix	1
	IPC gBlock (1000 copies/uL) - added in genomics room-	1

9 In a clear plastic bag, transfer the microcentrifuge tube with the first 3 ingredients of the master mix from the Reagent Preparation Clean Room to the PCR Genomic Room.

10 Remove the IPC gBlock and PCR gBlock controls from the -20 °C freezer to thaw.

See Materials section for gBlock sequences used.

11 When gBlocks are fully thawed vortex and spin down briefly.

12 Add the IPC gBlock to the master mix cocktail as per the recipe in step 20.

13 Vortex the master mix and briefly spin down.

14 Aliquot 15 µL of master mix into the required number of wells of a 96-well optical plate according to the plate map.

15 Order in rack and pipette in the following order:

1. **Initial NTC:** 5 µL of the same lot PCR grade water that was used to prepare the master mix.
2. **Patient Samples and Additional NTCs:**
   - 5 µL of each patient sample extract
   - 5 µL of additional NTCs of the same lot of PCR grade water that was used to prepare the master mix. Run approximately every 15th well.
3. **PEC*:** 5 µL of extract
4. **HBG-IS481-pIS1001 gBlock:** 5 µL
5. **NEC*:** 5 µL of extract

Note*: It is suggested to use a positive and negative extraction control as per individual laboratory practice. Please see "**Controls**" in the Materials section for more details on the PEC and NEC.

16 Apply an optical 96-well plate film to the plate using the 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 the 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

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

18 Check that the following cycling 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	

19 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.

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

	Target	Dye	Quencher
	HBG	NED	MGB
	IPC	CY5	None
	IS481	FAM	MGB
	pIS1001	VIC	None

21 Load the plate onto the ABI 7500 instrument.

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

Procedure D: 7500 FAST Run Analysis

23 Ensure that the run has completed successfully. Click OK.

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

25 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 now appear on the graph.

26 Under "Options" (at the bottom of the screen) Select "IS481"

27 Unclick Auto threshold and enter a manual value of **0.1.** Click "Auto Baseline".

28 Repeat steps 34 & 35 for targets "pIS1001", "HBG", and "IPC"

29 Click on the "Analyze" button.

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

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

32 Clinical Samples:

Examine results for the **HBG**, **IS481**, **pIS1001**, and **IPC** 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

	If Ct value for HBG is:	Then value for analysis is:
	Any Ct value	Positive
	Undetermined	Negative

	If Ct value for IS481 is:	Then value for analysis is:
	35 or lower	Positive for B. pertussis or B. holmesii*

	If Ct value for IS481 is:	Then value for analysis is:
	greater than 35 and less than or equal to 40	Indeterminate
	Undetermined	Negative

* Please see method limitation #1 in the Warning section.

	If Ct value for pIS1001 is:	Then value for analysis is:
	35 or lower	Positive for B. parapertussis
	greater than 35 and less than or equal to 40	Indeterminate
	Undetermined	Negative

	If Ct value for IPC is:	Then value for analysis is:
	Any Ct value	Positive
	Undetermined	Negative

Protocol references

1. Kamachi, K., Yoshino, S., Katsukawa, C., Otsuka, N., Hiramatsu, Y., & Shibayama, K. (2015). Laboratory-based surveillance of pertussis using multitarget real-time PCR in Japan: evidence for Bordetella pertussis infection in preteens and teens. New Microbes and New Infections, 70-74.

2. Tatti, K. M., Sparks, K. N., Boney, K. O., & Tondella, M. L. (2011). Novel Multitarget Real-Time PCR Assay for Rapid Detection of Bordetella Species in Clinical Specimens. Journal of Clinical Microbiology, 4059-4066.