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Real-time PCR amplification and genotyping of the 23s rRNA from *Bordetella pertussis* using the LightCycler 480

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We use this protocol and it's working

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Abstract

The method is an adaptation for LC480 (version II, Roche) of the one described by Kamachi *et al.,* EID 2020 (PMID: **32946738** - Supplementary Materials "Cycleave Real-Time PCR Assay for Detection of the A2047G Mutation"). qPCR targeting 23S rRNA differentiates two alleles of the ribosomal RNA of the 23S subunit of *Bordetella pertussis* (present in 3 copies per genome). The A2047G mutation distinguishes macrolide-susceptible isolates, with an A at position 2047, from macrolide-resistant *Bordetella pertussis* isolates (MRBP), with a G at position 2047.

Real-time amplification is performed from DNA extracted from a respiratory sample that has previously given a positive result for the detection of pertussis toxin (qPCR PTa, 1 copy per genome). Two distinct fluorescent probes are employed: one specific for the susceptible allele (FAM) and the other for the resistant allele (HEX). These probes specifically detect a 123 bp fragment of the 23S ribosomal RNA of *B. pertussis* and identify the A2047G mutation.

This qPCR is performed only for samples that have given a positive qPCR-PT result, which has a similar analytical sensitivity.

Attachments

MO_qPCR_23srRNA_Bp_L...
906KB

Guidelines

This qPCR is performed only for samples that have given a positive qPCR-PT result, which has a similar analytical sensitivity.

Materials

EQUIPMENT AND MATERIALS

- Sterile 1.5 mL Eppendorf tubes
- LightCycler 480 Multiwell plate 96, Roche, ref. no. 04 729 692 001
- Centrifuge for 1.5 mL Eppendorf tubes and centrifuge for microplates
- PCR refrigerated racks
- LightCycler 480 Thermal Cycler Version II, Roche

REAGENTS

- DNAaway, MBP, Ref. 038188
- LightCycler 480 Probes Master Kit, Roche, ref. no. 04 707 494 001
- Water, molecular biology quality, supplied with kit Ref. 04 707 494 001 or by default Sterile Distilled H2O (10 mL ampoule, Braun Medical B, Ref. 3511810)

POSITIVE OR STANDARD CONTROLS

Genomic DNA extracted from the **reference strain Tohama (CIP 8132),** either using the QIAGEN "DNeasy Blood & Tissue Kit (A69504)" or via the MAXWELL automatic extractor with the "Maxwell® CSC Blood DNA Kit (AS1321)". This DNA serves as a **susceptible positive control** and is used at the concentration of 100 pg/µL and 10 pg/µL. It is aliquoted to be stored between -15°C and -25°C. The tube is identified by the name of the strain and the date of aliquoting of the batch.

Genomic DNA extracted from **the FR4991 strain (CIP 112499**), either using the QIAGEN DNeasy Blood & Tissue Kit (A69504) or via the MAXWELL Automatic Extractor with the Maxwell® CSC Blood DNA Kit (AS1321). This DNA serves as a **resistant positive control** and is used at the concentration of 100 pg/µL and 10 pg/µL. It is aliquoted to be stored between -15°C and -25°C.

PRIMERS AND PROBES (HPLC quality, supplier TibMolBiol, Berlin, Germany)

They are supplied freeze-dried for "long-term" storage, protected from light, between +2°C and +8°C. Probes and primers are resuspended in H2O, molecular biology quality, and can be stored between -15°C and -25°C. Refer to the supplier's data sheets for quantities per tube.

Primers: 23S-rRNA-F and 23S-rRNA-R

23S-rRNA-F: 5'-GAATGGCGTAACGATG -3'
23S-rRNA-R: 5'- TGCAAAGCTACAGTAAAGG -3'

Probes:

23s_rRNA_SUS: 6FAM-CGGCTAGACGGAAAGACCCCA-BHQ2
23s_rRNA_RES: HEX-CGGCTAGACGGGAAGACCCCA-BHQ2

Troubleshooting

Safety warnings

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These strains (Tohama and FR4991) are deposited in the Institut Pasteur collection (CIP 8132 and CIP 112499, respectively) – https://catalogue-crbip.pasteur.fr/recherche_catalogue.xhtml – and can be ordered directly from their website.

QUALITY ASSURANCE

When the batch of the LightCycler 480 Probes Master Kit (Roche, Art. No. 04 707 494 001) expires, it can still be used as long as it is systematically revalidated with the positive amplification controls.

PRIMERS AND PROBES

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23S-rRNA-F: 5'-GAATGGCGTAACGATG -3'
23S-rRNA-R: 5'- TGCAAAGCTACAGTAAAGG -3'

Probes:

23s_rRNA_SUS: 6FAM-CGGCTAGACGGAAAGACCCCA-BHQ2
23s_rRNA_RES: HEX-CGGCTAGACGGGAAGACCCCA-BHQ2

SAMPLE PROCESSING

2 DNA from respiratory samples is extracted according to the protocol "Genomic DNA extraction with the High Pure PCR Template Preparation Kit (ROCHE) for use with the Bordetella R-gene and Parapertussis R-gene kits".

EXPERIMENTAL PROTOCOL

3 **Plate Plan**

The plate design must include:
- a negative amplification control (H2O tube, Roche kit)
- a negative H2O extraction control, if DNA extraction carried out in the lab
- positive amplification control for **susceptible *B. pertussis***: CIP 8132 at 100 pg/µL and 10 pg/µL
- positive amplification control for r**esistant *B. pertussis***: CIP 112499 at 100 pg/µL and 10 pg/µL
- samples to test (case n°1)

Case n°1
For a respiratory sample received already extracted or extracted with the procedure "Genomic DNA extraction by the High Pure PCR Template Preparation Kit (ROCHE) for use with the Bordetella R-gene and parapertussis R-gene kits", add 5 µL of the extracted DNA to the dedicated well (according to the plate plan).

4 **Preparation of the of the Assay Mix 20X**

In the reaction mix preparation room prepare the assay mix 20X

	A	B
	Reagent (final concentration)	**Volume**
	H2O	56 µL
	Primer 23srRNA-F (18 µM)	18 µL
	Primer 23srRNA-R (18 µM)	18µL
	23s_rRNA_SUS (4 µM)	4 µL
	23s_rRNA_RES (4 µM)	4 µL
	Total volume	**100 µL**

- Mix gently by aspiration/discharge with a pipette.
- Storage between -15°C and -25°C (maximum 12 months).

5 **Preparation of the reaction mixture (volumes are given for one sample)**

- Prepare the reaction mixture (by multiplying the quantity in the "Volume" column by the number of reactions to be performed, plus two additional reactions).

In a 1.5 mL Eppendorf tube:

	A	B	C
	Reagent	**Volume**	**Final Concentration**
	H2O	4 µL	
	Assay Mix 20X	1 µL	1X
	Probes Master 2X	10 µL	1X
	Total volume	**15 µL**	

- Mix gently by aspiration/discharge with a pipette.
- Place the 96-well plate in the refrigerated rack.
- Dispense **15 µL of the reaction mixture** into the wells following the plate plan.
- Cover the plate with aluminum foil to get out of the room.

- Don't forget to use the adhesive to seal the plaque later.

Reminder
When the reaction mix preparation part comes out, place the plate in the dedicated refrigerated rack.

6 **At a DNA sample repository:**

- Add **5 µL of extracted and control DNA** to the wells according to the plate plan.
- Immediately cover the plate with the appropriate adhesive.

7 **In PCR room where the thermal cycler is located:**

- Centrifuge the plate for 1 minute at about 1000 g at room temperature.
- Put the plate in the LightCycler 480 and start the "qPCR 23s rRNA" program

8 **Amplification Program**

Program Name	pre-incubation						
Cycles	1	Analysis Mode	None				
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:10:00	4.40		0	0	0
Program Name	amplification						
Cycles	30	Analysis Mode	Quantification				
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
95	None	00:00:10	4.40		0	0	0
60	Single	00:01:00	2.20		0	0	0
72	None	00:00:01	4.40		0	0	0
Program Name	cooling						
Cycles	1	Analysis Mode	None				
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp Rate (°C/s)	Acquisitions (per °C)	Sec Target (°C)	Step size (°C)	Step Delay (cycles)
40	None	00:00:30	2.20		0	0	0

DATA ANALYSIS

9 The analysis of the bacterial target is performed in **"Endpoint Genotyping"** mode, with FAM (465-510 nm filter) and HEX (533-580 nm filter) readout.

In the **"Sample Editor"** tab, negative and extraction controls should be set as **"Negative Control",** and positive controls as **"Standards"** in the **"EndPt Sample Type" column**.

- For standards, add **"susceptible"** for **CIP 8132** at 10 and 100 pg/µL **to the "**EndPt Genotype" column, and **"resistant"** for **FR4991** at 10 and 100 pg/µL.
- The samples to be tested must be set as **"Unknown"** in the **"Sample Type" column**.
- Finally, carry out the analysis.

Example:

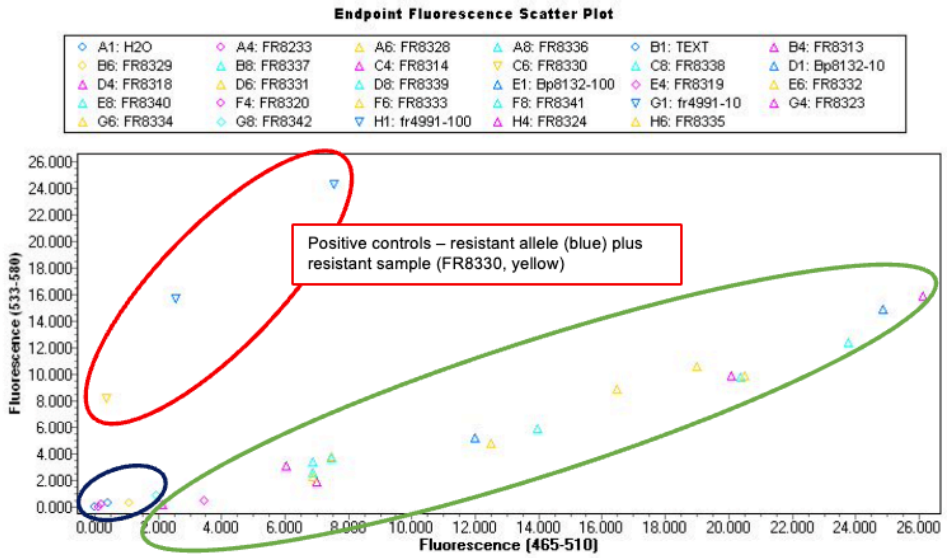
Results

Inc	Pos.	Sample Name	Endpoint Fluorescence Allele X	Endpoint Fluorescence Allele Y	Call	Score	Status
☑	A1	H2O	0.41	0.35	Negative		
☑	A4	FR8233	0.22	0.16	Negative		
☑	A6	FR8328	7.47	3.79	SENSIBLE	0.90	
☑	A8	FR8336	6.89	3.42	SENSIBLE	0.91	
☑	B1	TEXT	0.00	0.00	Negative		
☑	B4	FR8313	2.12	0.23	SENSIBLE	0.76	
☑	B6	FR8329	1.07	0.33	Negative		
☑	B8	FR8337	13.95	5.88	SENSIBLE	1.00	
☑	C4	FR8314	6.99	1.94	SENSIBLE	0.83	
☑	C6	FR8330	0.38	8.18	RESISTANT	0.80	
☑	C8	FR8338	20.40	9.79	SENSIBLE	0.94	
☑	D1	Bp8132-10	11.99	5.24	SENSIBLE	0.98	
☑	D4	FR8318	20.08	9.95	SENSIBLE	0.92	
☑	D6	FR8331	20.49	9.93	SENSIBLE	0.93	
☑	D8	FR8339	6.88	2.63	SENSIBLE	0.96	
☑	E1	Bp8132-100	24.89	14.88	SENSIBLE	0.81	
☑	E4	FR8319	3.44	0.53	Unknown	0.44	
☑	E6	FR8332	12.51	4.84	SENSIBLE	0.97	
☑	E8	FR8340	7.47	3.69	SENSIBLE	0.92	
☑	F4	FR8320	0.12	0.04	Negative		
☑	F6	FR8333	16.49	8.88	SENSIBLE	0.87	
☑	F8	FR8341	23.79	12.37	SENSIBLE	0.89	
☑	G1	fr4991-10	2.57	15.70	RESISTANT	0.95	
☑	G4	FR8323	26.13	15.92	SENSIBLE	0.79	
☑	G6	FR8334	6.87	2.34	SENSIBLE	0.91	

Negative Controls

Positive controls – susceptible allele

Positive controls – resistant allele



The final conclusion for a sample will be determined based on the alleles called. In general, the "**endpoint fluorescence"** for a susceptible sample is always higher for the susceptible allele (FAM) than for the resistant allele (HEX), and vice versa for a resistant sample.

It is important to note that for samples with cycle thresholds (Ct) greater than 35 in the PTa qPCR, the majority will be categorized as negative in the qPCR 23S rRNA PCR.

In all cases, the final decision on the interpretation of the results is taken by the NRC (deputy) head, who are also responsible for the diagnosis made.

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a **resistant positive control** and is used at the concentration of 100 pg/µL and 10 pg/µL. It is aliquoted to be stored between -15°C and -25°C.

Protocol references

Kamachi K, Duong HT, Dang AD, Hai T, Do D, Koide K, Otsuka N, Shibayama K, Hoang HTT. Macrolide-Resistant Bordetella pertussis, Vietnam, 2016-2017. Emerg Infect Dis. 2020 Oct;26(10):2511-2513. doi: 10.3201/eid2610.201035. PMID: 32946738; PMCID: PMC7510698.