

**Bordetellen-LC-PCR**

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## 1 Intended Use

This PCR is intended to detect *Bordetella pertussis* and *B. paraptussis*-DNA in nasopharyngeal swabs and other respiratory materials

The procedure is based on separate detection of *B. pertussis* and *B. paraptussis*-Genomes (simplex PCR). It is also possible to detect both bacteria by duplex PCR; however, this reduces the sensitivity (see references).

## 2 Summary and explanation of the test

Extracted DNA is amplified in a single reaction by PCR. Both primers and both fluorogenic probes hybridize during annealing with the target sequence. Detection is done by FRET (fluorescence resonance energy transfer), which means that both probes hybridize in very close position and energy is transferred from fluorescein to LightCycler-RED 640. The fluorescence emitted from LightCycler-RED 640 is measured continuously in every cycle.

The PCR-product of the *B. pertussis*-Genome is a 181 bp fragment of IS 481, and a 464 bp fragment of IS 1001 from *B. paraptussis*, respectively.

Analysis is done by melting curves.

## 3 Samples

Nasopharyngeal swabs, also sputum

Storage of samples: Samples are extracted immediately or stored at -20°C.

## 4 Reagents, instruments, and material

### 4.1 Reagents and instruments

- LightCycler - FastStart DNA Master Hybridization Probes (Roche):
  1. LightCycler - FastStart Enzyme (1a red lid): 15 x 64 µl (10x) ready to use „Hot Star“ reaction mix for PCR after mixing solution 1b and 1a
  2. LightCycler – FastStart Reaction Mix Hybridization Probes, 10x conc. (1b transparent lid): FastStart Taq DNA polymerase, reaction buffer, dNTPs (with dUTP instead of dTTP) and 10 mM MgCl<sub>2</sub>
  3. MgCl<sub>2</sub> 25 mM (2 blue lid): 2 x 1 ml
  4. Aqua dest (3 transparent lid): 7 x 1 ml
- Pertussis:
  - Forward Primer (BP-1)
  - Reverse Primer (BP-2)
  - Probe (BP-FLU)
  - Probe (BP-LCR)

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- Parapertussis:
  - Forward Primer (BPa-1)
  - Reverse Primer (BPa-2)
  - Probe (BPa-HP-1)
  - Probe (BPa-HP-2)
- LightCycler Capillaries (Roche)
- Desk centrifuge 5417 C with rotor F-45-30-11 (Eppendorf)
- 1,5 ml eppendorf caps sterile (Eppendorf)
- LightCycler Centrifuge Adapters: 32 Adapters in alum cooling block (Roche)

**Primers/Probes specification Pertussis:**

Primers and probes are taken from IS 481 of B.pertussis

**Primer** BP-1: 5` - gAT TCA ATA ggT TgT ATg CAT ggT T**Primer** BP-2: 5` - TTC Agg CAC ACA AAC TTg ATg ggC g**Probe** (Fluorescein): BP-FLU: 5` - TCg CCA ACC CCC CAg TTC ACT CA-F**Probe** (LC-Red 640): BP-LCR: 5' - LC-RED-640-AgC CCg gCC ggA TgA ACA CCC P

Accession Number:

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

771672 Genom-NT , Sanger Institute

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GCGAGGCCGG CTATCTGTGA AGATTCAATA GGTGTATGC ATGGTTCATC CGAACCCGGAT      60
TTGAGAAACT GGAAATCGCC GACCCCCCAG TTCACTCAAG GAGCCCGGCC GGATGAACAC      120
CCATAAGCAT GCCCGATTGA CCTTCCTACG TCGACTCGAA ATGGTCCAGC AATTGATCGC      180
CCATCAAGTT TGTGTGCCTG AAGCGGCCCG CGCCTATGGG GTCACCGCGC CGACTGTGCG      240
CAAATGGCTG GGCCGCTTCC TGGCTCAGGG CCAGGCGGGC TTGGCCGATG CGTCCTCGCG      300
CCCGACGGTC TCGCCCCGAG CGATTGCGCC GGCCAAGGCG CTGGCTATCG TGGAGCTGCG      360
CCGCAAGCGG CTGACCCAAG CGCGCATCGC CCAGGCGCTG GGCGTGTGAG CCAGCACCGT      420
CAGCCGCGTC CTGGCCCGCG CCGGTCTGTC GCACCTGGCC GACCTGGAGC CGGCCGAGCC      480

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**Primers/Probes specification Parapertussis:**

Primer and probe are taken from the IS1001 of B.parapertussis

**Primer** Bpa-1: 5` - CAC CgC CTA CgA gTT CgA gAT

**Primer** Bpa-2: 5` - CCT CgA CAA TgC Tgg TgT TCA

**Probe** (Fluorescein) BPa-HP-1: gTT CTA CCA AAg ACC TgC CTg ggC-F

**Probe** (LC-RED 705): BPa-HP-2: LCR-AgA CAA gCC Tgg AAC CAC Tgg TAC P

Accession Number:

Bordetella parapertussis 12822: ATCC BAA-587, NCTC 13253

TGCGGAACCGGATTGGTCCAAGATCGAGTATTTGGCGATGGACGAGTTTGCCCTGCACAAAGGGCAT  
 CGCTACGCGACAGTGGTGGTTCGATCCGATCGGCAGGCAGGTGCTGTGGATTGGCCCAGGACGCTCACG  
 CGAGACGGCCCGGGCGTTCTTCGAACAATTGCCGCTGGGGCCGCCAACGCATCAAGGCCGTTGCCA  
 TCGACATGAC**CACCGCTACGAGTTGGAGAT**CCAGGCCACAGCCACAGGCGGAGATCGTCTATGAC  
 TTGTTCCATGTTCGTGGCCAAGTATGGACGAGAGGTCATTGATCGGGTGGCGTGGATCAGGCCAATCA  
 ACTACGCCAGGATCGTCCCGCACGCAGGATCATCAAATCGAGTCGCTGGCTGCTGCTGCGCAACCGTG  
 ACAACCTGGATCGGCAGCAGGCCGTCCGGCTCGACGAATTGCTGCAAGCCAACCAGCCGCTGCTGACG  
 GTCTATGTCTGCGTGACGAACCTCAAACGGCTCTG**GTTCTACCAAAGACCTGCCTGGGC****AAGACAAGC**  
**CTGGAACCACTGGTAC**GAGCAGGCCGAGCAAAGCGGAATAGCCGCCTTGAACACCTTCGCTCAGCGCT  
 TGAAAGGCTATCTGCACGGCATCTGGCCAGATGCCGACATCCCC**TGAACACCAGCATTGTCGAGG**GC  
 ATCAACAACACTATCAAGGTCATCAAGCGGCGCGCTTACGGCTACCGCGACCAGGAATACTTCTTCTCCT  
 CAAAATCCGTGCCGCTTCCCCGGCAATGCGCGATGAACC

**4.2 Storage of reagents:**

	storage	Shelf life
Fast start enzyme (1a)	-15°C to -25°C	Until expiry date
Fast start reaction mix hybridisation probes (1b)	-15°C to -25°C	Until expiry date
FastStart DNA Master Hybridisation Probes (1a + 1b)	-15°C to -25°C, do not refreeze, protect from light! After thawing 2-8°C	3 months  1 week
MgCl <sub>2</sub>	-15°C to -25°C	Until expiry date
Primers und probes	lyophilised at 4 °C reconstituted: --15°C / -25°C	
A. bidest	Room temperature	Until expiry date
Control material, extracts	- 20 °C or - 80 °C	

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## 5 Quality control

Positive controls (low and high positive) and a no template control (NTC) is analyzed in every run.

## 6 Procedure of test

### 6.1 Sample preparation

Nucleic acids from respiratory samples is prepared with either:

QIAamp Min Elute Virus Vacuum-Kit

QIAamp Virus BioRot MDx Kit

or QIAamp DNA Mini Kit

#### 6.1.1 Positive controls

In every run, a high positive (Crossing point ca. 25) control (bacterial suspension) and a low positive (Crossing point ca. 35) control is analysed.

#### 6.1.2 Negative control

Per run at least one PCR-assay is done with water instead of DNA-extract.

### 6.2 Preparation of reagents

- „Hot Start“ reaction mix: 1a and 1b thaw tubes, centrifuge briefly, transfer 60 µl from 1b tube (LightCycler – FastStart Reaction Mix Hybridization Probes) into tube 1a (LightCycler – FastStart Enzyme) and mix carefully. DO NOT VORTEX ! Attach label: LightCycler – FastStart DNA Master Hybridization Probes to tube, and put red label with #1 on the lid.
- Primer BP-1, BP-2, Bpa-1 and Bpa-2 are diluted to 10 pmol/µl with Aqua dest
- Probe BP-FLU, BP-LCR, BPa-HP-1 and BPaHP-2 are diluted to 4 pmol/µl with Aqua dest

### 6.3 Assay procedure

1. Calculate amount of reagents needed.

#### 6.3.1 Working area I ( Mix )

2. Cover working area with sterile tissue.
3. Bring all reagents to room temperature, put them into the cooling block and put needed capillaries into the centrifuge adapter of the cooling block (Touch capillaries with gloves only !)
4. Pipet master-mix according to the following scheme in the cooling block (Volumes are meant for one sample):

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Master-mix for Bordetella pertussis-PCR:

Reagent	Volume	Final concentration
Aqua dest (transparent lid )	6,6 µl	
DNA Master (red lid)	2 µl	
MgCl <sub>2</sub> (blue lid) 25 mM	2,4 µl	4 mM
Primer BP-1 (10 pmol/µl)	1 µl	0,5 pmol/µl
Primer BP-2 (10 pmol/µl)	1 µl	0,5 pmol/µl
Probe BP-FLU (4 pmol/µl)	1 µl	0,2 pmol/µl
Probe BP-LCR (4 pmol/µl)	1 µl	0,2 pmol/µl
<b>Total volume</b>	<b>15 µl</b>	

Master-mix for Bordetella parapertussis-PCR:

Reagents	Volume	Final concentration
Aqua dest (transparent lid )	6,6 µl	
DNA Master (red lid)	2 µl	
MgCl <sub>2</sub> (blue lid) 25 mM	2,4 µl	4 mM
Primer BPa-1 (10 pmol/µl)	1 µl	0,5 pmol/µl
Primer BPa-2 (10 pmol/µl)	1 µl	0,5 pmol/µl
Probe BPa-HP-1 (4 pmol/µl)	1 µl	0,2 pmol/µl
Probe BPa-HP-2 (4 pmol/µl)	1 µl	0,2 pmol/µl
<b>Total volume</b>	<b>15 µl</b>	

5. Mix carefully.
6. Pipet 15 µl aliquot into LightCycler capillaries
7. For negative control pipet 5 µl Aqua dest into a capillary and seal with stopper.

**6.3.2 Working area III ( Pipetting )**

8. Add 5 µl DNA-extract (Patient samples, positive controls)
9. Seal capillaries with plugs. Put capillaries with inset into the centrifuge run at 3000 rpm for 5 sec
10. Put capillaries into sample rotor.

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Sequence of controls and patients:

1. Negative control Bordetella pertussis-PCR
2. Patient 1 and following patients Bordetella pertussis-PCR
3. Positive controls Bordetella pertussis-PCR
4. Negative control Bordetella parapertussis-PCR
5. Patient 1 and following patients Bordetella parapertussis-PCR
6. Positive controls Bordetella parapertussis-PCR

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

11. Put sample rotor into LightCycler and start instrument.

Thermocycler conditions for Bordetella pertussis and B. parapertussis:-PCR:

## Program 1 Denature

<b>Cycle Program Data</b>	<b>Value</b>
Cycles	1
Analysis Mode	None
<b>Temperatures Targets</b>	<b>Segment 1</b>
Target Temperature (°C)	95
Incubation Time (hrs:min:sec)	0:10:00
Temperature Transition Rate (°C/sec.)	20.0
Second Target Temperature (°C)	0
Step Size (°C)	0.0
Step Delay (Cycles)	0
Acquisition Mode	None

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## Program 2: Amplification

<b>Cycle Program Data</b>	<b>Value</b>		
Cycles	40		
Analysis Mode	Quantification		
<b>Temperatures Targets</b>	<b>Segment 1</b>	<b>Segment 2</b>	<b>Segment 3</b>
Target Temperature (°C)	95	60	72
Incubation Time (hrs:min:sec)	10	10	20
Temperature Transition Rate (°C/sec.)	20.0	20.0	20.0
Second Target Temperature (°C)	0	0	0
Step Size (°C)	0.0	0.0	0.0
Step Delay (Cycles)	0	0	0
Acquisition Mode	None	Single	None

## Program 3: Melting curve:

<b>Cycle Program Data</b>	<b>Value</b>		
Cycles	1		
Analysis Mode	Melting Curve		
<b>Temperatures Targets</b>	<b>Segment 1</b>	<b>Segment 2</b>	<b>Segment 3</b>
Target Temperature (°C)	95	45	95
Incubation Time (hrs:min:sec)	0	30	0
Temperature Transition Rate (°C/sec.)	20.0	20.0	0.1
Second Target Temperature (°C)	0	0	0
Step Size (°C)	0.0	0.0	0.0
Step Delay (Cycles)	0	0	0
Acquisition Mode	None	None	Cont.

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Program 4: Cooling:

<b>Cycle Program Data</b>	<b>Value</b>
Cycles	1
Analysis Mode	None
<b>Temperatures Targets</b>	<b>Segment 1</b>
Target Temperature (°C)	40
Incubation Time (hrs:min:sec)	0:02:00
Temperature Transition Rate (°C/sec.)	20.0
Second Target Temperature (°C)	0
Step Size (°C)	0.0
Step Delay (Cycles)	0
Acquisition Mode	None

## 7 Results

Analysis data are interpreted according to the amplification plot and the melting curve.

Bordetella pertussis PCR is analysed in fluorescence channel F2 (channel for LC-Red 640), Bordetella parapertussis PCR is analysed in fluorescence channel F3 (channel for LC-Red 705).

### 7.1 Validation criteria

B.pertussis positive controls must show a typical amplification plot and a melting point at 60°C +/- 2.5°C

Bordetella parapertussis positive controls must show a typical amplifikation plot and a melting point at 65°C +/- 2.5°C

Negative control must show a negative amplification plot.

### 7.2 Interpretation of results

Samples are regarded as "positive", when the fluorescence signal increases and shows a typical amplification kinetic.

Positive samples are also characterised by the typical melting point:

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Results	# of melting points	Temperature of melting point
B. pertussis	1	60°C +/- 2.5°C
B. parapertussis	1	65°C +/- 2.5°C

Samples are regarded as "negative", when they do not fulfil the criteria mentioned above.

Equivocal results may be repeated with the PCR in TaqMan-Format (MB\_SA\_BORD\_TAQ english).

## 8 Other documents

Pipetting plan Bordetella LC-PCR

Package insert

Literature:

K.Kösters et al

Real-Time LightCycler PCR for Detection and Discription of Bordetella pertussis and Bordetelle parapertussis.

J. Med. Microbiol. 40, 1719-1722 (2002)

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