February 2025

QIAprep&amp® Plasmodium Kit Handbook

For the detection of the parasite Plasmodium in whole blood or dried blood spots

Sample to Insight

Table of Contents

Kit Contents

Kit	**QIAprep&amp Plasmodium Kit**
Catalog no.	**223213**
Number of preps	**100 rxn**

Component	Volume	Qty
RNase-free Water	1.9 mL	1
PR Buffer	6 mL	1
DBS Wash Buffer	3 mL	1
Blood Lysis Buffer	10 mL	4
S-Solution	0.2 mL	1
QP&A DNA Mastermix	0.9 mL	1

Shipping and Storage

The QIAprep&amp Plasmodium Kit (cat. no. 223213) should be stored immediately upon receipt at –30 to –15°C in a constant temperature freezer and protected from light. Several components of the kit can be stored at room temperature (15–25°C): Blood Lysis Buffer, PR buffer, DBS Wash Buffer. The S-Solution is stored light-protected at –20°C. When stored correctly, the QIAprep&amp Plasmodium Kit is good until the expiration date printed on the kit box label.

This kit can be used for the detection of the Plasmodium parasite (in combination with the respective assay) in different workflows.

Intended Use

The QIAprep&amp Plasmodium Kit is intended for molecular biology applications only. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of QIAprep&amp Plasmodium Kit is tested against predetermined specifications to ensure consistent product quality.

Product Specifications

Table 1. QP&A DNA Mastermix

Component	Description
ROX Reference Dye	Optimized concentration of fluorescent dye for normalization of fluorescent signals on all instruments from Applied Biosystems® .
QuantiNova® DNA Polymerase	QuantiNova DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from Thermus aquaticus. QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient temperature. The enzyme is activated by a 2 minute, 95°C incubation step.
Buffer	Contains components enabling fast cycling, including Q-Bond®.
dNTP Mix	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality.

Table 2. Other components

Component	Description
S-Solution (Sensitivity Solution)	Enhances the sensitivity of the reaction.
DBS Wash Buffer	The buffer helps to wash off unwanted blood components.
Blood Lysis Buffer	Lyses red blood cells and releases Plasmodium cells.
PR Buffer (Plasmodium Release Buffer)	The buffer contains a proprietary list of additives making DNA molecules of *Plasmodium spp.* available.
RNase-free Water	Ultrapure quality, PCR-grade.

Introduction

The QIAprep&amp Plasmodium Kit is an innovative method optimized for the preparation and DNA-based detection of Plasmodium (such as *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*) from human samples. These are collected as whole blood samples (e.g. EDTA, Citrate, Heparin) or dried blood spots on paper cards (e.g. Whatman™ filter cards [GE, cat. no. 3030-917 or 3017-915], QIAcard® Bloodstain card [cat. no. WB100014], or QIAcard FTA Classic card [cat. no. WB 120205 or WB120305]).

Based on the sample type (liquid blood or dried blood spots) different protocols are applicable. Additionally for each sample type one ultrafast protocol and one ultrasensitive protocol are available (see Figure 1). Assays for detection of Plasmodium are separately available:

- *Pf/Non-Pf Detection Assay Kit Quick-Start Protocol*: **www.qiagen.com/HB-3669**
- *Pv/Pm/Po/Pk Detection Assay Kit Quick-Start Protocol*: **www.qiagen.com/HB-3671**

The QIAprep&amp Plasmodium Kit combines sample preparation with qPCR detection in one step. Therefore, QIAprep&amp Plasmodium Kit drastically accelerates time to result and simplifies the procedure, simultaneously addressing the need for highly sensitive detection of Plasmodium species.

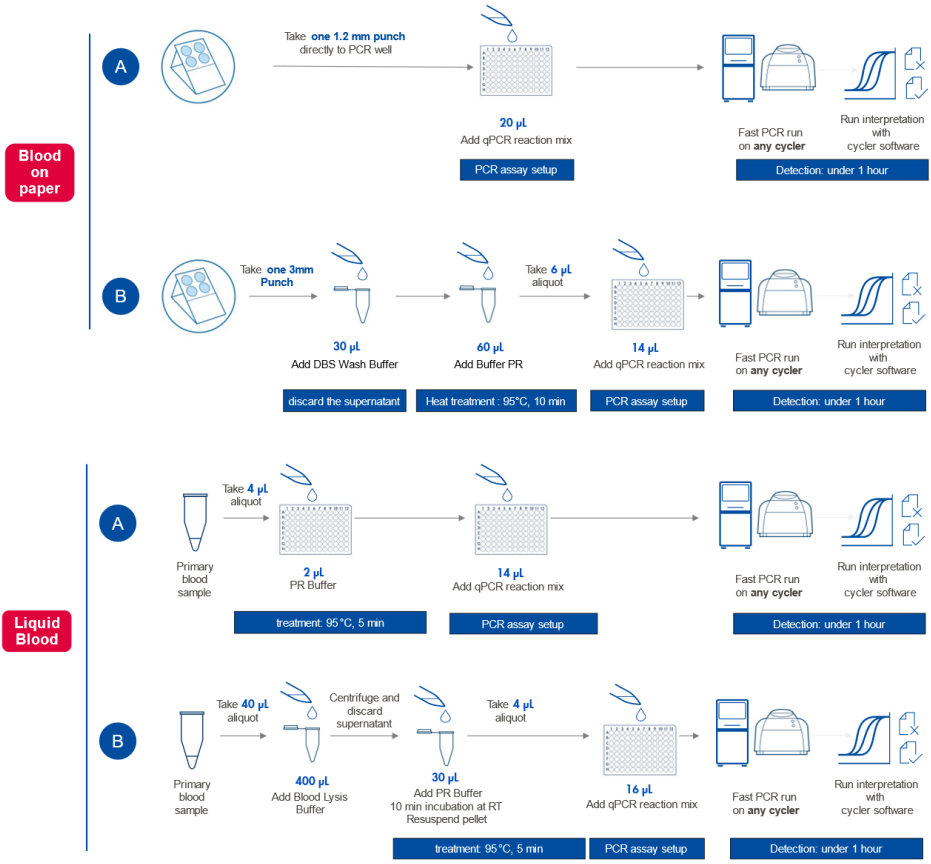

Figure 1. Overview of the workflows.

The kit is compatible with dual-labeled hydrolysis probes, such as the TaqMan® probes. High specificity and sensitivity in real-time PCR are achieved by a hot-start procedure. The QIAprep&amp Plasmodium Kit has been optimized for use with TaqMan probes in multiplex qPCR detection of one or more targets (altogether, up to 4 assays). The kit has been optimized for use with any real-time cyclers. The QN ROX Reference Dye is included in the mastermix.

Principle and procedure

Plasmodium sample preparation

The QIAprep&amp Plasmodium Kit is designed to be used with whole blood samples (e.g. EDTA, Citrate, and Heparin) as well as dried blood spots (DBS). Fresh, as well as frozen, whole blood samples are suitable. Dried blood spots generated with finger prick blood, as well as whole blood, are suitable. Before using whole blood samples, the blood samples need to be mixed by inversion or vortexing and briefly centrifuged. Optimized buffers for each workflow are provided to minimize inhibitory effects and maximize Plasmodium DNA yield. The QIAprep&amp Plasmodium Kit technology allows to use 20% of blood in the PCR reaction. Two workflows per sample type (whole blood or DBS) are provided. One workflow provides ultra-fast results with fewer steps, while the other workflow offers superior sensitivity with a few more handling steps.

PCR

The QIAprep&amp Plasmodium Kit contains a highly concentrated 2x Master Mix, which allows use of larger volumes of template in order to increase assay sensitivity. The assay setup can be done at room temperature.

Master Mix

The components of the QP&A DNA Mastermix include QuantiNova DNA Polymerase, buffer, and dNTPs. The optimized master mix ensures fast qPCR amplification with high specificity and sensitivity.

Novel, antibody-mediated hot-start mechanism

The QuantiNova DNA Polymerase is provided in an inactive state and has no enzymatic activity at ambient or higher temperatures. The antibody-mediated hot-start mechanism

prevents the formation and extension of nonspecific qPCR products and primer–dimers during reaction setup, and the first denaturation step. Therefore, this mechanism allows higher PCR specificity and accurate quantification. At low temperatures, the QuantiNova DNA Polymerase is kept in an inactive state by the QuantiNova Antibody and Guard, which stabilizes the complex and improves the stringency of the hot-start. Within 2 minutes of raising the temperature to 95°C, the QuantiNova Antibody and Guard are denatured and the QuantiNova DNA Polymerase is activated, enabling PCR amplification. This hot-start enables rapid and convenient room-temperature setup.

QIAprep&amp Plasmodium PCR Buffer

The PCR buffer from the QIAprep&amp Plasmodium Kit is specifically designed to facilitate fast real-time PCR using sequence-specific probes. The buffer additive, Q-Bond, allows for short cycling times. Q-Bond increases the affinity of the DNA Polymerase for short single-stranded DNA, reducing the time required for primer–probe annealing to a few seconds. In addition, the unique composition of the buffer supports the melting behavior of DNA, enabling short denaturation and annealing/extension times. The QP&A DNA Mastermix is also based on the unique QIAGEN PCR buffer system. The buffer composition is extremely robust to the inhibitory effects of blood and promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity. When using this master mix, primer annealing is only marginally influenced by the $MgCl_2$ concentration, so optimization by titration of Mg^2 is not required. The master mix also contains Factor MP, which facilitates multiplex PCR. This synthetic molecule increases the local concentration of primers and probes at the DNA template and stabilizes specifically bound primers and probes, allowing efficient annealing and extension. The combination of these various components of the QIAprep&amp Plasmodium PCR Buffer prevents multiple amplification reactions from affecting each other. The assay setup can be done at room temperature and should be processed immediately after sample addition.

Passive reference dye

For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR–related variations in fluorescence detection. Fluorescence from ROX dye does not change during the course of real-time PCR but provides a stable baseline to which PCR-related fluorescent signals are normalized. Thus, ROX dye compensates for differences in fluorescence detection either due to slight variations in well reaction volume or to differences in well position. ROX dye does not interfere with real-time PCR since it is not involved in the reaction and has an emission spectrum different from fluorescent dyes commonly used for probes. The use of ROX dye is necessary for instruments from Applied Biosystems. The QP&A DNA Mastermix of the QIAprep&amp Plasmodium Kit contains already ROX Reference Dye at a 200x concentration.

ROX dye should be used as a 20x concentrated solution when using an instrument requiring a high ROX dye concentration. For instruments requiring a low ROX dye concentration, use the dye as a 200x concentrate. Refer to Table 2 for details on real-time cyclers that require high or low ROX concentrations. If desired, ROX Reference dye can be added to the QIAprep&amp Plasmodium Kit for long term storage (Table 3).

Table 3. Real-time cyclers requiring high/low concentrations of ROX dye

High ROX dye concentration (1:20 dilution of ROX Reference Dye in 1x reaction)	Low ROX dye concentration (1:200 dilution of ROX Reference Dye in 1x reaction)
ABI PRISM 7000	Applied Biosystems 7500
Applied Biosystems 7300	Applied Biosystems ViiA™ 7
Applied Biosystems 7900	Applied Biosystems QuantStudio® Systems
Applied Biosystems StepOne™	
Applied Biosystems StepOne Plus	

Descriptions of protocols

The QIAprep&amp Plasmodium Kit is suitable for whole blood samples, but also for dried blood spots (DBS). For each sample type two workflows (WF) are available, one ultrafast as well as one ultrasensitive workflow.

- For **DBS samples**: the DBS direct WF provides ultrafast results, while the DBS Elution WF provides superior sensitivity with a minimal increase in handling steps and time.
- For **Whole Blood samples**: the Sedimentation WF provides the best sensitivity by incorporating additional steps, while the Whole Blood WF is characterized through its easy handling and quick results.

Protocol 1 DBS Direct WF

This ultrafast workflow is designated to be used with dried blood spots (DBS) directly in the reaction (Protocol: DBS Direct Workflow, page "Protocol: DBS Direct Workflow" on page 16).

Protocol 2 DBS Elution WF

This ultrasensitive workflow generates and assesses eluate from dried blood spots (DBS) (Protocol: DBS Elution Workflow, page "Protocol: DBS Elution Workflow" on page 19).

Protocol 3 Sedimentation WF

This workflow is designated for ultrasensitive detection of Plasmodium from whole blood (Protocol: Sedimentation Workflow, page "Protocol: Sedimentation Workflow" on page 23).

Protocol 4 Whole Blood WF

This ultrafast workflow is designated for whole blood (Protocol: Whole Blood Workflow, page "Protocol: Whole Blood Workflow" on page 26).

Equipment and Reagents to Be Supplied by User

- A programmable real-time PCR thermocycler with ideally five detection channels (a minimum of four detection channels is required) e.g. Rotor-Gene® Q 5plex System (cat. no. 9001640).
- Plastic PCR consumables compatible with the thermocycler in use.
- Calibrated micropipettes for volumes ranging from 1–500 µL and filter tips. Filter tips are recommended for all steps involving sample material.
- A probe-based assay for the detection of one or more targets from Plasmodium. This is referred to as primer–probe mix in the reaction setup table (Table 4). We recommend the Pf/Non-Pf Detection Assay (cat. no. 224113) for the detection of *Plasmodium falciparum* and discrimination from other non-falciparum Plasmodium species and the Pv/Pm/Po/Pk Detection Assay (cat. no. 225113) to distinguish between *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi* presence.
- For the generation of dried blood spots we recommend: the QIAcard Bloodstain card (cat. no. WB100014) or the QIAcard FTA Classic card (cat. no. WB 120205 or WB120305) or Whatman filter cards (GE, cat. no. 3030-917 or 3017-915).
- Puncher e.g. Uni-Core® Punch 1.2 mm for DBS direct WF (cat. no. WB100074), 3 mm for DBS Elution WF (cat. no. WB100078) or comparable punchers.
- Cutting mat (cat. no. WB100088).

Important Notes

- The QIAprep&amp Plasmodium Kit is an innovative method optimized for the preparation and detection of Plasmodium DNA targets from samples such as whole blood (EDTA, Citrate, and Heparin) and dried blood spots (DBS).
- Samples can be kept at room temperature during preparation steps and reaction setup. Sample preparation can conveniently be performed directly in the PCR vessel prior to the addition of the PCR reaction (DBS Direct WF or Whole Blood WF) or through few preparation steps (DBS Elute WF or Sedimentation WF). The assay setup can be done at room temperature and should be processed immediately after sample addition.
    - The qPCR protocol uses TaqMan probes in a multiplex reaction that works with any real-time cyclers. For fluorescence normalization, ROX dye might be required at the following concentrations:
    - Low concentration of ROX dye: Applied Biosystems 7500, ViiA 7, and QuantStudio Real-Time PCR Systems
    - High concentration of ROX dye: ABI PRISM 7000, Applied Biosystems 7300, 7900, and StepOne Real-Time PCR Systems
    - No requirement for ROX dye: Rotor-Gene Q, QIAquant, Bio-Rad® CFX, Roche® LightCycler® 480, and Agilent® Technologies Mx/AriaMx instruments.
    - The ROX Reference Dye should be used as a 20x concentrated solution for a 1x reaction when using an instrument requiring a high-ROX dye concentration. For instruments requiring a low ROX dye concentration, use the dye as a 200x concentrate. The QP&A DNA Mastermix already contains ROX at a 200x concentration.

- We recommend to use the assays provided in the kit, Pf/Non-Pf Detection Assay (cat. no. 224113) and Pv/Pm/Po/Pk Detection Assay (cat. no. 225113).
- Use the cycling conditions and primer amounts specified in the protocol.
- Before use, thaw the QP&A DNA Mastermix, the recommended PCR assay RNase-free water and S-Solution. Mix the individual solutions, including those that are stored at room temperature and workflow-dependent (PR Buffer, Blood Lysis Buffer, or DBS Wash Buffer). For both 96-well and 384-well block cyclers, we recommend a final reaction volume of 20 µL in order to maximize input volumes of template from the primary sample and increase assay sensitivity.

Protocol: DBS Direct Workflow

This protocol is intended to be used with whole blood samples collected as dried blood spots.

Notes before starting

- The following blood collection cards are compatible: Whatman filter paper (GE, cat. no. 3030-917 or 3017-915); QIAcard Bloodstain card (cat. no. WB100014) or any other blood collection cards (untreated, no preservatives); as well as the QIAcard FTA Classic card (cat. no. WB 120205 or WB120305).
- It is recommended to use a puncher such as the Uni-Core Punch 1.2 mm (cat. no. WB100074), or other comparable punchers.
- It is recommeded to use a Cutting mat (cat. no. WB100088).
- Thaw the QP&A DNA Mastermix, the PCR assay of choice, and RNase-free water.
- Additionally, this protocol uses S-Solution.
- Use the cycling conditions specified in this protocol.

Procedure

1. Vortex and centrifuge all reagents.
2. Take 1 paper punch from the blood sample collection card (preferably the middle of the blood spot) using a punching device (1.2 mm diameter of punch). Release the punch into the very bottom of a PCR plate/strip/tube.

   Note: Go to **www.qiagen.com/HB-3666** for a detailed instruction on how to use the puncher.

Important: Depending on the applied pressure, pieces of the mat can be transferred into the reaction. This does not have a negative effect on the result, however, coring of the mat should be avoided.

3. **PCR setup**: Prepare the PCR reaction mix for a multiplex PCR reaction as shown in Table 4. Vortex briefly and centrifuge to remove the liquid from the cap.

Table 4. Reaction mix setup

Component	Channel for detection	1 rxn (µL)	Final Concentration
QP&A DNA Mastermix	–	9	1x
20x Assay Mix	Select respective channels	1	1x
S-Solution	–	2	–
RNase-free Water	–	8	–
Total volume	**–**	**20**	**–**

4. Add 20 µL of PCR reaction mix (Table 4) to each well.
5. Seal the plate/tube thoroughly with a fresh foil/lid. Mix gently by vortexing with medium pressure (5–10 s). Place the plate in different positions while vortexing, to ensure an equal contact with the vortex platform.
6. Centrifuge the plate/tube briefly to collect the liquid at the bottom of the plate/tube. Place it in the real-time cycler and start the cycling program (with heated lid). Program the cycler as referred to in Table 5.

   Note: Data acquisition should be performed during the annealing/extension step.

Table 5. Cycling conditions

Step	Time	Temperature (°C)	Ramp rate
PCR initial heat activation	2 min	95	Maximal/fast mode
2-step cycling (40 cycles)			
Denaturation	5 s	95	Maximal/fast mode
Combined annealing/extension*	30 s	58	Maximal/fast mode

*Add data acquisition

Result interpretation

Refer to the following when using these assay kits:

a. *Pf/Non-Pf Detection Assay Kit Quick-Start Protocol*: **www.qiagen.com/HB-3669**

b. *Pv/Pm/Po/Pk Detection Assay Kit Quick-Start Protocol*: **www.qiagen.com/HB-3671**

Protocol: DBS Elution Workflow

This protocol is intended to be used with dried blood samples collected on paper.

Notes before starting

- The following blood collection cards are compatible for generating DBS: Whatman filter paper (GE, cat. no. 3030-917 or 3017-915); QIAcard Bloodstain card (cat. no. WB100014) or any other blood collection cards (untreated, no preservatives); as well as the QIAcard FTA Classic card (cat. no. WB 120205 or WB120305).
- It is recommended to use a puncher such as the Uni-Core Punch 3 mm (cat. no. WB100078), or other comparable punchers.
- It is recommeded to use a Cutting mat (cat. no. WB100088).
- Thaw the QP&A DNA Mastermix, the PCR assay of choice, and RNase-free water.
- Additionally, this protocol uses DBS Wash Buffer, PR Buffer, and S-Solution.
    - The PR buffer forms a precipitate upon storage below 15°C. If necessary, redissolve by mild agitation at 37°C and then place at room temperature.
- Use the cycling conditions specified in this protocol.

Procedure

1. Vortex and centrifuge all reagents.
2. Take 1 paper punch from the blood sample collection card (preferably the middle of the blood spot) using a punching device (3 mm diameter of punch). Release the punch into the very bottom of a PCR plate/strip/tube.

Note: Go to **www.qiagen.com/HB-3666** for a detailed instruction on how to use the puncher.

Important: Depending on the applied pressure, pieces of the mat can be transferred into the reaction. This does not have a negative effect on the result, however, coring of the mat should be avoided.

3. Add 30 µL DBS Wash Buffer to the plate/strip tube. Close the tubes and/or seal the plate/strip tube thoroughly to prevent cross-contamination. If working in a plate, make sure to apply pressure uniformly on the adhesive foil across the entire plate, to obtain a tight closure around each well.
4. Vortex the plate/strip/tube for 10 s.
5. Centrifuge the plate/strip/tube for 3 min (3400 x *g*).
6. Carefully open the strip caps or remove the foil from the plate to avoid cross-contamination.
7. Remove and discard the DBS Wash Buffer from each vial or well using a pipette.

   Important: Do not dump out the liquid.
8. Add 60 µL of the PR Buffer into the plate.

   Note: Close the tubes and/or seal the plate/tube thoroughly to prevent cross-contamination. If working in a plate, make sure to apply pressure uniformly on the adhesive foil across the entire plate, to obtain a tight closure around each well. Centrifuge it briefly to collect the liquid at the bottom of the plate/tube.
9. Incubate for 10 min at 95°C in a PCR cycler (with heated lid). Cool the samples down to room temperature.
10. Centrifuge briefly and transfer 6 µL to a fresh plate well/strip/tube.

    Important: If you plan further tests, keep the remaining eluate from step 9. It is recommended to store the eluate at –20°C for up to 1 month.

11. **PCR setup**: Prepare the PCR reaction mix for a multiplex PCR reaction as shown in Table 6. Vortex briefly and centrifuge to remove the liquid from the cap.

Table 6. Reaction mix setup

Component	Channel for detection	1 rxn (µL)	Final Concentration
QP&A DNA Mastermix	–	9	1x
20x Assay Mix	Select respective channels	1	1x
S-Solution	–	2	–
RNase-free Water	–	2	–
Total volume	**–**	**14**	**–**

12. Add 14 µL of PCR reaction mix (Table 6) to the 6 µL eluate prepared in step 10.

13. Seal the plate/tube thoroughly with a fresh foil/lid. Mix gently by vortexing with medium pressure (5–10 s). Place the plate in different positions while vortexing, to ensure an equal contact with the vortex platform.

14. Centrifuge the plate/tube briefly to collect the liquid at the bottom of the plate/tube. Place it in the real-time cycler and start the cycling program (with heated lid). Program the cycler as referred to in Table 7.

    Note: Data acquisition should be performed during the annealing/extension step.

Table 7. Cycling conditions

Step	Time	Temperature (°C)	Ramp rate
PCR initial heat activation	2 min	95	Maximal/fast mode
2-step cycling (40 cycles)			
Denaturation	5 s	95	Maximal/fast mode
Combined annealing/extension*	30 s	58	Maximal/fast mode

*Add data acquisition

Result interpretation

Refer to the following when using these assay kits:

a. *Pf/Non-Pf Detection Assay Kit Quick-Start Protocol*: **www.qiagen.com/HB-3669**

b. *Pv/Pm/Po/Pk Detection Assay Kit Quick-Start Protocol*: **www.qiagen.com/HB-3671**

Protocol: Sedimentation Workflow

This protocol is intended to be used with whole blood samples collected in EDTA, citrate or heparin tubes.

Notes before starting

- Thaw the QP&A DNA Mastermix, the PCR assay of choice, and RNase-free water.
- Additionally, this protocol uses Blood Lysis Buffer, PR Buffer, and S-Solution.
    - The PR buffer forms a precipitate upon storage below 15°C. If necessary, redissolve by mild agitation at 37°C and then place at room temperature.
- Use the cycling conditions specified in this protocol.

Procedure

1. Vortex and centrifuge all reagents and each blood sample.
2. Pre-dispense 400 µL Blood Lysis Buffer into a new tube (e.g. 1.5–2 mL tube)
3. Add 40 µL blood to the tube. Vortex for 5 s.
4. Put the tubes into a centrifuge with the hinges facing the exterior, this will help in ensuring that the sediment forms at the same spot in every tube. Centrifuge for 2 min at 4300 x *g*.

   Note: After centrifugation, a small pellet may become visible at the bottom of the tube. If the pellet is very small it may not be visible.
5. Carefully remove and discard the supernatant completely using a pipette. Do not decant the supernatant.
6. Add 30 µL PR Buffer to the pellet and incubate for 10 min at room temperature.
7. Pipet up and down thoroughly 10–20 times to resuspend the pellet.

8. Transfer 4 µL of dissolved pellet to a new tube or well of the PCR reaction plate and seal the tube/plate.

   Important: Seal the plate/tube thoroughly to prevent cross-contamination. In case an adhesive film is used, make sure to apply pressure uniformly across the entire plate, to obtain a tight seal across individual wells. Centrifuge it briefly to collect the liquid at the bottom of the plate/tube.

   Note: Keep the remaining dissolved pellet (e.g. for a reflex test). It is recommended to store the dissolved pellet at –20°C for up to 1 month. The dissolved pellet can be subjected up to 8 freeze–thaw cycles.

9. Incubate for 5 min at 95°C in a PCR cycler (with heated lid). After incubation, let the samples cool down to room temperature. Centrifuge the plate/tube briefly.

10. **PCR setup**: Prepare the PCR reaction mix for a multiplex PCR reaction as shown in Table 8. Vortex briefly and centrifuge.

Table 8. Reaction mix setup

Component	Channel for detection	1 rxn (µL)	Final Concentration
QP&A DNA Mastermix	–	9	1x
20x Assay Mix	Select respective channels	1	1x
S-Solution	–	2	–
RNase-free Water	–	4	–
Total volume	**–**	**16**	**–**

11. Remove the cover of the plate/tube and add 16 µL of PCR reaction mix (Table 8) to each well.

12. Seal the plate/tube thoroughly with a fresh foil/lid. Mix gently by vortexing with medium pressure (5–10 s). Place the plate in different positions while vortexing, to ensure an equal contact with the vortex platform.

13. Centrifuge the plate/tube briefly to collect the liquid at the bottom of the plate/tube. Place it in the real-time cycler and start the cycling program (with heated lid).

    Program the cycler as referred to in Table 9.

    Note: Data acquisition should be performed during the annealing/extension step.

Table 9. Cycling conditions

Step	Time	Temperature (°C)	Ramp rate
PCR initial heat activation	2 min	95	Maximal/fast mode
2-step cycling (40 cycles)			
Denaturation	5 s	95	Maximal/fast mode
Combined annealing/extension*	30 s	58	Maximal/fast mode

*Add data acquisition

Result interpretation

Refer to the following when using these assay kits:

a. *Pf/Non-Pf Detection Assay Kit Quick-Start Protocol*: **www.qiagen.com/HB-3669**

b. *Pv/Pm/Po/Pk Detection Assay Kit Quick-Start Protocol*: **www.qiagen.com/HB-3671**

Protocol: Whole Blood Workflow

This protocol is intended to be used with whole blood samples collected in EDTA, citrate or heparin tubes.

Notes before starting

- Thaw the QP&A DNA Mastermix, the PCR assay of choice, and RNase-free water.
- Additionally, this protocol uses PR Buffer and S-Solution.
    - The PR buffer forms a precipitate upon storage below 15°C. If necessary, redissolve by mild agitation at 37°C and then place at room temperature.
- Use the cycling conditions specified in this protocol.

Procedure

1. Vortex and centrifuge all reagents and each blood sample.
2. Pre-dispense 2 µL of PR Buffer into each PCR tube or well of a PCR plate.
3. Transfer 4 µL of blood to the tube or well of the PCR plate. Mix by pipetting up and down, or vortex for 5–10 s. Make sure to add the blood to the PR Buffer and not vice versa.
4. **Important**: Seal the plate/tube thoroughly to prevent cross-contamination. If an adhesive film is used, make sure to apply pressure uniformly across the entire plate, to obtain a tight seal across individual wells. Centrifuge it briefly to collect the liquid at the bottom of the plate/tube.
5. Incubate for 5 min at 95°C in a PCR Cycler (with heated lid). After incubation, let the samples cool down to room temperature.

   Note: The blood starts clotting during the heating step. This is normal and expected.

6. Centrifuge the plate/tube briefly.
7. **PCR setup**: Prepare the PCR reaction mix for a multiplex PCR reaction as shown in Table 10. Vortex briefly and centrifuge.

Table 10. Reaction mix setup

Component	Channel for detection	1 rxn (µL)	Final Concentration
QP&A DNA Mastermix	–	9	1x
20x Assay Mix	Select respective channels	1	1x
S-Solution	–	2	–
RNase-free Water	–	2	–
Total volume	**–**	**14**	**–**

8. Remove the cover of the plate/tube and add 14 µL of PCR reaction mix (Table 10) to each well.
9. Seal the plate/tube thoroughly with a fresh foil/lid. Mix gently by vortexing with medium pressure (5–10 s). Place the plate in different positions while vortexing, to ensure an equal contact with the vortex platform.
10. **Important**: In the heating step (step 5) the blood can curdle a bit. It is perfectly normal if some wells do not show a perfect mixture of blood and PCR reaction mix. This does not affect performance.
11. Centrifuge the plate/tube briefly to collect the liquid at the bottom of the plate/tube. Place it in the real-time cycler and start the cycling program (with heated lid). Program the cycler as referred to in Table 11.

    Note: Data acquisition should be performed during the annealing/extension step.

Table 11. Cycling conditions

Step	Time	Temperature (°C)	Ramp rate
PCR initial heat activation	2 min	95	Maximal/fast mode
2-step cycling (40 cycles)			
Denaturation	5 s	95	Maximal/fast mode
Combined annealing/extension*	30 s	58	Maximal/fast mode

*Add data acquisition

Result interpretation

Refer to the following when using these assay kits:

a. *Pf/Non-Pf Detection Assay Kit Quick-Start Protocol*: **www.qiagen.com/HB-3669**

b. *Pv/Pm/Po/Pk Detection Assay Kit Quick-Start Protocol*: **www.qiagen.com/HB-3671**

Result Interpretation

Depending on the amount of blood, the fluorescent signal of the amplification curves may be quenched. This effect is most prominent in the Whole Blood WF. Some variation in the amount of quenching among different blood samples is expected, but this does not impact the interpretation. Please see below for examples to aid in the interpretation of amplification signals.

Examples of positive amplification signals:

Regular signal

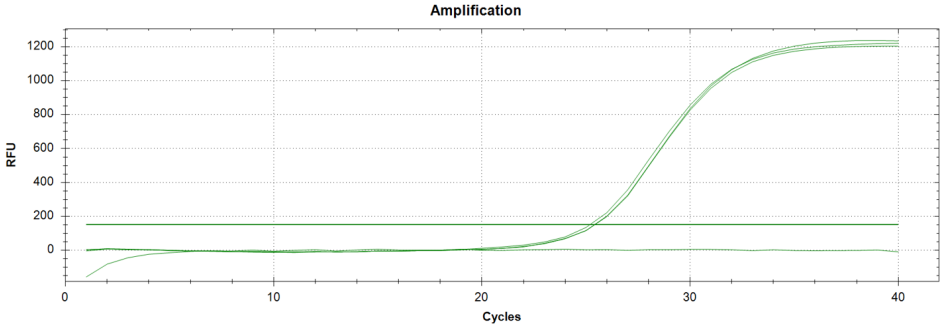

Figure 2. A regular amplification signal. A Plasmodium culture was diluted in negative blood and analyzed with the Whole Blood WF. The depicted amplification curve shows the signal for *Plasmodium falciparum*.

Weak Fluorescence

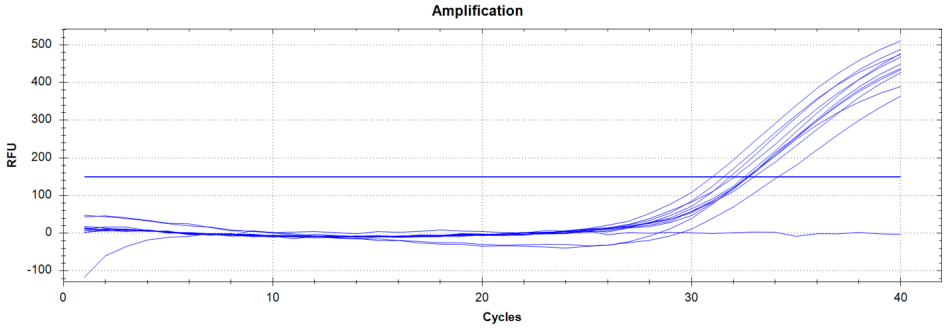

Figure 3. Quenching of fluorescent signal. A Plasmodium culture was diluted in negative blood and analyzed with the Whole Blood WF. The signal for human RNase P is quenched by the presence of blood, resulting in low fluorescence values.

Late positive signal

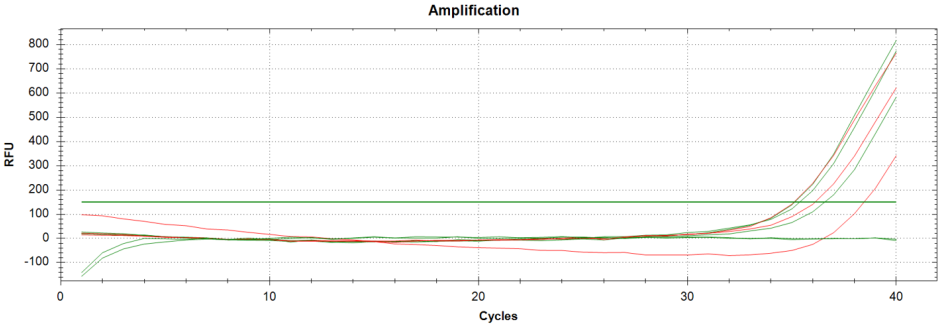

Figure 4. A late positive signal. Two Plasmodium cultures were diluted in negative blood and analyzed using the Whole Blood WF. The signal for *Plasmodium falciparum* generates a late Ct value due to the low concentration of template DNA present in the *Plasmodium falciparum* assay.

Noisy data

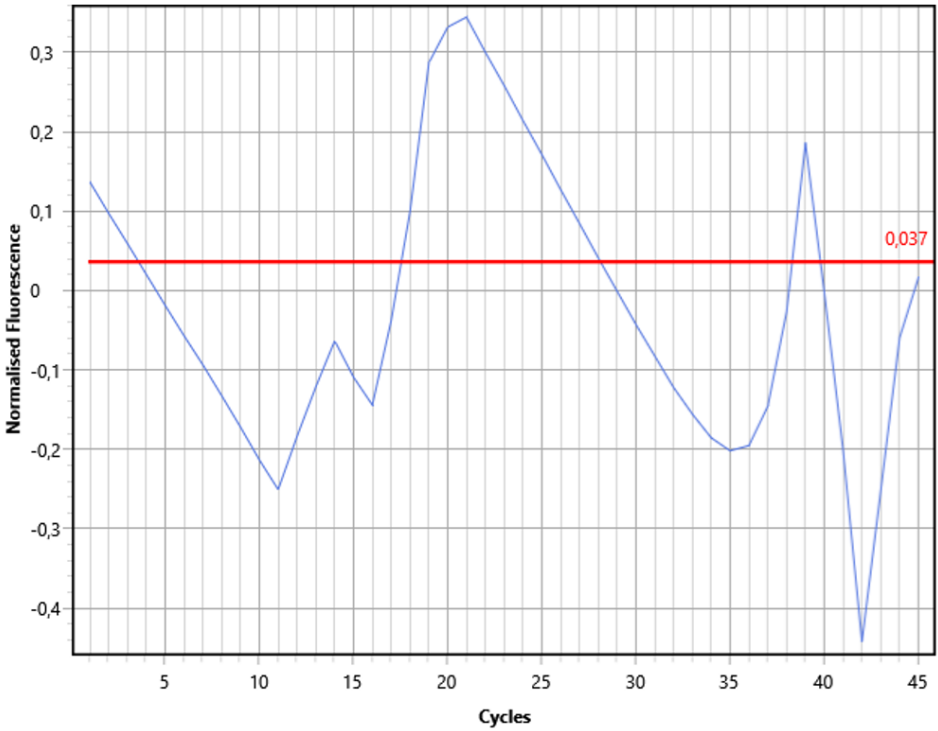

Figure 5. An artifact. In this sample, abrupt jumps in fluorescent signal were recorded. No true signal is present; only an artifact.

Example of negative amplification signals:

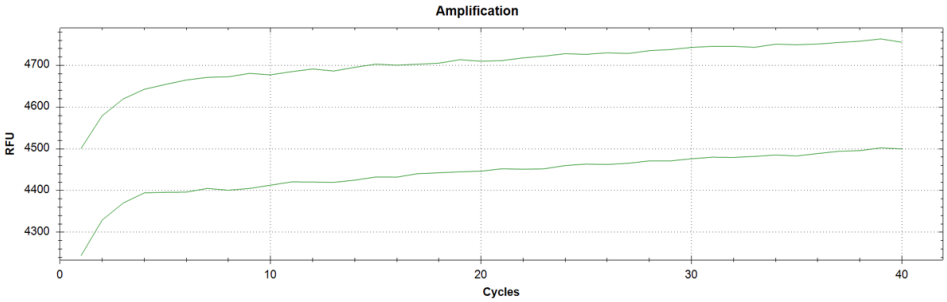

Figure 6. A negative signal. Two wells for the non-template control (NTC) are depicted. Background fluorescence is visible, but no amplification curve is present. An artifact is present in the first two cycles, leading to a step-like increase. However, since no S-shaped amplification curve is present (with the expected exponential increase in fluorescent signal) and the artifact occurs very early (artifacts are more common in the first few cycles), these wells are clearly negative. To remove artifacts like these from the data analysis, the first few cycles can be cropped.

Analysis guidelines for common PCR instruments and their software

All cyclers:

Due to the expected variance in amplification signals across different sample types, workflows, and instruments, the use of fixed thresholds is not feasible. Thresholds must be set individually to capture true positive signals while excluding potential background noise. Generally, cropping the first few cycles (e.g., 5) can help avoid baseline calculation artifacts. Typically, even very high parasitemia samples will not result in Ct values below 10. Analyzing raw data is helpful in determining whether a signal reflects amplification or is an artifact. Below are some examples comparing analyzed versus raw data.

One specific baseline artifact has been observed for the DBS Direct workflow (Figure 7). These artifacts are likely caused by air trapped in the blood spot, which gets released during PCR cycling. Raising the threshold above the baseline artifact, or, if available in the corresponding

analysis software, setting the baseline calculation window outside the affected areas, prevents such artifacts from being detected as signals crossing the threshold.

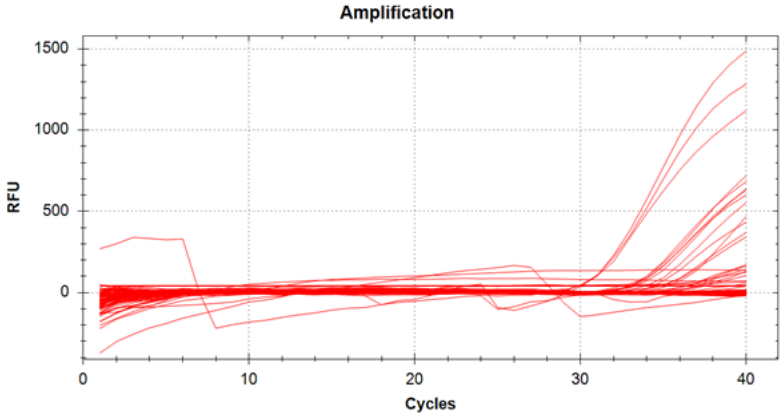

Figure 7. Baseline artifacts caused by dried blood spots. The DBS Direct workflow was followed. Some samples show strong drops in fluorescence between cycle 1 and 30. Depending on thresholds and analysis settings, these might be picked up as signals leading to early Cts or false positives.

Biorad CFX:

It is recommended to use the "Apply Fluorescence Drift Correction" function for baseline setting. It has been found to effectively remove different kinds of background artifacts. See Figure 8 and Figure 9 as examples.

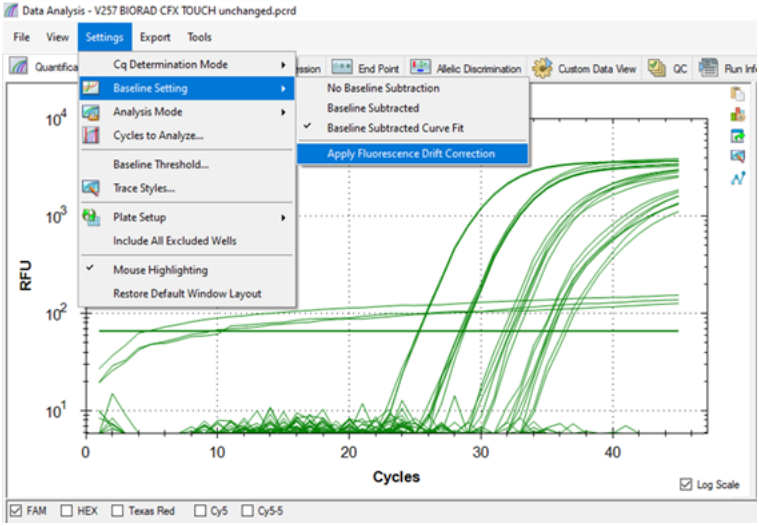

Figure 8. Baseline correction for Biorad CFX cyclers. Some negative samples show a fluorescence drift resulting in early Ct values when using the "Baseline Subtracted Curve Fit" mode. These artifacts are removed using the "Apply Fluorescence Drift Correction" (see Figure 9).

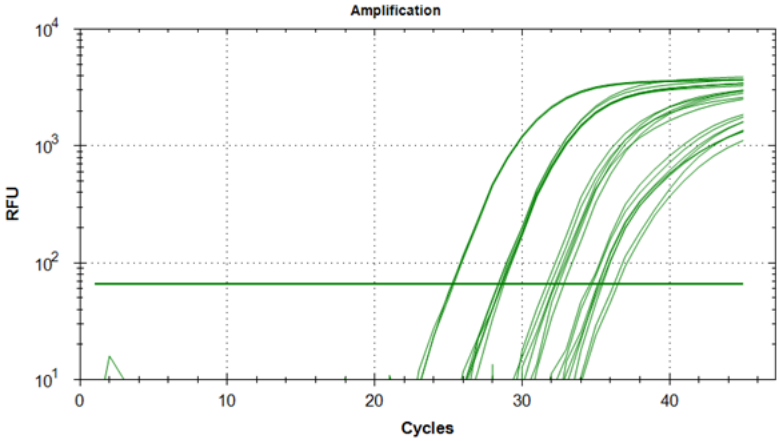

Figure 9. Baseline correction for Biorad CFX cyclers. Data from Figure 8 after drift correction.

Bio Molecular Systems Mic cycler:

Use either the "Auto Hide Excluded" setting or the "Ignore cycles before" setting in solving baseline artifact issues.

Applied Biosystems Quantstudio 5 and 7500 instruments:

Baseline artifacts can be removed by adjusting the baseline start and end for individual samples (see Figure 10). Generally, we recommend to inspect the raw data using the "Multicomponent Plot" in case of unclear results.

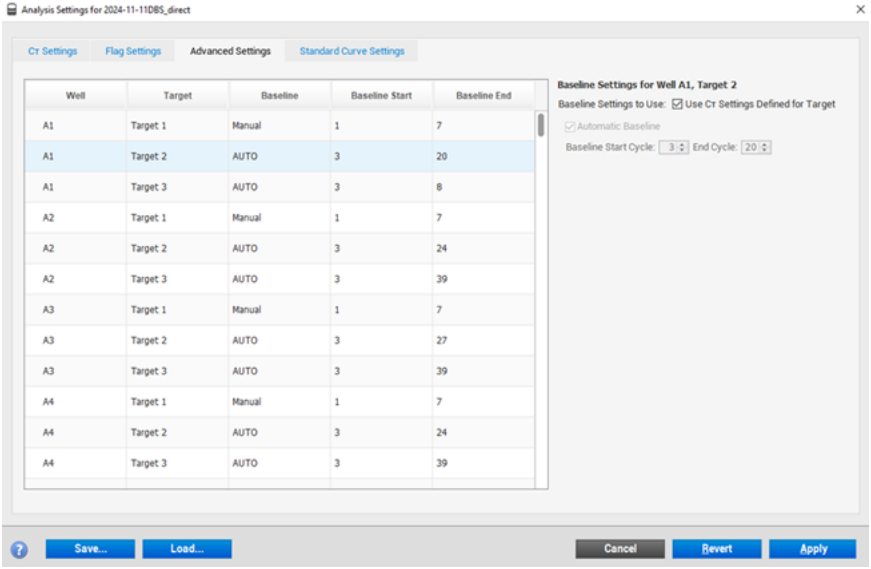

Figure 10. Baseline adjustment for Applied Biosystems instruments. Use "Advanced Settings" to define the baseline start and end for individual samples.

QIAquant:

Using the crosstalk compensation function is strongly recommended.

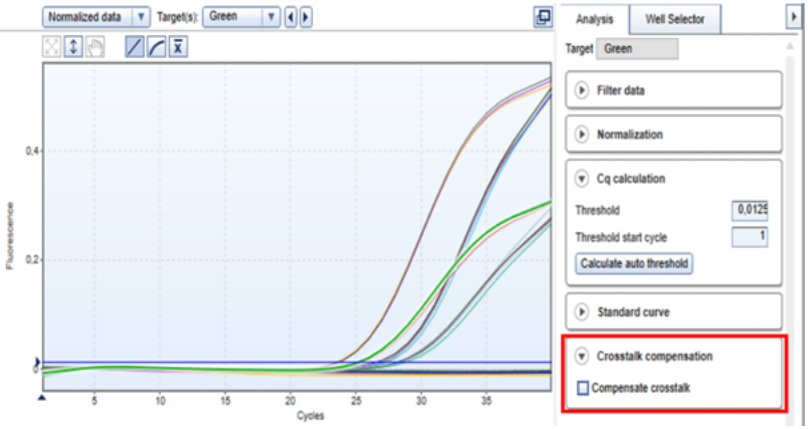

Figure 11. Crosstalk compensation for QIAquant. The function, highlighted in red, helps prevent crosstalk artifacts, especially between the blue and green channels.

Rotor-Gene Q:

In some cases, a high starting signal is detected as a very early Ct. We recommend cropping the first few cycles to exclude these signals (see Figure 12).

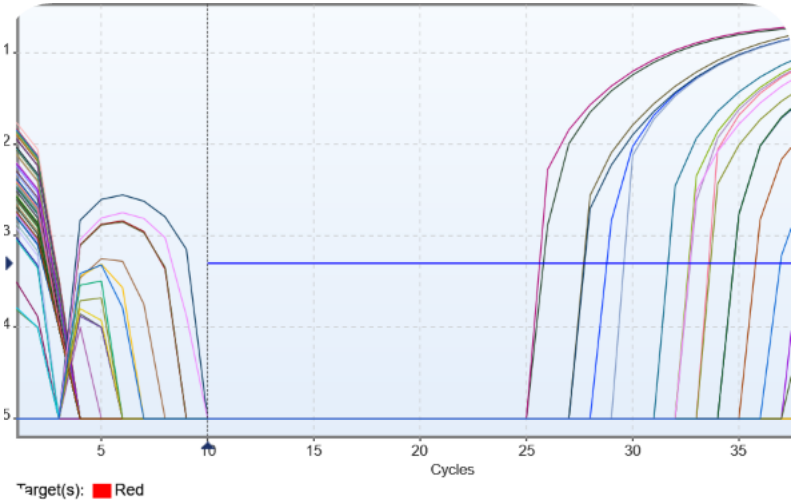

Figure 12. High starting fluorescence on Rotor-Gene Q. First 10 cycles have been cropped to exclude early signals.

Reviewing the raw data helps distinguish between a true amplification signal and baseline artifacts (see Figure 13 and Figure 14).

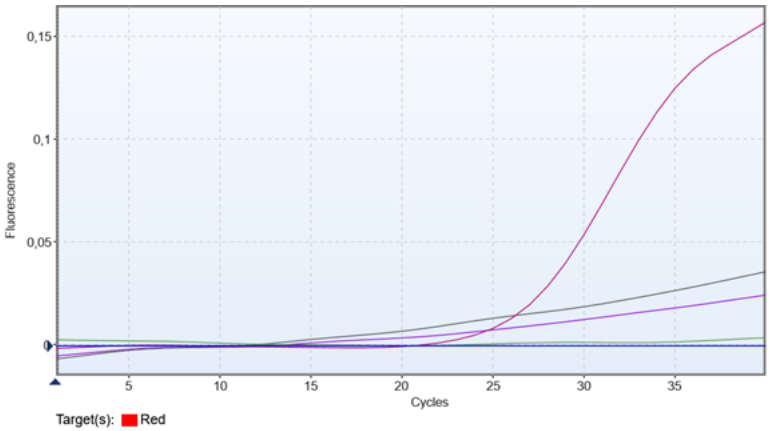

Figure 13. Rising baselines of negative samples on Rotor-Gene Q. Along with one positive signal, three negative samples were detected. Analyzing the raw data (Figure 14) helps identify false positive signals.

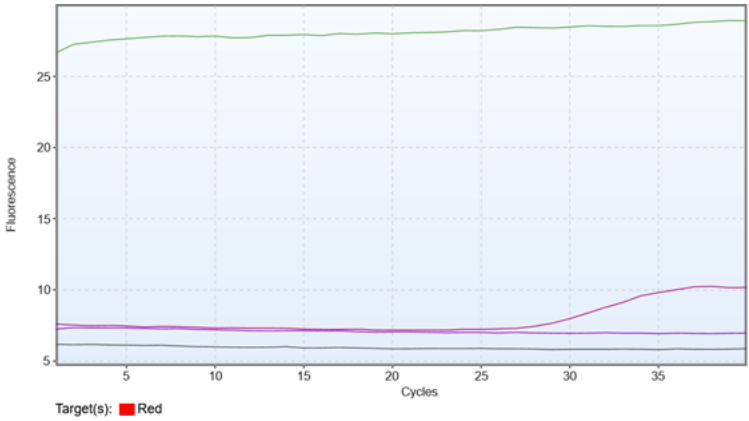

Figure 14. Rising baselines of negative samples on Rotor-Gene Q. Raw data of Figure 13. Only the positive samples display a typical amplification curve.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx** (for contact information, visit **www.qiagen.com**).

	Comments and suggestions
No signal or one or more signals detected late in PCR	
a) Incorrect cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the PCR step of your cycling conditions include the initial step for activation of the QuantiNova DNA Polymerase (95°C for 2 minutes) and the specified times for denaturation and annealing/extension.
b) QuantiNova DNA Polymerase not activated	Ensure that the cycling program includes the QuantiNova DNA Polymerase activation step (2 minutes at 95°C) as described in the protocols.
c) Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers, probes, and template nucleic acid. Repeat the PCR.
d) Wrong or no detection step	Ensure that fluorescence detection takes place during the combined annealing/extension step when using hybridization probes.
e) Problems with starting template	Check the concentration, storage conditions, and quality of the starting template .
f) Insufficient amount of starting template	Increase the amount of template, if possible. Ensure that sufficient copies of the target nucleic acids are present in your sample. If necessary, concentrate or make new serial dilutions of template nucleic acid from the stock solutions. Repeat the PCR.
g) Insufficient number of cycles	Increase the number of cycles. We recommend 40 cycles.
h) Reaction volume is too high	For both 96-well and 384-well block cyclers, we recommend a final reaction volume of 20 µL.
i) Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter setting is chosen for the reporter dye.
j) No detection activated	Check that fluorescence detection was activated in the cycling program.

	Comments and suggestions
k) Primers degraded	Check for possible degradation of primers on a denaturing polyacrylamide gel.
l) High concentration of PCR inhibitors	We recommend repeating the experiment with lower template input volume. This may dilute potential inhibitors to a concentration they do not significantly inhibit the PCR.
m) Too much blood in reaction	Blood is inhibitory to PCR. Do not use more than the recommended amount of blood. If needed reduce amount of blood.
n) No detection of RNase P	If a Plasmodium or non-Plasmodium signal was obtained, the result is valid. In case the sample gave no signal at all, sample preparation and PCR should be repeated.
o) Carry over of Blood Lysis Buffer	In the Sedimentation workflow, carry over of the buffer into the PCR reaction can cause a shift in Ct values. Ensure to remove the buffer completely from the pellet with a pipette.
p) Loss of cell pellet	In the Sedimentation workflow, ensure the supernatant is removed carefully and in a timely manner. The lack of RNase P detection is an indication for pellet loss.
Increased fluorescence or CT value for "No Template" control	
a) Contamination of reagents	Discard all the components of the assay (e.g., master mix, primers, and probes). Repeat the assay using new components.
b) Contamination during reaction setup	Take appropriate precautions during reaction setup, such as using aerosol-barrier pipette tips.
c) Minimal probe degradation, leading to sliding increase in fluorescence	Check the amplification plots, and adjust the threshold settings.
Varying fluorescence intensity	
a) Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
b) Real-time cycler is no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.
c) Quenching effect of whole blood	The presence of blood in the reaction is know to quench the fluorescent signal to some degree. Adjust the detection threshold accordingly.

Comments and suggestions	
All cycler systems:	
a) Wavy curve at high template amounts for highly expressed targets	In the analysis settings, reduce the number of cycles used for background calculation (if your real-time cycler allows you to do so) or reduce the amount of template.
b) Carry-over contamination	If the negative control (without template) shows an qPCR product or a smear, exchange all reagents. Use disposable pipette tips containing hydrophobic filters to minimize cross-contamination. Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
c) Unusually low Ct values	Even high parasitemia samples typically do not result in Ct values below 10. Check amplification plots for regular signals corresponding to these reported Ct values.
d) Samples positive for two or more species	Double infections occur and due to the sensitivity of the assay are likely detected. However, we recommend to check such samples for potential detection artifacts, e.g. cross talk.
Applied Biosystems instruments only:	
ΔRn values are unexpectedly too high or too low	The concentration of the ROX Reference Dye is incorrect. To choose the right ROX concentration for your cycler, refer to Table 3, page "Real-time cyclers requiring high/low concentrations of ROX dye" on page 11.

Contact Information

For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support**, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**).

Ordering Information

Product	Contents	Cat. no.
QIAprep&amp Plasmodium Kit	4 x 10 mL Blood Lysis Buffer, 1 x 6 mL PR Buffer, 1 x 3 mL DBS Wash Buffer, 1 x 0.2 mL S-Solution, 1 x 0.9 mL QP&A DNA Mastermix, 1 x 1.9 mL RNase-free Water For 100 reactions.	223213
Pf/Non-Pf Detection Assay	1 x 0.1 mL PF/Non-PF RNaseP, 20x Oligomix.	224113
Pv/Pm/Po/Pk Detection Assay	1 x 0.1 mL Pv/Pm/Po/Pk, 20x Oligomix.	225113
Relative Products		
QIAcard Bloodstain (100)	1 x 100 cards, 4 sample areas per card.	WB100014
QIAcard FTA Classic (100)	1 x 100 cards, 4 sample areas per card.	WB120205
QIAcard FTA Classic (25)	1 x 25 cards, 4 sample areas per card.	WB120305
UniCore Punch 1.2 mm (25)	1 x 25 FTA Sample Punchers.	WB100074
UniCore Punch 3.0 mm (25)	1 x 25 FTA Sample Punchers.	WB100078
Cutting Mat 2.5" x 3.0"	1 x cutting mat for clean sample cuts from FTA/FTA Elute Cards.	WB100088
Rotor-Gene Q 5plex System	Real-time PCR cycler with 5 channels (green, yellow, orange, red, crimson), laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training.	9001640

Document Revision History

Date	Changes
02/2025	Initial release

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