TECHNICAL DOCUMENT

Guidance and protocol for the use of real-time PCR in laboratory diagnosis of human infection with *Bordetella pertussis* or *Bordetella parapertussis*

As part of the EUpert-Labnet surveillance network

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ECDC TECHNICAL DOCUMENT

Guidance and protocol for the use of real-time PCR in laboratory diagnosis of human infection with *Bordetella pertussis* or *Bordetella parapertussis*

As part of the EUpert-Labnet surveillance network

The report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Adoración Navarro Torné and produced by the members of the European *Bordetella* expert group 'EuPert-Labnet', as part of the project contract *Coordination of activities for laboratory surveillance of whooping cough in Member States and EEA countries* (OJ/26/05/2011-PROC/2011/037).

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Intended use

Guidance and protocol for the use of real-time PCR for diagnosis of whooping cough in EU Member States and EEA countries.

The guidance can be used for several different real-time formats, while the protocol is intended for use with the LightCycler® capillary based system. The protocol can serve as a basis on which to develop an in-house real-time PCR method.

This protocol is intended to serve as a starting point for laboratories aiming to introduce real-time PCR methods for the diagnosis of human *Bordetella (pertussis)* infections. Depending on the PCR apparatus available in laboratories, the protocol will need to be adapted.

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Disclaimer: this technical guidance is based on the most recently published data on diagnostic test performance available at the time of writing. ECDC does not endorse any particular commercial product or instrument.

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Contents

1 Introduction

Whooping cough is a bacterial respiratory infection caused by *Bordetella pertussis*. It is characterised by paroxysmal cough, whoop and post-tussive vomiting. It is spread through air droplets produced by coughs or sneezes. The most severe disease occurs in infants and young children, while in adolescents and adults, who constitute a reservoir and are a source of the infection spreading to young children, the disease is usually milder. Pertussis remains endemic worldwide and tends to be a cyclic disease, peaking every three to five years.

Since 2011, the reported number of pertussis cases has increased in different regions of the world, even in those with sustained high vaccination coverage. In Europe, the situation is similar, with many countries observing an increase in cases, mostly in infants, adolescents and adults.

Laboratory diagnosis of whooping cough is difficult due to variations in the specificity and sensitivity of the different methods. Differences in methods and protocols among European countries have also been detected.

ECDC is addressing the harmonisation and improvement of pertussis diagnosis for surveillance and outbreak detection/monitoring in order to assure quality and comparability of data. In 2011, ECDC launched the project 'Coordination of activities for laboratory surveillance of whooping cough in Member States and EEA countries'. One of the main aims of this project was to produce a consensus document on laboratory guidance relating to the use of real-time PCR on DNA extracted from clinical specimens obtained from patients with suspected whooping cough.

The guidance and protocol are intended for real-time PCR on DNA extracted from clinical specimens obtained from patients with suspected whooping cough (i.e. infection with *Bordetella pertussis* or *B. parapertussis*). Suitable samples are either nasopharyngeal swabs (NPS) or nasopharyngeal aspirates (NPA) which have been used to sample specimens from the posterior nasopharynx of a patient.

As other *Bordetella* species such as *B. holmesii* and *B. bronchiseptica* can also be detected by PCR from DNA extracted from human NPS or NPA, and several amplification targets currently used for *Bordetella* are present in more than one *Bordetella* species, it is imperative that PCR results are interpreted correctly.

2 Samples

2.1 Indications for sampling

Clinical symptoms of pertussis are typical in non-vaccinated children and include coughing spasms, whooping and vomiting. However, cases in neonates and unvaccinated infants often present with apnea as the only symptom. *B. pertussis* infections in older children, adolescents and adults represent non-primary contacts with pertussis antigens, and thus the symptoms can vary widely. These are often atypical and may only present as prolonged coughing without other typical indications [1]. Consequently, the clinical diagnosis must be confirmed by laboratory tests.

As the sensitivity of PCR decreases significantly after three to four weeks of symptoms [2], it is important to perform the sampling for PCR in the early stages of the disease (during the first three weeks after the beginning of the cough). If the individual has experienced symptoms for more than four weeks, serology (IgG-anti PT test) is recommended [3] or a PCR from secondary cases since the disease is highly contagious.

2.2 Sampling techniques

It is very important to sample for a PCR test by collecting either a posterior nasopharyngeal swab or a nasopharyngeal aspirate. Throat swabs or anterior nasal swabs should not be taken since the *B. pertussis* bacteria are mainly attached to the ciliated epithelium in the nasopharynx.

The recommended procedure for swabbing is sampling through the nostril using a thin flexible swab. Swabs made of dacron or rayon can be used, but swabs made of cotton or calcium alginate are not suitable for use with PCR techniques [3].The Copan flexible twisted wire swab is frequently used and can be recommended. Gloves should be used to avoid contamination. Examples of suitable transport media for the swabs are Amies medium with charcoal and Stuart's medium [4].

A video of the recommended sampling technique is available on YouTube: http://youtu.be/d6d-y7SX_dY or http://www.youtube.com/watch?v=d6d-y7SX_dY&feature=youtu.be (Title: '*Prelevcoqueluche-desktop.m4v*' produced by Institut Pasteur, Paris, France).

If using swabs in liquid transport media it is imperative to avoid contamination of the swab shaft since such contamination can be transferred to the medium. See the following link on best practices: http://www.cdc.gov/pertussis/clinical/diagnostic-testing/diagnosis-pcr-bestpractices.html.

As some pertussis vaccines may contain *B. pertussis* DNA, it is recommended that vaccinations and specimen sampling be carried out in different rooms [5].

2.3 DNA extraction

Several commercial kits are available for DNA extraction. In particular, the QIAamp DNA mini kit (Qiagen) and High Pure PCR template preparation kit (Roche) are frequently used [4, 6-10]. Extraction using the Roche MagNA Pure system (Roche) [11] or by Chelex are also suitable options.

3 PCR apparatus

3.1 Real-time PCR

Real-time PCR has several advantages compared to the traditional qualitative block-based PCR using agarose gels and ethidium bromide staining under UV light for visualisation of results.

Real-time PCR is less time consuming (both in terms of technical workload and turnaround time as there is no need for analysis of gels after the PCR run) and can detect lower concentrations of DNA than the gel-based detection methods. Moreover, the results can be monitored during the run. With real-time PCR there are no post-PCR manipulations, thereby avoiding potential DNA contamination with opened tubes containing high concentrations of amplicons. An additional advantage is that real-time PCR also allows for quantitative determination of DNA concentrations.

Real-time PCR facilitates specific confirmation of the amplified target. Sequence-specific detection can be performed by dual-labelled hybridisation probes (fluorescence resonance energy transfer, FRET), hydrolysis probes (TaqMan technology) or molecular beacons. Although non-sequence-specific detection using SYBR Green is possible, detection of amplicons should be made by sequence-specific probes to enhance the specificity of results. Real-time PCR is therefore recommended.

3.2 Real-time PCR apparatus

Real-time PCR instruments allow simultaneous detection of multiple targets because they have variable excitations of up to six or more different colour detections. Many apparatus are available for real-time PCR. Among the most frequently used are LightCycler®(Roche), Applied Biosystems, Stratagene, Rotor-Gene™ (Qiagen), SmartCycler (Cepheid) and ICycler (Bio-Rad). All instruments are considered suitable.

The selection of real-time PCR platform depends on your application. Some instruments are designed for low capacity and others are made for high-throughput application. The first-generation LightCyclers (Roche) use thin-walled glass capillary tubes. At present, many instruments employ standard plates (e.g. 96-well plates). The real-time PCRs are usually optimised for a small reaction volume (e.g. 20 to 25-µl).

3.3 Kits

Several commercial kits are available for *Bordetella pertussis* and/or *Bordetella parapertussis* detection in clinical specimens and these include an internal control to detect inhibitors. Of these kits, the Simplexa Bordetella assay (Focus Diagnostics), the SmartCycler Bordetella pertussis/parapertussis assay (Cepheid), and the Bordetella R-gene (Argene) have been evaluated and proven suitable, whereas the Bordetella pertussis Real Time PCR kit (Shanghai ZJ Bio-Tech) was found to be unsuitable [9]. New PCR kits for *Bordetella pertussis* and/or *Bordetella parapertussis* are frequently introduced onto the market. As a general rule, the amplification targets and interpretation of results should follow the guidance below regarding the selection of amplification targets.

4 Amplification targets

Several amplification targets are possible for Bordetella PCR diagnostics. However, most of these targets are present in multiple *Bordetella* species, and most target combinations will therefore not be able to identify a single *Bordetella* species. Sequential or multiplex PCRs are therefore crucial in order to be able to identify the infecting organism. However, using multiplex PCR rather than singleplex can in some cases reduce the analytical sensitivity [12].

Table 1. Frequently used amplification targets for *Bordetella*-related PCRs [13]

IS*481*	High copy number target, present in *B. pertussis*, *B. holmesii*, some *B. bronchiseptica*
IS*1001*	High copy number target, present in *B. parapertussis*, *B. bronchiseptica*, (some homology to *B. holmesii*)
ptxA-Pr	Single copy number target, present in *B. pertussis* , *B. parapertussis* and *B. bronchiseptica*. *B.pertussis*-specific assays targeting this region are amongst the most well-characterised
ptxS1	Single copy number target, present in *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*
IS*1002*	Low copy number target present in *B. parapertussis* and *B. pertussis*
hIS*1001*	Low copy number target present in *B. holmesii*
RecA	Single copy number target. Depending on primer/probe design either 1) *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* specific or 2) *B. holmesii* specific.

Different combinations of these amplification targets in either multiplex or sequential PCRs can be used to identify the *Bordetella* species. However, optimum performance is only possible if the technology used is carefully designed to deal with the different targets since there is a difference in sensitivity between a high copy number target and a single copy number target.

For example, a multiplex IS*1001*/IS*481* PCR can be followed by a *ptxA*-Pr specific PCR in case of a positive outcome, and thereby several results can be obtained:

IS*1001*-positive: *B. parapertussis* or *B. bronchiseptica*

IS*481*-positive, *ptxA*-Pr-negative: *Bordetella* species

IS*481*-positive, *ptxA*-Pr- positive: *B.pertussis*

IS*481*-positive and IS*1001*-positive: possible co-infection with different *Bordetella* species.

For practical purposes, a positiveIS*481* PCR can be considered as a probable *B. pertussis* infection, when the clinical symptoms are in accordance with this result. Similarly, a positive IS*1001*PCR result can be considered as a probable *B. parapertussis* infection. In the case of epidemiological studies with unknown clinical data, positive results from an IS*481*real-time PCR should only be regarded as evidence of infection with *Bordetella* spp.

Table 2. Recently published *Bordetella* PCR methods and amplification targets used

Grogan *et al* [6]	IS*481* + IS*1001* + PTpromoter
Njamkepo *et al* [14]	PtxA-Pr + RecA + BP3385
Roorda *et al* [15]	IS*481* + IS*1001* + IS*1002*
Slinger *et al* [16]	IS*481* + IS*1001*
Tatti *et al* [7]	ptxS1 + IS*481* + IS*1001* + hIS*1001*
Xu *et al* [8]	IS*481* + IS*1001* + PtxP
Fry *et al* [11]	*ptxA*-Pr + IS*481*
Guiso N [17]	IS*481* + IS*1001*
Kösters *et al* [12]	IS*481* + IS*1001*

5 Quality assurance

Several general guidance documents have been published on quality assurance and quality control when using PCR methods. Among these are guidance from the United States Environmental Protection Agency (http://www.epa.gov/microbes/qa_qc_pcr10_04.pdf) and from the UK Health Protection Agency (2011): 'Good Laboratory Practice when Performing Molecular Amplification Assays. UK Standards for Microbiology Investigations'. G 4 Issue 4.1 (http://www.hpa.org.uk/SMI/pdf).

5.1 Establishing an in-house PCR method

When establishing an in-house PCR method on the ABI platform, a good starting-point could be the method published by Grogan *et al* [6] and/or Kösters *et al* [18]. For the LightCycler capillary system the WHO manual (http://whqlibdoc.who.int/hq/2004/WHO_IVB_04.14_eng.pdf), Kösters *et al* [12], or Fry *et al* [11] could be used.

5.2 Prevention of crossover contamination

All real-time PCR formats minimise crossover contamination by design. When using the uracil-DNA glycosylase system, additional low-level crossover contamination prevention is possible, and some commercially available generic reaction mixtures contain the basic reagents (i.e. LightCyclerFastStart DNA master hybridisation probes; Roche).

5.3 Controls

PCR assays for human genes (e.g. the mitochondrial cytochrome oxidase (HMCO) gene) can be used to quality control extracted samples [4, 9].

An internal process control (IPC) should be included in each sample to test for PCR inhibition. These can be exogenous, e.g. phocine herpes virus, or competitive.

Internal amplification controls

For real-time PCR formats, an internal amplification control is recommended to avoid false-negative reporting. Internal controls, based on the separate amplification of a phocine herpes virus or on an alternative system to the PT promoter PCR or the IS*481* PCR, have been described [9, 11, 19].

Almost all samples tested so far harboured human gene sequences, and thus their amplification can also be used as an inhibition control.

It is essential to test for the presence of any inhibition to the PCR reaction to avoid false-negative reporting. If an IPC is not readily available, an alternative is to 'spike' a replicate sample with a dilution of positive control DNA (e.g. 0.1pg for a single copy and 0.01pg for a multiple copy target such as IS*481*). For each test sample reaction mix a duplicate is prepared, but the 'spiked' sample contains a low dilution of purified positive control DNA in addition to the extracted test sample DNA. These two reactions are then run and analysed in parallel. If the test sample alone does not contain detectable levels of *Bordetella* (i.e. PCR negative), and the spiked reaction produces a signal comparable with that obtained from the same dilution used in the standard curve, then the result can be reported as a valid negative result. If, when compared to the same dilution in the standard curve, the spiked sample yields no signal then the result should be reported as inhibitory.

Positive and negative in-run controls

In-run controls should include negative controls (PCR grade water) and positive controls. Typically, non-standardised purified DNA (controlling for amplification and extraction) or a bacterial suspension (also controlling for bacterial lysis) is used as a positive control. A standardised total DNA extract from a sequenced strain, *B. pertussis* Tohama I or *B. parapertussis* ATCC 12822, would be desirable.

5.4 External quality assessment programmes

In order to ensure continued high performance of the assay, participation in external quality assessment (EQA) programmes is encouraged. Such programmes could be the INSTAND (http://www.instandev.de/en/about-instand-ev.html) or the QCMD (http://www.qcmd.org/).

6 Protocol

Diagnosis of *Bordetella* infections: detection of *Bordetella* DNA in nasopharyngeal samples using real-time PCR

Purpose

This protocol is an appendix to the document 'Guidance and protocol for the use of real-time PCR in laboratory-diagnosis of human infection with *Bordetella pertussis* or *Bordetella parapertussis*'.

The guidance and protocol are intended for the use of real-time PCR on DNA extracted from clinical specimens obtained from patients with suspected whooping cough (i.e. an infection with *B. pertussis* or *B. parapertussis*). Suitable samples are either nasopharyngeal swabs (NPS) or nasopharyngeal aspirates (NPA) sampled from the posterior nasopharynx.

The protocol details three different real-time PCR methods for the detection of *Bordetella* DNA: IS481, ptxA-Pr and IS1001.

This protocol is based on the LightCycler®capillary technology (Roche), since many laboratories are already using LightCycler for detection of *B. pertussis* and *B. parapertussis* and the previous guideline protocols have been based on the LightCycler[17]. Although there are many different choices for PCR machines, it was necessary to select one in order to create a proper protocol. However, the protocol can be used as a basis on which to build an in-house method for other real-time formats.

Summary

The details for real-time capillary based PCR are presented for three different targets.

Each PCR should be run separately.

- IS*481*: High copy number target, present in *B. pertussis*, *B. holmesii*, some *B. bronchiseptica*
- IS*1001*: High copy number target, present in *B. parapertussis*, *B. bronchiseptica*
- *ptxA*-Pr: Single copy number target, present in *B. pertussis, B. parapertussis and B. bronchiseptica,* but assay designed to be specific for *B.pertussis*

Materials

Sample material

Nasopharyngeal swabs or aspirates from patients suspected of having an infection with either *B. pertussis* or *B. parapertussis*.

Apparatus

LightCycler instrument using capillaries. (LightCycler® 2.0, Roche)

LightCycler capillaries and capillary-related tools and adapters (Roche)

Microcentrifuge

Sterile Eppendorf 1.5ml tubes

Pipettes

Pre-sterilised aerosol resistant pipette tips.

Reagents

LightCycler®FastStart DNA Master HybProbe kit and MgCl2 (Roche) (IS481 and ptxA-Pr)

LightCycler®FastStart DNA Master PLUS HybProbe kit (Roche) (IS1001)

PCR grade water (contained in the FastStart kit)

AmpErase® Uracil N-glycosylase (Applied Biosystems) (IS481 and ptxA-Pr)

Uracil-DNA Glycosylase, heat-labile (Roche) (IS1001).

Controls

Positive control

It is recommended that a sample of *Bordetella* DNA be included as a positive control. For IS481 and ptxA-Pr this should be *B. pertussis*. For IS1001 this should be *B. parapertussis*.

Negative control

It is recommended that a sample of PCR grade water should be included as a negative control.

Internal process control (IPC)

It is recommended that an internal process control should be included, as described in the guidance section.

Prevention of contamination

The synthetic deoxynucleotide dUTP is used instead of dTTP in the PCR mix, enabling Uracil-DNA Glycosylase (UNG) to be applied prior to a new assay to prevent carry-over of amplicons. UNG catalyses the removal of uracil from single- and double-stranded DNA that has been synthesised in the presence of dUTP.

Primers and probes

For each of the three PCR protocols (IS*481*, *ptxA*-Pr, IS*1001*), two primers and two probes are needed. Detailed sequences are set out in the section entitled 'Details'.

Procedure

DNA extraction

Sample material from dry swabs can be re-suspended in sterile PBS before DNA extraction by vortexing the swab in 500µl of PBS in a closed sterile container. The wire shaft is cut with sterile scissors to enable the lid to be replaced before vortexing.

If necessary, nasopharyngeal aspirate and sputum can be liquefied with a fluidisation preparation in the ratio 1:1 before DNA extraction.

Fluidisation preparation

- Sodium citrate (2.9%) 5 ml
- N-actyl L-cysteine 0.05g
- H2O to make 10 ml

DNA extraction from nasopharyngeal samples (swab or aspirate) may be carried out manually using the QIAamp DNA mini kit (Qiagen), High Pure PCR template preparation kit (Roche) or Chelex. Automated methods such as the MagNA Pure Compact (Roche) are also an option.

Prepare a 1:10 dilution of each DNA sample in PCR grade water. Each DNA sample should be analysed in both undiluted and 1:10 dilution in order to detect potential inhibition.

Preparation of reagents

FastStart DNA Master

Following the instructions in the kit, insert for either the LightCycler®FastStart DNA Master HybProbe kit (IS*481* and *ptx4*-Pr) or the LightCycler®FastStart DNA Master ^{PLUS} HybProbe kit (IS*1001*) and mix the contents of vial 1a and 1b accordingly.

IMPORTANT: Do NOT vortex, but mix by gently pipetting up and down.

Master mix

Prepare a master mix (on ice or in a cooling block) in a sterile 1.5 ml tube by multiplying the amount of mix for one test by the number of reactions to be cycled, plus one additional reaction. Remember that each DNA sample is analysed twice, in undiluted as well as 1:10 dilution. Remember to also leave room for positive and negative controls.

The detailed volumes for the master mix are presented in the 'Details' section.

Pre-PCR

The LightCycler cooling block with centrifuge adapters and room for reagents should be stored at 2-8°C so it is cold when in use. Place the master mix in the reagents section and place the acquired number of capillaries in the adapters.

Pipette 15µl of master mix into each of the cooled capillaries.

Then add 5µl of the DNA sample to the capillaries: For each sample, two capillaries are needed: one with 5µl undiluted DNA and one with 5µl of 1:10 diluted DNA.

For the negative control, pipette 5µl of PCR grade water into a capillary.

For the positive control, pipette 5µl of *B. pertussis* DNA (IS*481*, *ptxA*-Pr) or *B. parapertussis* DNA (IS*1001*) into a capillary.

Seal each capillary with a stopper and place the adapters containing the capillary in a standard bench top centrifuge. Centrifuge at 700g for 5 seconds (or place the capillaries in the rotor and use a LightCycler Carousel Centrifuge).

Place the capillaries in the rotor of the LightCycler instrument.

PCR cycles

The detailed cycle-programmes for the PCR reactions are presented in the 'Details' section.

Results

Analyse the amplification curves according to the LightCycler manual. Use channel 640 for the samples.

Analysis data are interpreted according to the amplification plot. Samples are regarded as positive when the fluorescence signal increases and shows a typical amplification kinetic curve (see Figure 1). Samples are regarded as negative when they do not fulfil the criteria mentioned above.

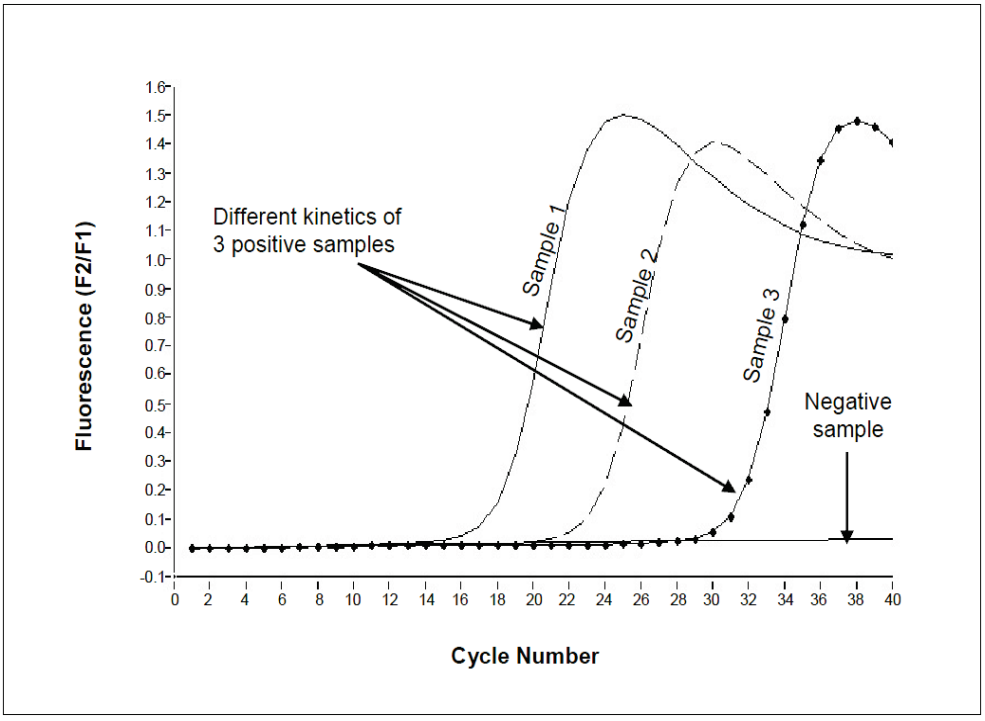

Figure 1. Example of amplification curves (WHO laboratory protocol)

Interpretation of results

Interpretation of results from the three separate PCR runs is based on the combination of positive/negative results in the three analyses (Table 3).

Table 3. Interpretation of results

IS*481*	*ptxA*-Pr	IS*1001*	Result
+	-	-	*Bordetella* spp. positive(*B pertussis* , *B. holmesii, B. bronchiseptica, B. parapertussis*)
-	+	-	Equivocal
+	+	-	*B. pertussis* positive
-	-	+	*B. parapertussis* or *B. bronchiseptica*
+	+	+	Co-infection with different *Bordetella* spp.
+	-	+	Co-infection with different *Bordetella* spp.
-	-	-	Negative

Details, IS481 real-time PCR

Primers and probes (Fry *et al* [11])

Forward primer: BP-1

5′ –GAT TCA ATA GGT TGT ATG CAT GGT T

Reverse primer: BP-2

5′ –TTC AGG CAC ACA AAC TTG ATG GGC G

Probe 1: BP-FLU

5′–TCG CCA ACC CCC CAG TTC ACT CA(F)

Probe 2: BP-LCR

5′ –(LC-Red 640) AGC CCG GCC GGA TGA ACA CCC(P)

Master mix

Reagents	Conc. of stock	Conc. per reaction	Vol. (µl) per capillary
PCR grade water			8.2
FastStart DNA Master	x10	x1	2
$MgCl_2$	25 mM	3.0 mM	1.6
BP-1	10 pmol / µl	0.5 pmol / µl	1
BP-2	10 pmol / µl	0.5 pmol / µl	1
BP-FLU	10 pmol / µl	0.25 pmol / µl	0.5
BP-LCR	10 pmol / µl	0.25 pmol / µl	0.5

Cycle programme

	Segment	Target (°C)	Hold	Slope (°C/sec)	Acquisition mode	Cycles
Pre-incubation		50	10			
Denature		95	10 min		None	1
PCR	1	95	10 sec	20	None	50
	2	50	10 sec	20	Single	
	3	72	20 sec	20	None	
Cooling		40	30 sec	20	None	1

Details ptxA-Pr real-time PCR

Primers and probes (Fry *et al* [11])

Forward primer: BPpr-1

5' – CGC CAA GCT GAA GTA GCA

Reverse primer: BPpr-2

5' – AAG GAG CGT TCA TGC CG

Probe 1: BPpr-FLU

5'–CAT GCG TGC AGA TTC GTC GTA CA(F)

Probe 2: BPpr-LCR

5'–(LC-Red 640)ACC CTC GAT TCT TCC GTA CAT CC(P)

Master mix

Reagents	Conc. of stock	Conc. per reaction	Vol.(µl) per capillary
PCR grade water			7.4
FastStart DNA Master	x10	x1	2
$MgCl_2$	25 mM	4.0 mM	2.4
BPpr-1	10 pmol / µl	0.5 pmol / µl	1
BPpr-2	10 pmol / µl	0.5 pmol / µl	1
BPpr-FLU	10 pmol / µl	0.25 pmol / µl	0.5
BPpr-LCR	10 pmol / µl	0.25 pmol / µl	0.5
AmpErase UNG	500 units/ µl	0.2 unit	0.2
Total volume			15

Cycle programme

	Segment	Target (°C)	Hold	Slope (°C/sec)	Acquisition mode	Cycles
Pre-incubation		50	10 min		None	1
Denature		95	10 min		None	1
PCR	1	95	10 sec	20	None	50
	2	50	10 sec	20	Single	
	3	72	20 sec	20	None	
Cooling		40	30 sec	20	None	1

Details IS1001 real-time PCR

Primers and probes (Kösters *et al* [12], WHO protocol [17])

Forward primer: BPpara-1

5'– CAC CGC CTA CGA GTT CGA GAT

Reverse primer: BPpara-2

5'– CCT CGA CAA TGC TGG TGT TCA

Probe 1: BPpara-FLU

5'– GTT CTA CCA AAG ACC TGC CTG GGC-(F)

Probe 2: BPpara-LCR

5'– (LC-Red 640) AGA CAA GCC TGG AAC CAC TGG TAC-(P)

Master mix

Reagents	Conc. of stock	Conc. per reaction	Vol. (µl) per capillary
PCR grade water			7
FastStart PLUS DNA Master	x5	x1	4
BPpara-1	10 pmol / µl	0.5 pmol / µl	1
BPpara-2	10 pmol / µl	0.5 pmol / µl	1
BPpara-FLU	10 pmol / µl	0.25 pmol / µl	0.5
BPpara-LCR	10 pmol / µl	0.25 pmol / µl	0.5
UNG (Roche)	1 U / µl	1 units	1
Total volume			15

Cycle programme

	Segment	Target (°C)	Hold	Slope (°C/sec)	Acquisition mode	Cycles
Denature		95	10 min	20	None	1
PCR	1	95	10 sec	20	None	40
	2	60	10 sec	20	Single	
	3	72	20 sec	20	None	
Cooling		40	30 sec	20	None	1

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