

**Bordetellen -TaqMan PCR**

SOP Nr: MB\_SA\_BORD\_TAQ Version: 01

Gültig ab: 01.02.2003

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## 1 Intended Use

This PCR is intended to detect *Bordetella pertussis*- and *Bordetella parapertussis*-specific DNA from nasopharyngeal swabs.

## 2 Summary and Explanation of the test

Amplification and detection of *Bordetella*-specific DNA is based on the TaqMan® format.

Extracted DNA is amplified by PCR. During annealing both specific primers and a specific fluorogenic probe hybridize to the amplified sequence. The fluorogenic probe is cleaved during the simultaneous extension (two-step-PCR) by the 5'-3' exonuclease-activity of the Taq-polymerase, thereby increasing the fluorescence signal. If this signal surpasses a cut-off value, which is calculated by the machine's software, the sample will be regarded as "positive" and the corresponding PCR-cycle is defined as "threshold cycle" ( $C_T$ ).

## 3 Samples

Nasopharyngeal swabs, also sputum samples

Storage of samples: the samples are analysed immediately after arrival, or stored at -20°C.

## 4 Reagents, apparatus

- 10 x PCR buffer (Qiagen)
- ROX solution (ABI)
- MgCl<sub>2</sub>, 25 mM (Qiagen)
- Nukleotide Set, PCR-grade, 100 mM (Roche)
- HotStar Taq DNA Polymerase 5 U/μl (Qiagen)
- twin.tec PCR plate 96 (Eppendorf)
- MicroAmp Optical Caps (ABI)
- Bench centrifuge 5417 C with rotor F-45-30-11 (Eppendorf)
- ABI Prism 7700 SDS (with thermocycler 9600) (ABI)

Pertussis PCR:

- Forward primer (Sense 2)
- Reverse primer (ASense 2)
- Fluorogenic probe (Pert Probe 2)

Parapertussis PCR:

- Forward primer (Para s 1)
- Reverse primer (Para as 1)
- Fluorogenic probe (Para Probe 1)

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**Primers/probe specification for B.pertussis:**

Primers and probe are taken from the IS 481-sequence

**Primer** Sense 2: 5` - ATC AAg CAC CgC TTT ACC C

**Primer** Asense 2: 5` - TTg ggA gTT CTg gTA ggT gTg

**Probe** (5'-reporter dye: FAM; 3'-quencher dye: TAMRA) Pert-Probe 2:

5` - FAM- AAT ggC AAg gCC gAA CgC TTC A

Accession number:

Bordetella pertussis Tohama I: ATCC BAA-589, NCTC 13251

```

GGGACGTATC CAGCGCCCTG GCCACCGGGT CACGGGCAAC CGACGCGATA CCGTTGAGGG 600
GGCCGGCTGG GACTTCGTCT TCGTGGCCAT CGATGACCAC GCCCGCGTGG CCTTCACCGA 660
CATCCCCCCC GACGAGCGCT TCCCCAGCGC CGTCCAGTTC CTCAAGGACG CAGTGGCCTA 720
CTACCAGCGC CTGGGCGTGA CCATCCAGCG CTTGCTCACC GACAATGGCT CGGCCTTTTCG 780
CAGCCGCGCC TTCGCCGCGC TGTGCCATGA GCTGGGCATC AAGCACCCTTACCCGACC 840
TTACCGCCCA CAGACC AATG GCAAGGCCGA ACGCTTCAATC CAGTCGGCCT TGCCTGAGTG 900
GGCTTACGCT CACACCTACC AGAACTCCCA ACACCGAGCC GATGCCATGA AATCCTGGCT 960
ACACCACTAC AACTGGCATC GACCCACCA AGGCATCGGG CGCGCTGTAC CCATCTCCAG 1020
ACTCAACCTG GACGAATACA ACCTATTGAC AGTTCACAG TATCCGGACC GGC(nt772744)1073

```

**Primers/probe specification for B.parapertussis:**

Primer and probe are taken from the IS1001-sequence

**Primer** Para s 1: 5` - gAT ATC AAC ggg TgA Cgg ATC

**Primer** Para as 1: 5` - gTA TgC CAA CCC AgT TCg AA

**Probe** (5'-reporter dye: FAM; 3'-quencher dye: TAMRA) Para-Probe 1:

5` - FAM- TgC TgC AAT CgA gCA ACg TgC A

Accession number:

Bordetella parapertussis 12822: ATCC BAA-587, NCTC 13253

```

CAGGAGTGCAGGGAGATGCTGGATCGCAAGTTGATGGAGTCGCTGGGAGGCTGGCAGGGCTATGGCGTCGAACGC
GTGGAATGGCCGAAGACCCAGGGCGCACGCTGTTCGATCTATTTGAAGCCAACGGCCAAGGTGATGCTGTGCGAG
CAGTGCGGCGCGCGGTGTCCGAGGTGCATGAGACCACGGTTCGACGGGTGCGAGATCTGCCGATATTCGAGTAT
CGGGTCGTTCTGCACGTGCCGCGCCGACGCTTGTGGTGTGAGCAATGCGGCGGCCCGCCTGGAGCGGCTTGCC
TGGCTGGGGC GATATCAACGGGTGACGGATCGGCTGGCGCAGGCCTGCAGCCAAT TCGTGCATTCGAGCAACGTC
CAGGCGGTGGCGAGGTTTC TCGAACTGGGTGGCATAACGCTCAAGACGCTGGACAAGGCTCGGCTGCGTGCGTCG
GTGCGCGAACCAGGATTGGTCCAAGATCGAGTATTTGGCGATGGACGAGTTTGCCTGCACAAAGGGCATCGCTAC
GCGACAGTGGTGGTTCGATCCGATCGGCAGGCAGGTGCTGTGGATTGGCCAGGACGCTCACGC

```

**4.1 Storage of reagents**

	storage	shelf life
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10fold PCR buffer	at – 20 °C	until expiry date
ROX-solutions	undiluted at 4 °C diluted at –20 °C	
MgCl <sub>2</sub>	at – 20 °C	until expiry date
Nucleotides	undiluted at – 20 °C as mix at – 20 °C	until expiry date until expiry date
Probes	lyophilised at 4 °C diluted at – 20 °C	
Primers	lyophilised at 4 °C diluted at – 20 °C	
A. bidest	room temperature	until expiry date
Controls and extracts of controls	at – 80 °C	

## 5 Quality control

In every run, positive controls (low and high positive) and no template controls (NTC) are analysed.

## 6 Test procedure

### 6.1 Preparation of reagents

- Nucleotide mix: 30 µl of nucleotides dATP, dCTP, dGTP, dTTP each are mixed with 1080 µl A. bidest
- Primer master solution: dissolve primers in A.bidest to get a concentration of 50 µM (pmol/µl).
- Primer working solution: dilute primer master solution 1 : 5 with A. bidest to get a concentration of 10 µM (pmol/µl)
- Probe working solution: dissolve with A. bidest to get a concentration of 2 µM (pmol/µl).
- ROX-solution: The concentration of ROX working solution must be set to give a fluorescence signal of ROX in the multicomponent-display between 2000 – 3000 Units as follows:
- ROX is diluted with A. bidest:

Aqua bidest (µl)	30	31	32	33	34	35	35,4	36
Rox (µl)	8	7	6	5	4	3	2,6	2

Rox (µl/test)	0,4	0,35	0,3	0,25	0,2	0,15	0,13	0,1
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1,9 µl of the Aqua bidest. ROX-mixture is given to a regular PCR mix in duplicate.

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5 µl of water are added instead of a DNA extract

Fluorescence intensity is measured with an abridged thermal cycler program:

10 min at 95 °C  
 5 cycles like HCV-PCR

## 6.2 Preparation of samples

Samples and controls can be prepared by the following kits:

- QIAamp min elute virus vacuum kit
- QIAamp virus BioRobot MDx kit
- QIAamp DNA mini kit

## 6.3 Controls

### 6.3.1 Positive controls

In every PCR run a high positive (Ct appr. 25) control (bacterial suspension) and a low positive control (Ct appr. 35) are included.

### 6.3.2 No template control (NTC)

In every run three PCR mixes with water instead of DNA are included (in triplicate for Pertussis and in triplicate for Parapertussis).

## 6.4 Procedure of test

1. Calculate the amount of reagents needed for pertussis PCR and parapertussis PCR

### 6.4.1 Working area I (M i x)

2. Cover working area with sterile tissue.
3. Bring all reagents to room temperature.
4. Pipet master mix according to following table (Volumes are set for one sample):

**For Pertussis PCR:**

Reagent	Concentration	Volume (µl)	Final concentration
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Taq Puffer	10 x	5	1x
MgCl <sub>2</sub>	25 mM	8	4 mM
Nucl.-Mix	2,5 mM	4	0,2 mM
forw. Primer (Sense 2)	10 pmol/μl	1,5	0,3 mM
rev. Primer (ASense 2)	10 pmol/μl	1,5	0,3 mM
Probe (Pert-Probe 2)	2 pmol/μl	0,75	0,3 mM
Hot Star Taq	5 U/μl	0,25	1,25 U
Aqua bidest. plus ROX		24	

**For Parapertussis PCR:**

Reagent	Concentration	Volume (μl)	Final concentration
Taq Puffer	10x	5	1 x
MgCl <sub>2</sub>	25 mM	8	4 mM
Nucl.-Mix	2,5 mM	4	0,2 mM
Forw. Primer (Para s 1)	10 pmol/μl	1,5	0,3 mM
Rev. Primer ( Para as 1)	10 pmol/μl	1,5	0,3 mM
Probe (Para Probe 1)	2 pmol/μl	0,75	0,3 mM
Hot Star Taq	5 U/μl	0,25	1,25 U
Aqua bidest. Plus ROX		24	

5. Mix well

6. Give a 45 μl aliquot into the twin.tec PCR reaction plate.

**6.4.2 Working area III (S a m p l e p i p e t t i n g )**

7. Add 5 μl of DNA extracts (i.e. samples, controls) or water for NTC

8. Close reaction plate with optical caps

9. Shake carefully.

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**6.4.3 Working area IV (A m p l i f i c a t i o n)**

10. Put plate into ABI Prism 7700 and start program.

Thermocycler conditions

Hold	95 °C	15 min	
Cycle	95 °C	15 s	} 45 x
	57 °C	60 s	

**7 Results****7.1 Definitions**

R<sub>n</sub> (normalized reporter signal): Quotient of the emission intensity of the reporter dye (FAM) divided by the emission intensity of the passive reference dye (ROX).

ΔR<sub>n</sub>: R<sub>n</sub>-value minus background signal

C<sub>T</sub>- value: number of cycle in which the reporter fluorescence crosses the cut off line (threshold)

Threshold: standard deviation of normalized measured value of cycles 3 to 15 multiplied by 10.

**7.2 Validation of results**

NTC-runs should have no positive signal (contamination control).

Both positive controls must be positive.

**7.3 Interpretation of results**

Samples are regarded as "positive", if the ΔR<sub>n</sub>-value surpasses the threshold during the 45 PCR-cykles and if it shows a typical amplification curve of a positive PCR-run.

Samples are regarded as "negative", when their threshold-cycle is 45.

**8 Other documents needed**

Pipetting plan Bordetella TaqMan PCR

Reference: K.Kösters et al: Evaluation of a real-time PCR assay for detection of Bordetella pertussis and B. parapertussis in clinical samples.  
J. Med. Microbiol. 50, 436-440 (2001)

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