PathoFinder®

RealAccurate® Quadruplex Respiratory PCR Kits

PF0970-R RealAccurate® Quadruplex Influenza PCR Kit
PF0971-R RealAccurate® Quadruplex Corona PCR Kit
PF0971B-R RealAccurate® Quadruplex Corona-*plus* PCR Kit
PF0971C-R RealAccurate® Quadruplex Sars-CoV-2 PCR Kit
PF0972-R RealAccurate® Quadruplex Parainfluenza PCR Kit
PF0973-R RealAccurate® Quadruplex RSV/hMPV PCR Kit
PF0974-R RealAccurate® Quadruplex Adeno/Boca/Rhino/Entero PCR Kit

50 reactions/kit

Instructions For Use

June 2020

CE IVD

Disclaimer

The results obtained from these or any other diagnostic panels should be used and interpreted only in the context of the overall clinical picture. PathoFinder BV cannot accept responsibility for any clinical decisions that are made. PathoFinder BV does not represent this guide as a comprehensive summary of all possible outcomes from using the RealAccurate® Quadruplex Respiratory PCR products. This guide is intended for use solely as an aid to memory. It is not for use in any clinical interpretation of the results of the assay. Laboratories must interpret and report the results of the assay in accordance with their own locally developed procedures.

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Customer is responsible for validation of assays and compliance with regulatory requirements that pertain to their procedures and uses of the RealAccurate® Quadruplex Respiratory PCR Kits.

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1. Introduction

Acute respiratory tract infection is the most widespread type of acute infection in adults and children. The number of pathogens involved is numerous. Ageing of the population and the increased number of immunocompromised patients have raised the number of individuals at risk.

Respiratory tract infections (RTI) are commonly divided into upper respiratory tract infections (URTI) and lower respiratory tract infections (LRTI). The URTI include rhinitis, pharyngitis and laryngitis (common cold) which may be complicated by otitis media, sinusitis and conjunctivitis. LRTI include pneumonia, bronchiolitis and bronchitis. Both viruses and bacteria cause acute RTI, and the number of different causative agents is large, which provides a great challenge for diagnostics.

The RealAccurate® Quadruplex Respiratory PCR Kits are seven separate multiplex PCR tests for detection and (partial[$]) differentiation of 20 viral respiratory pathogens. They are one-step reverse transcriptase PCR assays designed for fast pathogen detection on real-time PCR instruments using 4 different detection channels (Green, Yellow, Orange and Red). An overview of the kits is given in Table 1.

[$]Remark: Parainfluenza virus 2 and 4 can be detected but not differentiated
Coronavirus NL63 and HKU1 can be detected but not differentiated
Rhinovirus and Enterovirus can be detected but not differentiated

Table 1: Overview RealAccurate® Quadruplex Respiratory PCR Kits

PF0970-R Influenza	**PF0971-R** Corona	**PF0971B-R** Corona-*plus*	**PF0971C-R** SARS-CoV-2	**PF0972-R** Parainfluenza	**PF0973-R** RSV/hMPV	**PF0974-R** Adeno/Boca/ Rhino/Entero
Influenza A virus	Coronavirus 229E	SARS-CoV-2*	SARS-CoV-2* N gene	Parainfluenza virus 1	RSV[†] A	Adenovirus
Influenza B virus	Coronavirus OC43	MERS-CoV[#]		Parainfluenza virus 2/4	RSV[†] B	Bocavirus
Influenza A(H1N1)pdm09 virus	Coronavirus NL63/HKU1		SARS-CoV-2* RdRp gene	Parainfluenza virus 3	hMPV[$] A+B	Rhino/Entero virus
Internal Control	Internal Control	Internal Control	Internal Control	Internal Control	Internal Control	Internal Control

*SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2 (causative agent of COVID-19)
[#]MERS-CoV: Middle East Respiratory Syndrome Coronavirus
[†]RSV: Respiratory Syncytial Virus
[$]hMPV: Human metapneumovirus

Intended Use

RealAccurate® Quadruplex Influenza PCR Kit is a multiplex PCR-based test to detect and differentiate within 2 hours three influenza viruses that can cause respiratory tract infections in humans: influenza virus A, B and A(H1N1)pdm09.

RealAccurate® Quadruplex Corona PCR Kit is a multiplex PCR-based test to detect and differentiate within 2 hours four coronaviruses that can cause respiratory tract infections in humans: coronavirus 229E, coronavirus OC43, coronaviruses NL63/HKU1. Coronavirus NL63 and HKU1 can be detected, but not differentiated.

RealAccurate® Quadruplex Corona-*plus* PCR Kit is a multiplex PCR-based test to detect and differentiate within 2 hours two coronaviruses that can cause respiratory tract infections in humans: SARS-CoV-2 and MERS-CoV.

RealAccurate® Quadruplex SARS-CoV-2 PCR Kit is a multiplex PCR-based test to detect within 2 hours coronavirus SARS-CoV-2 which can cause respiratory tract infections in humans, using 2 different genes: Nucleocapsid protein (N) gene and RNA-dependent RNA polymerase (RdRp) gene.

RealAccurate® Quadruplex Parainfluenza PCR Kit is a multiplex PCR-based test to detect and differentiate within 2 hours four parainfluenza viruses that can cause respiratory tract infections in humans: parainfluenza virus 1, parainfluenza viruses 2/4 and parainfluenza virus 3. Parainfluenza virus 2 and 4 can be detected, but not differentiated.

RealAccurate® Quadruplex RSV/hMPV PCR Kit is a multiplex PCR-based test to detect and differentiate within 2 hours three RNA viruses that can cause respiratory tract infections in humans: respiratory syncytial virus A, respiratory syncytial virus B and human metapneumovirus (subtype A and B).

RealAccurate® Quadruplex Adeno/Boca/Rhino/Entero PCR Kit is a multiplex PCR-based test to detect and differentiate within 2 hours 2 RNA and 2 DNA viruses that can cause respiratory tract infections in humans: rhino-/enterovirus, adenovirus and bocavirus. Rhinovirus and enterovirus can be detected, but not differentiated.

Other laboratory testing and assessment of clinical presentation must be included in the final diagnosis. Negative results do not necessarily indicate absence of viral respiratory tract infection. Negative results should not be used as the sole basis for diagnosis, therapy, other clinical management decisions or infection prevention measures. Positive results do not exclude co-infection with other pathogens. The pathogen(s) detected may not be the definite cause of disease.

The products are for use by laboratory professionals only.

2. Principle of the tests

The RealAccurate® Quadruplex Respiratory PCR Kits are designed for direct detection of viral respiratory tract infections caused by influenza viruses A, B and A(H1N1)pdm09, coronaviruses (CoVs) 229E, OC43, NL63, HKU1, SARS-CoV-2 and MERS-CoV, parainfluenza viruses 1, 2, 3 and 4, respiratory syncytial viruses A and B, human metapneumovirus A and B, adenovirus, bocavirus, enterovirus and rhinovirus. The kits contain ready to use primer and probe mixes (unique in each kit – see Table 1) and the Master mix containing reverse transcriptase and *Taq* DNA polymerase. The RealAccurate® Quadruplex Respiratory PCR Kits have been extensively validated to ensure a reliable detection of low copy numbers of pathogens' nucleic acid. As most of the pathogens detected by RealAccurate® Quadruplex Respiratory PCR Kits are RNA viruses, the assay begins with a reverse transcription step in which the viral RNA is converted into cDNA. Subsequently, the cDNA is subjected to real-time PCR in the same reaction vessel (one-step RT-PCR).

Detection is performed on a real-time PCR instrument, capable of detection of Green, Yellow, Orange and Red fluorescent labels (see Table 5), such as LightCycler® 480 (Roche), Rotor-Gene® Q (QIAGEN), CFX96™ (Bio-Rad), Mic qPCR Cycler (Bio Molecular Systems) or QuantStudio™ 5 (ThermoFisher).

In addition, each RealAccurate® Quadruplex Respiratory PCR Kit contains a dedicated Positive Control (PC) and an Internal Control (IC). The PC consists of synthetic nucleic acid fragments representing each pathogen detected by this kit. The IC allows for discrimination between true negative and false negative sample results that may occur due to nucleic acid degradation, PCR inhibition or test failure.

The input sample is total nucleic acids extracted and purified from nasopharyngeal swabs obtained from patients suspected of respiratory tract infections. Nucleic acid extraction is a separate process, outside of the scope of this product. The RealAccurate® Quadruplex Respiratory PCR Kits have been validated using NucliSENS® easyMAG® system (bioMérieux) and InviGenius® PLUS (Invitek Molecular GmbH) for nucleic acid extraction.

3. Target genes

The sequences targeted by the RealAccurate® Quadruplex Respiratory PCR Kits have been selected from conserved genomic regions of the pathogen of interest. Details are presented in Table 2.

Table 2: Target genes selected for the detection of pathogens with the RealAccurate® Quadruplex Respiratory PCR Kit

Product	Pathogen	Gene
PF0970-R	Influenza virus type A Influenza virus type B Influenza virus type A(H1N1)pdm09 Internal Control (MS2 phage)	Matrix protein gene Nucleoprotein gene Neuraminidase gene Phage coat / Lysis protein genes
PF0971-R	Coronavirus 229E Coronavirus OC43 Coronavirus NL63/HKU1 Internal Control (MS2 phage)	Nucleocapsid protein (N) gene Nucleocapsid protein (N) gene Nucleocapsid protein (N) gene Phage coat / Lysis protein genes
PF0971B-R	SARS-CoV-2 MERS-CoV Internal Control (MS2 phage)	Nucleocapsid protein (N) gene Envelope (E) gene Phage coat / Lysis protein genes
PF0971C-R	SARS-CoV-2 SARS-CoV-2 Internal Control (MS2 phage)	Nucleocapsid protein (N) gene RNA-dependent RNA polymerase (RdRp) gene Phage coat / Lysis protein genes
PF0972-R	Parainfluenza virus 1 Parainfluenza virus 2/ Parainfluenza virus 4 Parainfluenza virus 3 Internal Control (MS2 phage)	Hemagglutinin-neuraminidase gene Hemagglutinin-neuraminidase gene/ Major nucleocapsid protein gene Hemagglutinin-neuraminidase gene Phage coat / Lysis protein genes
PF0973-R	RSV A RSV B hMPV A+B Internal Control (MS2 phage)	Major nucleocapsid protein gene Major nucleocapsid protein gene Major nucleocapsid protein gene Phage coat / Lysis protein genes
PF0974-R	Adenovirus Bocavirus Rhinovirus / enterovirus Internal Control (MS2 phage)	Hexon gene Noncapsid gene 5' untranslated region Phage coat / Lysis protein genes

The target amplicons of RealAccurate® Quadruplex Respiratory PCR Kits are detected by measuring the Green, Yellow, Orange and Red fluorescence that is emitted following hydrolysis of the respective probes.
Table 3 gives an overview of the fluorescent labels that are used for the detection of the viral respiratory pathogens in each RealAccurate® Quadruplex Respiratory PCR assay.

Table 3: Pathogens and corresponding fluorescent detection labels

Product	Pathogen	Fluorescent label
PF0970-R	Influenza virus type A Influenza virus type B Influenza virus type A(H1N1)pdm09 Internal Control	Green Yellow Orange Red
PF0971-R	Coronavirus 229E Coronavirus OC43 Coronavirus NL63/HKU1 Internal Control	Green Yellow Orange Red
PF0971B-R	SARS-CoV-2 MERS-CoV Internal Control	Green Yellow Red
PF0971C-R	SARS-CoV-2 N gene SARS-CoV-2 RdRp gene Internal Control	Green Orange Red
PF0972-R	Parainfluenza virus 1 Parainfluenza virus 2/4 Parainfluenza virus 3 Internal Control	Green Yellow Orange Red
PF0973-R	RSV A RSV B hMPV A+B Internal Control	Green Yellow Orange Red
PF0974-R	Adenovirus Bocavirus Rhino/Enterovirus Internal Control	Green Yellow Orange Red

4. Contents of the kits

The following materials are provided per kit (Table 4).

Table 4: Materials provided in each RealAccurate® Quadruplex Respiratory PCR Kit

Components	Volume	Color of tube	Color of screw cap
Primer/Probe mix (specific for each kit)	>400 µl	Amber	Specific* for PCR kit
Master mix	>650 µl	Transparent	White
Internal Control	>800 µl	Transparent	Black
Positive Control	>125 µl	Transparent	Specific* for PCR kit
Negative Control	>1500 µl	Transparent	Transparent
			*in PF0970-R: Red *in PF0971-R: Blue *in PF0971B-R: Green *in PF0971C-R: Brown *in PF0972-R: Yellow *in PF0973-R: Orange *in PF0974-R: Purple

5. Equipment and reagents to be provided by user

The following equipment and materials are needed to perform the assays:

- RNA/DNA extraction reagents as outlined in section 10
- Real-time PCR instrument* suitable for detection of fluorescent labels as indicated in Table 5, including suitable sterile RNase/DNase free PCR tubes, strips or plates
- Disposable gloves
- Adjustable pipettes*: 0.1–2 µl, 2–20 µl, 20–200 µl, 100–1000 µl
- Disposable tips containing hydrophobic filters
- Vortex mixer
- Sterile RNase/DNase free 1.5 ml vials
- Centrifuge* capable of centrifuging PCR tubes, strips or plates and 1.5 ml vials
- Cooling block or ice

* Remark: these instruments must be checked and calibrated according to the manufacturer's recommendations

6. Storage and handling

The components of the RealAccurate® Respiratory Quadruplex PCR Kits should be stored in the dark at –30 °C to –15 °C. The expiration date is indicated on the label. Repeated thawing and freezing (>10x) should be avoided.
To avoid contamination, we recommend performing the experimental activities in three separate areas.

Area 1:	- Preparation PCR mix
Area 2:	- Nucleic acid extraction from samples
	- Addition nucleic acid extracts to the mix
	- Addition of Positive Control to the mix
Area 3:	- PCR reactions

7. General recommendations

The following precautions should be taken to both avoid contamination and allow optimal reproducibility of the assays:

- This molecular diagnostic assay should only be performed by qualified laboratory personnel.
- Physically separate the workplaces as outlined in section 6.
- Wear disposable gloves when performing the assay.
- Use **disposable tips** containing hydrophobic **filters** to prevent cross-contamination.
- Use RNase/DNase free PCR vials.
- Thaw **RNA/DNA samples** always on ice and keep them on **ice** or on a **cooling block**.
- Keep **enzymes** always on **ice** or on a **cooling block** when taken out of the freezer. Handle enzymes with care and mix very gently.
- When thawed, **spin down the reagents** for 5 seconds in a centrifuge and mix by gently pipetting up and down.
- The cycling program should be entered in the real-time PCR instrument before performing the assay.
- Always centrifuge PCR vials and plates briefly; open with care to avoid aerosols.

8. Real-time PCR instrument settings

The RealAccurate® Quadruplex Respiratory PCR assays are one-step reverse transcriptase PCR assays which are designed for fast pathogens' nucleic acid detection on real-time instruments using 4 different detection channels: Green, Yellow, Orange and Red. In Table 5 the optimal filter settings are given for detection of the 4 fluorescent labels. For each real-time PCR instrument, select the optimal filters for detection of the fluorescent labels used in the RealAccurate® Quadruplex Respiratory PCR Kits.

Table 5: Filter settings for real-time PCR instruments for a RealAccurate® Quadruplex PCR reaction

Fluorescent label	Source	Detector
Green	495 nm	516 nm
Yellow	524 nm	557 nm
Orange	598 nm	617 nm
Red	645 nm	665 nm

Programming of the instruments should be carried out according to the manufacturer's instructions. Detection in the 4 detection channels should be activated. (See section 13 for PCR instrument-specific detection channels).

Measurement data are displayed as sigmoid-shaped plots (when using a linear scale), in which the fluorescence is plotted against the number of cycles. The threshold cycle (C_t) value increases with a decreasing with a decreasing initial amount of template in the reaction. The threshold is set above the baseline, in the log-linear range of the plot. Before determining the C_t value, check whether the threshold is positioned correctly and adjust if necessary.

9. Clinical specimen

Respiratory pathogen diagnosis depends on the collection of high-quality specimens, their rapid transport to the laboratory and appropriate storage before laboratory testing.

Clinical specimens should be transported to the laboratory as soon as possible, aliquoted and processed. The specimens should be kept at 2–8 °C. If specimens cannot be processed within 48 hours, they should be kept frozen at or below –20 °C, preferably –70 °C. Validation of RealAccurate® Quadruplex Respiratory PCR Kits has been performed on nasopharyngeal swabs suitable for the detection of viral and/or bacterial infections of the respiratory tract.

10. Preparation of specimen RNA/DNA extraction

10.1 Commercial extraction systems

Sample preparation is a separate process, outside of the scope of this product, therefore suitable methods or products must be used to handle specimens and extract and purify nucleic acids.

The following RNA/DNA extraction methods have been validated in combination with the RealAccurate® Quadruplex Respiratory PCR Kits:

- NucliSENS® easyMAG® (bioMérieux).

For nucleic acid extraction on NucliSENS® easyMAG® system, the 'Generic 2.0.1' protocol must be used according to manufacturer's instructions. Two hundred (200) µl of sample material is used with on-board lysis and an elution volume of 100 µl is selected. The Internal Control is added according to the manufacturer's instructions (see section 11).

- InviGenius® PLUS (Invitek Molecular GmbH)

For nucleic acid extraction on the InviGenius® PLUS system, the InviMAG® Universal Kit /IG must be used according to manufacturer's instructions. Two hundred (200) µl of sample material is used and an elution volume of 100 µl. The Internal Control is added according to the manufacturer's instructions (see section 11).

10.2 Heat-treatment

Only validated in combination with RealAccurate® Quadruplex SARS-CoV-2 PCR Kit!

The robust composition of the RealAccurate® Quadruplex SARS-CoV-2 PCR Kit allows replacement of a full nucleic acid extraction procedure by a simple heat-treatment. This decreases sample processing time and saves on nucleic acid extraction reagents and disposables.
Samples in eSwab™ medium can be used directly in the heating step, samples in Universal Transport Medium should be diluted 1:1 with RNase free water.
For this purpose, incubate an aliquot of the (diluted) sample at 99°C for 10 minutes. We recommend to do this in PCR tubes or strips with individual caps. After the heating step, centrifuge briefly and use 5 µl of the supernatant in the PCR reaction.

Prevent freeze-thaw cycles of the extracted DNA/RNA and store nucleic acid extracts at 2–8 °C when processed within one day. For longer periods, store the extracted RNA/DNA at –20 °C or –70 °C.

11. Controls

The RealAccurate® Quadruplex Respiratory PCR Kits contain the following assay controls.

- **Internal Control (IC)**

The Internal Control (IC) supplied in the kits, is an MS2 bacteriophage suspension. It serves as a control for lysis, RNA/DNA extraction, the RealAccurate® Quadruplex Respiratory PCR assay performance, and to check for possible PCR inhibition. Five (5) µl IC needs to be added to each clinical sample (200 µl) in the nucleic acid extraction procedure

<u>Addition of IC in NucliSENS® easyMAG® procedure</u>

In the NucliSENS® easyMAG® nucleic acid extraction procedure, the IC provided with the RealAccurate® Quadruplex Respiratory PCR Kits is added to the silica solution.

For 8 samples, the following mix is prepared using step 1 of the NucliSENS® easyMAG® multi-pipet for addition of the silica:

550 µl silica + 55 µl IC + 495 µl H_2O

Using step 2 of the multi-pipet, 125 µl of the mix is dispensed into each of 8 wells of a micro-titer plate. With step 3 of the multi-pipet, 100 µl of the dispensed silica/IC mix is transferred to each NucliSENS®® easyMAG® vessel containing sample material. In this way 5 µl of the IC are added per sample.

If less than 8 samples are processed, the volumes stated in Table 6 can be used.

Table 6. Protocol for spiking the IC to the silica solution.

Samples	Sterile water (µl)	IC (µl)	Magnetic silica (µl)	Total (µl)
1	63	7	70	140
2	126	14	140	280
3	189	21	210	420
4	252	28	280	560
5	315	35	350	700
6	378	42	420	840
7	441	49	490	980
8	495	55	550	1100

Addition of IC in InviGenius® PLUS procedure

In the InviGenius® PLUS nucleic acid extraction procedure, the IC is added to the PKC tube. 700 µl RNAse free water is added to the PKC tube. The tube is thoroughly mixed and additionally 100 µl IC is added to the PKC tube. Again, the solution is mixed well.
The InviGenius® PLUS instrument uses 40 µl of the PKC solution (including IC) per sample, which represents 5 µl RealAccurate® Quadruplex IC per extraction.

Addition of IC using heat-treatment with RealAccurate® Quadruplex SARS-CoV-2 PCR Kit

When the RealAccurate® Quadruplex SARS-CoV-2 assay is performed without nucleic acid extraction, but with the heat-treatment method described in section 10.2, the IC is added directly to the PCR mix (see table 7).

In case of a strong infection, the IC PCR curve might not be visible in the final analysis. This is explained by the fact that high amounts of pathogenic nucleic acids consume most of the reagents in the assay. Consequently, when the IC signal is absent in the presence of one or more positive PCR curves, indicating an infection, the assay is still valid.

- **Positive Control (PC)**

Each RealAccurate® Quadruplex Respiratory PCR Kit contains a Positive Control.
The Positive Control consists of synthetic DNA fragments of all the targets that can be detected by that assay, including the Internal Control. The Positive Control is handled as a regular nucleic acid extract and controls for PCR reagents and real-time PCR protocol. Positive Control should not be subjected to the nucleic acid extraction procedure.

- **Negative Control (NC)**

A negative control in a RealAccurate® Quadruplex Respiratory PCR run consists of 200 µl Negative Control (provided in the kit) or 200 µl negative sample.
When the RealAccurate® Quadruplex SARS-CoV-2 assay is performed without nucleic acid extraction, but with the heat-treatment method 5 µl Negative Control (provided in the kit) or 5 µl negative sample is used. (See 10.2 for details).
Negative Control is handled as a regular sample, including the addition of IC, and is a control for contamination in the extraction or test procedure.

12. RealAccurate® Quadruplex Respiratory PCR protocol

12.1 Preparation of the PCR mix

The PCR reaction is performed in a final volume of 25 µl.

Table 7: Preparation of PCR mix

Component	Volume/reaction	Volume/reaction using heated samples*
Master mix Primer/Probe mix IC	12.5 µl 7.5 µl	12.5 µl 7.5 µl 0.25 µl
Total Volume	20 µl	20.25 µl

*Only validated in combination with RealAccurate® Quadruplex SARS-CoV-2 PCR Kit

- Mix the PCR mix gently but thoroughly and dispense 20 µl per sample or control into a PCR vial or well of a PCR strip or plate.
- Add 5 µl of nucleic acid extract of each sample, including extracted Negative Control and add 5 µl Positive Control (no extraction needed) to the PCR mix(es).
  Or **(only for RealAccurate® Quadruplex SARS-CoV-2 PCR Kit)**: Add 5 µl of each heated (diluted) sample, including Negative Control and add 5 µl Positive Control (no extraction needed) to the PCR mix.
- Close the PCR vials, strips or plates, centrifuge briefly and place the PCR vials, strips or plates in the real-time PCR instrument.

12.2 PCR program

Table 8 shows the PCR cycling program for the RealAccurate® Quadruplex Respiratory PCR reactions.

Table 8: RealAccurate® Quadruplex Respiratory PCR program

Time	Temperature	Function	
10 min	50 °C	Reverse transcription	
1 min	95 °C	Activation of *Taq* DNA polymerase, inactivation of reverse transcriptase	
10 sec 60 sec	95 °C 60 °C*	Denaturation Annealing and extension	PCR 40 cycles

* Fluorescence acquisition in Green, Yellow, Orange and Red channel

13. PCR instrument related issues

13.1 LightCycler® 480 (Roche)

When a LightCycler® 480 II is used in combination with the RealAccurate® Quadruplex Respiratory PCR Kits, the detection channels as stated in Table 9 must be selected:

Table 9: Detection channels LightCycler® 480 II

Product	Pathogen	Fluorescent label	Excitation	Emission
PF0970-R	Influenza virus type A	Green	465nm	510nm
	Influenza virus type B	Yellow	533nm	580nm
	Influenza virus type A(H1N1)pdm09	Orange	533nm	610nm
	Internal Control	Red	618nm	680nm
PF0971-R	Coronavirus 229E	Green	465nm	510nm
	Coronavirus OC43	Yellow	533nm	580nm
	Coronavirus NL63/HKU1	Orange	533nm	610nm
	Internal Control	Red	618nm	680nm
PF0971B-R	SARS-CoV-2	Green	465nm	510nm
	MERS-CoV	Yellow	533nm	580nm
	Internal Control	Red	618nm	680nm
PF0971C-R	SARS-CoV-2 N gene	Green	465nm	510nm
	SARS-CoV-2 RdRp gene	Orange	533nm	610nm
	Internal Control	Red	618nm	680nm
PF0972-R	Parainfluenza virus 1	Green	465nm	510nm
	Parainfluenza virus 2/4	Yellow	533nm	580nm
	Parainfluenza virus 3	Orange	533nm	610nm
	Internal Control	Red	618nm	680nm
PF0973-R	RSV A	Green	465nm	510nm
	RSV B	Yellow	533nm	580nm
	hMPV A+B	Orange	533nm	610nm
	Internal Control	Red	618nm	680nm
PF0974-R	Adenovirus	Green	465nm	510nm
	Bocavirus	Yellow	533nm	580nm
	Rhino/Enterovirus	Orange	533nm	610nm
	Internal Control	Red	618nm	680nm

Result analysis is performed using “Abs Quant/2nd Derivative Max”.
Color Compensation is applied to the LightCycler® 480 runs to correct for possible cross-talk between detection channels, using a CC Object created with the RealAccurate® Quadruplex Color Compensation Kit (PFCC-R).

In rare cases, the LightCycler® software calls C_p (crossing point) values which are not in line with the observed real-time PCR results (e.g. a C_p value of 9 in a background fluorescence part of the PCR graph). Therefore, it is recommended to check the C_p values that are generated by LightCycler® 480 software manually.

Although a color compensation file is applied to a LightCycler® 480 run, residual cross-talk may occasionally remain. To be able to discriminate between a weak positive sample and a weak signal caused by remaining cross-talk, the fluorescent signal can be analyzed with and without color compensation. A signal that decreases significantly by applying the color compensation is most probably caused by cross-talk coming from one of the neighboring detection channels.

13.2 Rotor-Gene® Q (QIAGEN)

When a Rotor-Gene® Q is used in combination with the RealAccurate® Quadruplex Respiratory PCR Kits, the detection channels as stated in Table 10 must be selected. Gain 5 is selected for all channels.

Table 10: Detection channels Rotor-Gene® Q

Product	Pathogen	Channel	Source	Detector	Gain
PF0970-R	Influenza virus type A	Green	470nm	510nm	5
	Influenza virus type B	Yellow	530nm	555nm	5
	Influenza virus type A(H1N1)pdm09	Orange	585nm	610nm	5
	Internal Control	Red	625nm	660nm	5
PF0971-R	Coronavirus 229E	Green	470nm	510nm	5
	Coronavirus OC43	Yellow	530nm	555nm	5
	Coronavirus NL63/HKU1	Orange	585nm	610nm	5
	Internal Control	Red	625nm	660nm	5
PF0971B-R	SARS-CoV-2	Green	470nm	510nm	5
	MERS-CoV	Yellow	530nm	555nm	5
	Internal Control	Red	625nm	660nm	5
PF0971C-R	SARS-CoV-2 N gene	Green	470nm	510nm	5
	SARS-CoV-2 RdRp gene	Orange	585nm	610nm	5
	Internal Control	Red	625nm	660nm	5
PF0972-R	Parainfluenza virus 1	Green	470nm	510nm	5
	Parainfluenza virus 2/4	Yellow	530nm	555nm	5
	Parainfluenza virus 3	Orange	585nm	610nm	5
	Internal Control	Red	625nm	660nm	5
PF0973-R	RSV A	Green	470nm	510nm	5
	RSV B	Yellow	530nm	555nm	5
	hMPV A+B	Orange	585nm	610nm	5
	Internal Control	Red	625nm	660nm	5
PF0974-R	Adenovirus	Green	470nm	510nm	5
	Bocavirus	Yellow	530nm	555nm	5
	Rhino/Enterovirus	Orange	585nm	610nm	5
	Internal Control	Red	625nm	660nm	5

For a correct analysis of the results, the Yellow channel needs to be normalized to the Green channel: Open the raw channel Cycling A. Yellow. Click on the “Options” button and select “Normalise to Cycling A. Green”.

The analysis of the results must be performed with fluorescence presented in logarithmic scale (“Log. Scale” button under the PCR graph) and with activated “dynamic tube” function (default setting). Thresholds must be set per channel using the background fluorescence level of the Negative Control reaction.

It has been noticed that in some cases the fluorescence signal of a PCR curve obtained on a Rotor-Gene® Q instrument decreases during the first cycles of the PCR reaction. An example is given in figure 1. To avoid incorrect C_t calling, the first cycles can be eliminated in the Rotor-Gene® Q C_t calculation during result analysis.

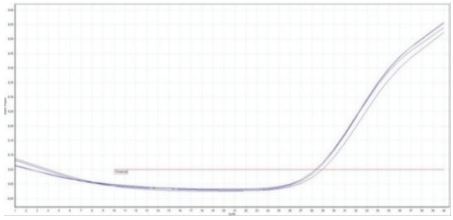

Figure 1. Example of decrease in fluorescence signal in first PCR cycles of a Rotor-Gene® Q

13.3 CFX96™ (Bio-Rad)

When a CFX96™ is used in combination with the RealAccurate® Quadruplex respiratory PCR Kits, the fluorophores as stated in Table 11 must be selected as detection channels.

Table 11: Detection channels CFX96™

Product	Pathogen	Fluorescent label	Fluorophore
PF0970-R	Influenza virus type A Influenza virus type B Influenza virus type A(H1N1)pdm09 Internal Control	Green Yellow Orange Red	FAM HEX Texas Red CY5
PF0971-R	Coronavirus 229E Coronavirus OC43 Coronavirus NL63/HKU1 Internal Control	Green Yellow Orange Red	FAM HEX Texas Red CY5
PF0971B-R	SARS-CoV-2 MERS-CoV Internal Control	Green Yellow Red	FAM HEX CY5
PF0971C-R	SARS-CoV-2 N gene SARS-CoV-2 RdRp gene Internal Control	Green Orange Red	FAM Texas Red Cy5
PF0972-R	Parainfluenza virus 1 Parainfluenza virus 2/4 Parainfluenza virus 3 Internal Control	Green Yellow Orange Red	FAM HEX Texas Red CY5
PF0973-R	RSV A RSV B hMPV A+B Internal Control	Green Yellow Orange Red	FAM HEX Texas Red CY5
PF0974-R	Adenovirus Bocavirus Rhino/Enterovirus Internal Control	Green Yellow Orange Red	FAM HEX Texas Red CY5

Clear well Hard-shell®96-Well PCR Plates (Bio-Rad art: HSP9641) must be used. (White well reaction plates should not be used due to a-specific fluorescence)

For result analysis use in in “Settings” Cq Determination Mode: “Single Threshold” and in “Baseline settings”: “Baseline subtracted Curve Fit” and “Apply Fluorescence Drift Correction”.

13.4 Mic qPCR Cycler (Bio Molecular Systems)

When a Mic qPCR Cycler is used in combination with the RealAccurate® Quadruplex Respiratory PCR Kits, the fluorophores as stated in Table 12 must be selected as detection channels. Before appointing the channels, select "Hydrolysis Probe" as chemistry type in the "Information" tab. This results in an auto-gain setting of 10 for all channels.

Table 12: Detection channels Mic qPCR Cycler

Product	Pathogen	Fluorescent label	Fluorophore
PF0970-R	Influenza virus type A Influenza virus type B Influenza virus type A(H1N1)pdm09 Internal Control	Green Yellow Orange Red	FAM HEX Texas Red CY5
PF0971-R	Coronavirus 229E Coronavirus OC43 Coronavirus NL63/HKU1 Internal Control	Green Yellow Orange Red	FAM HEX Texas Red CY5
PF0971B-R	SARS-CoV-2 MERS-CoV Internal Control	Green Yellow Red	FAM HEX CY5
PF0971C-R	SARS-CoV-2 N gene SARS-CoV-2 RdRp gene Internal Control	Green Orange Red	FAM Texas Red Cy5
PF0972-R	Parainfluenza virus 1 Parainfluenza virus 2/4 Parainfluenza virus 3 Internal Control	Green Yellow Orange Red	FAM HEX Texas Red CY5
PF0973-R	RSV A RSV B hMPV A+B Internal Control	Green Yellow Orange Red	FAM HEX Texas Red CY5
PF0974-R	Adenovirus Bocavirus Rhino/Enterovirus Internal Control	Green Yellow Orange Red	FAM HEX Texas Red CY5

In Table 13 an overview is given for the additional Cycling Analysis settings using the Mic qPCR Cycler in combination with the RealAccurate® Quadruplex Respiratory PCR Kits.

Table 13: Software settings Mic qPCR Cycler

Analysing settings	Channel			
	FAM	HEX	Texas Red	Cy5
Auto Set Threshold	Check mark	Check mark	Check mark	Check mark
Method	Dynamic	Dynamic	Dynamic	Dynamic
Threshold Level	0,100*	0,100*	0,100*	0,100*
Threshold Start	1,00	1,00	1,00	1,00
Ignore Cycles Before	5	5	5	5
Exclusion	Extensive	Extensive	Extensive	Extensive
Fluorescence Cut-off Level	5,0%	5,0%	5,0%	5,0%
Initial Y-axis Scale	Linear	Linear	Linear	Linear
Auto generate Analysis	Check mark	Check mark	Check mark	Check mark

** standard value, which is ignored if Auto Set Threshold is check marked*

Select the correct "assay" for the samples to be analyzed in the "Samples" tab.
The "Assay" which contains all settings to perform a RealAccurate® Quadruplex Respiratory PCR assay as described above, can also be obtained at PathoFinder.

13.5 QuantStudio™ 5 (ThermoFisher)

When a QuantStudio ™ 5 is used in combination with the RealAccurate® Quadruplex Respiratory PCR Kits, the detection channels as stated in Table 14 must be selected.

Table 14: Detection channels QuantStudio™ 5

Product	Pathogen	Fluorescent label	Excitation	Emission
PF0970-R	Influenza virus type A	Green	470nm	520nm
	Influenza virus type B	Yellow	520nm	558nm
	Influenza virus type A(H1N1)pdm09	Orange	580nm	623nm
	Internal Control	Red	640nm	682nm
PF0971-R	Coronavirus 229E	Green	470nm	520nm
	Coronavirus OC43	Yellow	520nm	558nm
	Coronavirus NL63/HKU1	Orange	580nm	623nm
	Internal Control	Red	640nm	682nm
PF0971B-R	SARS-CoV-2	Green	470nm	520nm
	MERS-CoV	Yellow	520nm	558nm
	Internal Control	Red	640nm	682nm
PF0971C-R	SARS-CoV-2 N gene	Green	470nm	520nm
	SARS-CoV-2 RdRp gene	Orange	580nm	623nm
	Internal Control	Red	640nm	682nm
PF0972-R	Parainfluenza virus 1	Green	470nm	520nm
	Parainfluenza virus 2/4	Yellow	520nm	558nm
	Parainfluenza virus 3	Orange	580nm	623nm
	Internal Control	Red	640nm	682nm
PF0973-R	RSV A	Green	470nm	520nm
	RSV B	Yellow	520nm	558nm
	hMPV A+B	Orange	580nm	623nm
	Internal Control	Red	640nm	682nm
PF0974-R	Adenovirus	Green	470nm	520nm
	Bocavirus	Yellow	520nm	558nm
	Rhino/Enterovirus	Orange	580nm	623nm
	Internal Control	Red	640nm	682nm

- Apply the correct PCR profile in the **Method** tab.
- Select in **Plate** tab in Quick Setup: Passive Reference: None
- Select in the **Plate** tab Advanced Setup to Add the targets Green (Reporter FAM / Quencher non), Yellow (Reporter VIC / Quencher non), Orange (Reporter ROX / Quencher non) and Red (Reporter CY5 / Quencher non), or apply the names of the targets of the RealAccurate® Quadruplex Respiratory PCR Kit to be tested.

14. Data analysis

Interpretation RealAccurate® Quadruplex Respiratory PCR data is described below, separately for each kit:

14.1 PF0970-R: RealAccurate® Quadruplex Influenza PCR Kit

- If a Green fluorescence signal is detected, the sample contains RNA of Influenza A targeted by the Green-labelled probe.
- If a Yellow fluorescence signal is detected, the sample contains RNA of Influenza B targeted by the Yellow-labelled probe.
- If an Orange fluorescence signal is detected, the sample contains RNA of Influenza A(H1N1)pdm09 targeted by the Orange-labelled probe.
- If a Red fluorescence signal is detected, the sample contains RNA of the IC targeted by the Red-labelled probe.
- If no fluorescence signal is detected see 14.8.

14.2 PF0971-R: RealAccurate® Quadruplex Coronavirus PCR Kit

- If a Green fluorescence signal is detected, the sample contains RNA of coronavirus 229E targeted by the Green-labelled probe.
- If a Yellow fluorescence signal is detected, the sample contains RNA of coronavirus OC43 targeted by the Yellow-labelled probe.
- If an Orange fluorescence signal is detected, the sample contains RNA of coronavirus NL63 and/or HKU1, both targeted by Orange-labelled probes.
- If a Red fluorescence signal is detected, the sample contains RNA of the IC targeted by the Red-labelled probe.
- If no fluorescence signal is detected see 14.8.

14.3 PF0971B-R: RealAccurate® Quadruplex Corona-*plus* PCR Kit

- If a Green fluorescence signal is detected, the sample contains RNA of SARS-CoV-2 targeted by the Green-labelled probe.
- If a Yellow fluorescence signal is detected, the sample contains RNA of coronavirus MERS-CoV targeted by the Yellow-labelled probe.
- If a Red fluorescence signal is detected, the sample contains RNA of the IC targeted by the Red-labelled probe.
- If no fluorescence signal is detected see 14.8.

14.4 PF0971C-R: RealAccurate® Quadruplex SARS-CoV-2 PCR Kit

- If a Green and an Orange fluorescence signal is detected, the sample contains RNA of SARS-CoV-2 targeted by the Green-labelled and/or Orange-labelled probes.
- If a Green or an Orange fluorescence signal is detected, the sample contains RNA of SARS-CoV-2 targeted by the Green-labelled or Orange-labelled probe. Most likely the obtained Ct value is relatively high (>37), which means that the sample contains very little SARS-CoV-2 RNA, at the borderline of detection, resulting in a single positive PCR reaction.
  Note: In LightCycler 480 weak signals are called with Cp 35 and comment “Late Cp call (last five cycles) has higher uncertainty”.
- If a Red fluorescence signal is detected, the sample contains RNA of the IC targeted by the Red-labelled probe.
- If no fluorescence signal is detected see 14.8.

14.5 PF0972-R: RealAccurate® Quadruplex Parainfluenza PCR Kit

- If a Green fluorescence signal is detected, the sample contains RNA of parainfluenza virus type 1 targeted by the Green-labelled probe.
- If a Yellow fluorescence signal is detected, the sample contains RNA of parainfluenza virus type 2 and/or type 4, both targeted by Yellow-labelled probes.
- If an Orange fluorescence signal is detected, the sample contains RNA of parainfluenza virus type 3 targeted by the Orange-labelled probe.
- If a Red fluorescence signal is detected in the PCR assay, the sample contains RNA of the IC targeted by the Red-labelled probe.
- If no fluorescence signal is detected see 14.8.

14.6 PF0973-R: RealAccurate® Quadruplex RSV/hMPV PCR Kit

- If a Green fluorescence signal is detected, the sample contains RNA of RSV A targeted by the Green-labelled probe.
- If a Yellow fluorescence signal is detected in the PCR assay, the sample contains RNA of RSV B targeted by the Yellow-labelled probe.
- If a Orange fluorescence signal is detected, the sample contains RNA of hMPV A or B targeted by the Orange-labelled probe.
- If a Red fluorescence signal is detected in the PCR assay, the sample contains RNA of the IC targeted by the Red-labelled probe.
- If no fluorescence signal is detected see 14.8.

14.7 PF0974-R: RealAccurate® Quadruplex Adeno/Boca/Rhino/Entero PCR Kit

- If a Green fluorescence signal is detected in the PCR assay, the sample contains RNA of Adenovirus targeted by the Green-labelled probe.
- If a Yellow fluorescence signal is detected in the PCR assay, the sample contains RNA of Bocavirus targeted by the Yellow-labelled probe.
- If an Orange fluorescence signal is detected in the PCR assay, the sample contains RNA of Rhinovirus and/or Enterovirus targeted by the Orange-labelled probe.
- If a Red fluorescence signal is detected, the sample contains RNA of the IC targeted by the Red-labelled probe.
- If no fluorescence signal is detected see 14.8.

14.8 No signal in a RealAccurate® Quadruplex Respiratory PCR reaction

If no fluorescence signal is present in any of the detection channels in a RealAccurate® Quadruplex Respiratory PCR reaction, the sample is inhibited, one of the assay steps has failed or a manual error has occurred. See section 16 for troubleshooting.

14.9 RealAccurate® Quadruplex Respiratory PCR sample and control results

- **Samples**

C_t (cycle threshold) values obtained in real-time PCR depend on the used real-time PCR instrument and C_t calculation method.
In general, a strong positive sample is characterized by a C_t value lower than 25, whereas a C_t value between 35 and 40 indicates a weak positive sample.

- **Internal Control**

The IC is added in a low concentration to the RealAccurate® Quadruplex Respiratory PCR reaction in order not to compete with pathogen amplification but should be present when no pathogens are detected in the reaction. Depending on the real-time PCR instrument used for the reaction, the C_t value of the IC reaction varies between 32 and 35. Only in samples with a high pathogen load, the IC can be outcompeted. In absence of an IC signal in positive samples with a relatively high pathogen Ct value (≥35), it is recommended to repeat the nucleic acid extraction to exclude inhibiting factors in the sample.

- **Positive Control**

The Positive Controls of a RealAccurate® Quadruplex Respiratory PCR Kit consists of synthetic targets representing each of the pathogens detected by that RealAccurate® Quadruplex Respiratory PCR Kit. These synthetic targets are present in the Positive Controls in moderate concentrations and should reveal C_t values of approximately 30 (depending on the used real-time PCR instrument). The IC target in the Positive Control reaction should reveal a slightly higher C_t value than those of the pathogen targets, to be more in line with the low IC concentration in a RealAccurate® Quadruplex Respiratory PCR reaction.

Note: Due to the specific optics in a Rotor-Gene Q instrument, normalization of "Cycling A. Yellow to Cycling A. Green" in a RealAccurate® Quadruplex Corona-*plus* PCR run might remove the PC signal in the Yellow channel.

15. Performance Characteristics

Performance characteristics were obtained using reference strains and samples of different origin. In this section, the following abbreviations are used for the sources:

ATCC	American Type Culture Collection
CHU de Caen	Centre Hospitalier Universitaire, Caen, France
RIVM	Netherlands National Institute for Public Health and the Environment (Dutch: *Rijksinstituut voor Volksgezondheid en Milieu*)
ZeptoMetrix	ZeptoMetrix corporation, Buffalo, USA
QCMD	Quality Control for Molecular Diagnostics, Glasgow, Scotland

The performance characteristics as described in this section were obtained using NucliSENS® easyMAG® (bioMérieux) for nucleic acid extraction as described in section 10 and LightCycler® 480 for RealAccurate® Quadruplex Respiratory PCR as described in section 13.1. using LightCycler® software release 1.5.1.

Equivalent nucleic acid extraction was proven for InviGenius® PLUS (Invitek Molecular GmbH).
Equivalent RealAccurate® Quadruplex Respiratory PCR performance was proven using:

Rotor-Gene® Q with software version 2.3.1 (build 49) – 2.3.4
Mic qPCR Cycler with software version 2.6.4 – 2.8.10
CFX96™ with software version 3.0 – 3.1
QuantStudio™ 5 with software version 1.5.1

Limit of detection (LoD)

The limit of detection (LoD) in consideration of the purification (sensitivity limit) was assessed for the RealAccurate® Quadruplex Respiratory PCR Kits using whole virus specimens in Universal Transport Medium (UTM™) in combination with nucleic acid extraction on NucliSENS® easyMAG® system (bioMérieux). The limit of detection in consideration of the purification of the RealAccurate® Quadruplex Respiratory PCR Kits was determined using a dilution series of viral strains (ATCC®, ZeptoMetrix®, QCMD, viral culture) in UTM™. These were subjected to RNA/DNA extraction using NucliSENS® easyMAG® system and 'Generic 2.0.1' protocol (extraction volume: 200 µl, elution volume: 100 µl). Each of 5 extractions was analyzed in quadruplicate with the corresponding RealAccurate® Quadruplex Respiratory PCR Kit. The results were determined by hit rate (≥95% hit rate was defined as the analytical detection limit for the respective pathogen). An overview of the limit of detection for all pathogens tested is shown in Table 15. Limit of detection was calculated using stock concentrations given by the supplier ($TCID_{50}$, $CEID_{50}$, CFU, fg) but are also given as absolute copy numbers obtained by in-house quantification of stock solutions using QX200™ Droplet Digital™ PCR, Bio-Rad.

Table 15. Limit of detection in consideration of the purification (NucliSENS® easyMAG®) of the RealAccurate® Quadruplex Respiratory PCR Kits using LightCycler® 480

PCR panel	Pathogen	Subtype/ Strain	Source	Mat#	LoD		
					titer/ml	co/ml	co/react.
Influenza	Influenza A	H3N2 Victoria	ATCC	VR-822	50 CEID50	7552	76
	Influenza A	H1N1 Virginia	ATCC	VR-1737	$5x10^2$ TCID50	8869	89
	Influenza B	Maryland	ATCC	VR-296	$1x10^3$ CEID50	10955	110
Corona	Coronavirus	229E	ATCC	VR-740	0.9 TCID50	1017520	10175
	Coronavirus	OC43	ATCC	VR-1558	$2.8x10^{-2}$ TCID50	1893	19
	Coronavirus	NL63	Zepto-Metrix	0810228CF	0.14 TCID50	2407	24
	Coronavirus	HKU1 (viral culture)	CHU Caen, France	n.a.	4 pg	384	4
Corona-*plus*	Coronavirus	SARS-CoV-2	ATCC	VR-1986D	n.a.	1050	5
	Coronavirus	MERS	QCMD	MERS19S-06	n.a.	316	1.6
SARS-CoV-2	Coronavirus	SARS-CoV-2	ATCC	VR-1986D	n.a.	N gene 1050	N gene 5
	Coronavirus	SARS-CoV-2	ATCC	VR-1986D	n.a.	RdRp gene 710	RdRp gene 3.5
Parainfluenza	Parainfluenza	1	ATCC	VR-94	0.1 TCID50	8890	89
	Parainfluenza	2	ATCC	VR-92	2.8 TCID50	10533	105
	Parainfluenza	3	ATCC	VR-93	89 TCID50	24964	250
	Parainfluenza	4a	ATCC	VR-1378	0.9 TCID50	693	7
RSV/hMPV	RSV-A	2	ATCC	VR-1540	2.8 TCID50	96	1
	RSV-B	18537	ATCC	VR-1580	0.9 TCID50	35	1
	hMPV	B3	Zepto-Metrix	0810156CF	4.2 TCID50	350	4
Adeno/Boca/ Rhino/Entero	Adenovirus	B3	ATCC	VR-3	158 TCID50	1700	17
	Bocavirus	n.a. (recombinant plasmid)	In-house	n.a.	100 fg	18300	183
	Coxsackievirus	A9	ATCC	VR-1311	1.6 TCID50	18400	184
	Rhinovirus	16	ATCC	VR-283	15.8 TCID50	181	2

Relatively equivalent LoD values were obtained using InviGenius® PLUS for nucleic acid extraction. Overall, InviGenius® PLUS shows a slightly lower nucleic acid extraction efficiency. For a few strains given in Table 15 sensitivity was 0.5-1 log lower with InviGenius® PLUS extraction compared with easyMAG® extraction.
Equivalent LoD values were obtained using Rotor-Gene® Q, CFX96™, Mic qPCR Cycler and QuantStudio™ 5 for the RealAccurate® Quadruplex PCR reactions.

An in-house validation study showed that the sensitivity of the heat-treatment protocol (only to be used with RealAccurate® SARS-CoV-2 PCR Kit) is 93% for UTM samples and near 100% for eSwab samples when compared with NucliSens® easyMAG® extraction. This is due to the slightly higher Ct/Cp values obtained with the heat-treatment protocol in comparison to NucliSens® easyMAG®. Figure 2 and 3 show this for eSwab and UTM samples, respectively.

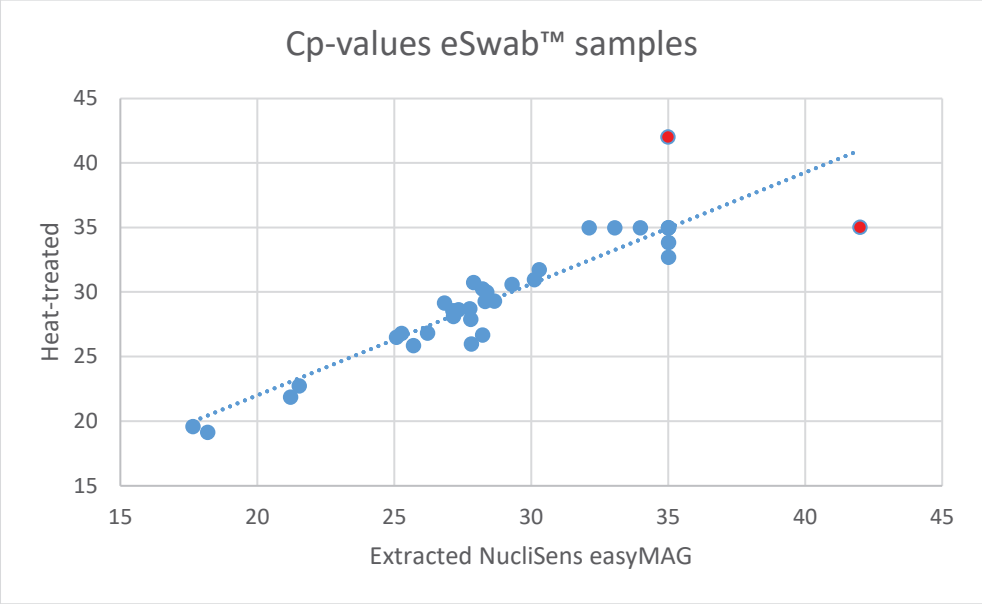

Figure 2. Relation between Cp-values for the N- and RdRp-target obtained with the RealAccurate® SARS-CoV-2 PCR Kit on eSwab samples (n=17) extracted with NucliSens® easyMAG® versus the heat-treatment protocol. The 2nd Derivative analysis of LightCycler® 480 was used, hence weak signals (Cp ≥ 35) are called with Cp 35. Note that in the higher Cp range, the variation becomes more pronounced. To allow inclusion in the graph, an arbitrary Cp-value of 42 was assigned to two negative results; these belong to the same sample (marked red) which was positive for the N- and negative for the RdRp-target with easyMAG® and vice versa for the heat-treatment protocol.

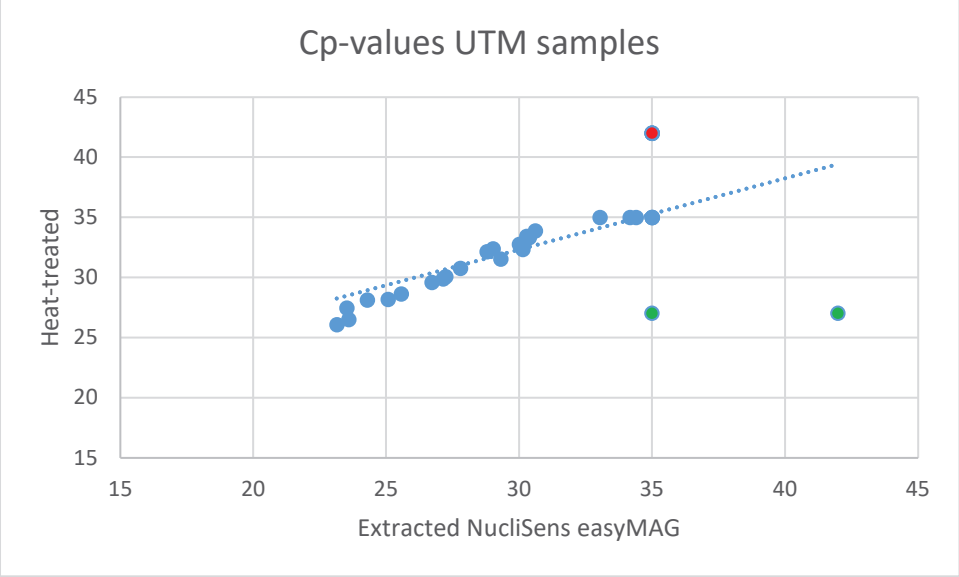

Figure 3. Relation between Cp-values for the N- and RdRp-target obtained with the RealAccurate® SARS-CoV-2 PCR Kit on UTM samples (n=15) extracted with NucliSens® easyMAG® versus the heat-treatment protocol. The 2nd Derivative analysis of LightCycler® 480 was used, hence weak signals (Cp ≥ 35) are called with Cp 35. To allow inclusion in the graph, an arbitrary Cp-value of 42 was assigned to negative results. The red dot represents 4 data points from 3 samples for which the NucliSens® easyMAG® resulted in Cp 35, yet the result was negative after the heat-treatment protocol. Two of these samples were positive for both targets with easyMAG® extraction and for one target with the heat-treatment, hence SARS-CoV-2 was still detected; the third sample was positive for both targets with easyMAG® extraction (Cp 35) and negative for both targets with the heat-treatment, hence false negative with the heat-treatment. The green dots represent the N-target (left) and RdRp-target (right) for a sample of which easyMAG® extraction resulted in inhibition due to clotting and heat-treatment gave positive results.

Inclusivity

The inclusivity (analytical reactivity) of the RealAccurate® Quadruplex Respiratory PCR Kits is first and foremost ensured by the selection of the primers, as well as the selection of stringent reaction conditions. The primers were checked for possible homologies to all sequences published in databases by sequence comparison analysis. The detectability of all relevant genotypes has further been confirmed by the RealAccurate® Quadruplex Respiratory PCR Kits runs on a LightCycler® 480 instrument using nucleic acid extracts from reference strains of the genotypes presented in Table 16. These strains were tested at a concentration of 10x LoD of the corresponding pathogen from the LoD study, as determined by Droplet Digital™ PCR.

Table 16. Testing of the inclusivity of relevant strains: different subtypes detected by the different RealAccurate® Quadruplex Respiratory PCR Kits

PCR panel	Pathogen	Subtype/Strain	Source	Mat#
Influenza	Influenza A	H1N1 New Jersey	ATCC	VR-897
	Influenza A	Netherlands/522/2014 H3 clade 3C.2a	RIVM	4311401094
	Influenza A	Netherlands/525/2014 H3 clade 3C.3	RIVM	4311401108
Parainfluenza	Parainfluenza	4b	ATCC	VR-1377
RSV/hMPV	RSV-A	RSVA long	ATCC	VR-26
	RSV-B	RSVB WV	ATCC	VR-1400
Adeno/Boca/ Rhino/Entero	Adenovirus	B7	ATCC	VR-7
	Adenovirus	B11	ATCC	VR-12
	Adenovirus	B14	ATCC	VR-15
	Adenovirus	E4	ATCC	VR-1572
	Adenovirus	C1	ATCC	VR-1
	Adenovirus	C5	ATCC	VR-5
	Adenovirus	C6	ATCC	VR-6
	Coxsackievirus	B1	ATCC	VR-28
	Coxsackievirus	B2	ATCC	VR-29
	Coxsackievirus	B3	ATCC	VR-30
	Coxsackievirus	B5	ATCC	VR-185
	Coxsackievirus	A10	ATCC	VR-168
	Coxsackievirus	A24	ATCC	VR-1662
	Echovirus	30	ATCC	VR-1660
	Echovirus	11	ATCC	VR-41
	Echovirus	4	ATCC	VR-1734
	Rhinovirus	1A	ATCC	VR-1559
	Rhinovirus	1B	ATCC	VR-1645
	Rhinovirus	2	ATCC	VR-482
	Rhinovirus	30	ATCC	VR-505
	Rhinovirus	39	ATCC	VR-340
	Rhinovirus	60	ATCC	VR-1473

Specificity

A potential cross-reactivity of the RealAccurate® Quadruplex Respiratory PCR Kits was tested using samples known to contain one of the species listed in Table 17. None of the tested pathogens was reactive with any of the kits.

Table 17. Testing the specificity of the kit with potentially cross-reactive pathogens

Pathogen	Pathogen
Haemophilus influenzae	Herpes Simplex Virus
Stenotrophomonas maltophilia	Epstein–Barr virus
Achromobacter xylosoxidans	Varicella zoster virus
Pseudomonas aeruginosa	*Aspergillus fumigatus*
Serratia marcescens	*Candida krusei*
Escherichia coli	*Candida albicans*
Klebsiella oxytoca	*Mycoplasma pneumoniae*
Staphylococcus aureus	*Chlamydia pneumoniae*
Klebsiella pneumoniae	*Legionella pneumophila*
Mycobacterium tuberculosis	*Bordetella pertussis*
Cytomegalovirus	

Additionally, all pathogen strains included in the LoD study with one of the RealAccurate® Quadruplex Respiratory PCR panels (as stated in Table 15) were tested with the other kits. None of the pathogen strains listed in Table 15 was reactive or showed a positive signal due to cross-reactivity with one of the pathogens of the other RealAccurate® Quadruplex Respiratory PCR panels.

Robustness

The verification of the robustness allows the determination of the total failure rate of the Internal Control of the RealAccurate® Quadruplex Respiratory PCR Kits. To verify the robustness, 30 negative nasopharyngeal swab samples from asymptomatic individuals were collected in Universal Transport Medium (UTM™) and tested with the RealAccurate® Quadruplex Respiratory PCR Kits. After extraction on the NucliSENS® easyMAG® or InviGenius® PLUS system, the samples were analyzed with the kits. The Internal Control was detected in all samples. Thus, the RealAccurate® Quadruplex Respiratory PCR Kits are considered to be robust.
Robustness of the RealAccurate® Quadruplex Respiratory PCR Kits was verified using LightCycler® 480, Rotor-Gene® Q, CFX96™, Mic qPCR Cycler and QuantStudio™ 5.

An additional Robustness study was done to verify the heat-treatment protocol for the RealAccurate® Quadruplex SARS-CoV-2 PCR Kit. For this purpose, negative nasopharyngeal swab samples from asymptomatic individuals were collected (n=30 in UTM™ and n=30 eSwab™). Subsequent to the heat-treatment protocol, the samples were analyzed with the RealAccurate® Quadruplex SARS-CoV-2 PCR Kit. The Internal Control was detected in all samples. Thus, the RealAccurate® Quadruplex SARS-CoV-2 PCR Kit is considered robust for heat-treated samples.

Interfering substances

The RealAccurate® Quadruplex Respiratory PCR Kits were tested for interference with human genomic DNA. It is very likely that sample material to be used for analysis with one of the RealAccurate® Quadruplex Respiratory PCR Kits obtained with a nasopharyngeal swab contains human genomic DNA from epithelial cells from the nasopharynx. Most pathogens listed in Table 15 were tested at a concentration of 0.5 log above the respective LoD in the presence of 1 µg human DNA per sample. No negative influence of additional human DNA was seen in any sample tested with the Influenza, Corona, Parainfluenza and RSV/hMPV assay showing no interference of human DNA with the performance of these RealAccurate® Quadruplex Respiratory PCR Kits. In the Adeno/Boca/Rhino/Entero assay, both Adeno and Bocavirus amplification reactions showed no interference of human DNA with the performance at the low test concentration of 0.5 log above the respective LoD. For Rhinovirus and Enterovirus, negative interference of human DNA was seen at a target concentration of 0.5 log above LoD, but testing at 1 log above LoD (which is still far below clinically relevant viral loads) showed no interference of human DNA. The Internal Control was detected in all reactions of all PCR panels where 1 µg human DNA was spiked to the samples, showing no interference of human DNA with the performance of the Internal Control.

Reproducibility

The reproducibility was tested on five targets of the RealAccurate® Quadruplex Respiratory PCR Kits. Target concentrations were selected to obtain C_p values of 28–30 on a LightCycler® 480 instrument. Five nucleic acid extractions of each of the five targets tested was used for duplicate amplification reactions on 2 different LightCycler® 480 and 2 different Rotor-Gene® Q instruments. Each instrument was used for 2 amplification runs. The total of 8 runs were executed on 3 different days by 3 different operators.

The average C_p/C_t values and the standard deviations were calculated for 10 replicates tested on each instrument and are given in Table 18.

Table 18: Assay reproducibility. Each instrument was tested 2x by 2 different operators. Average C_p/C_t values and standard deviations were calculated. The colors indicate the detection channels that were used per PCR panel

PCR panel	Pathogen	LC480 (1)		LC480 (2)		RGQ (1)		RGQ (2)	
		avg	stdev	avg	stdev	avg	stdev	avg	stdev
Influenza	Influenza A H3N2 Victoria	30.62	0.64	30.58	0.65	27.85	0.69	27.54	0.70
		30.81	0.56	30.75	0.62	28.22	0.69	27.66	0.70
Corona	OC43	28.56	0.87	28.47	0.87	25.88	1.11	26.78	1.10
		28.71	0.73	28.68	0.75	26.03	1.08	26.53	1.19
Parainfluenza	PIV 3	29.48	0.23	29.03	0.91	28.15	1.03	28.91	0.85
		29.72	0.85	29.81	0.87	29.13	0.95	29.05	1.04
RSV/hMPV	RSV A2	30.62	0.16	30.50	0.17	26.,75	0.17	26.76	0.18
		30.91	0.15	30.95	0.14	27.05	0.18	26.71	0.19
Adeno/Boca/ Rhino/Entero	Bocavirus	28.79	0.06	28.76	0.06	24.73	0.13	26.02	0.12
		28.15	0.04	28.19	0.10	25.12	0.07	25.12	0.07
Operator		3		1		3		3	
		2		2		1		1	

The automatic C_p calling, using the Abs Quant / 2nd Derivative Max option in the LightCycler® software for the LightCycler® 480 amplification runs, and the C_t calculation as obtained by the Rotor-Gene® Q software using a threshold of 0.05 for all channels for the

Rotor-Gene® Q runs, revealed overall higher C_p values than C_t values, but C_p values as well as C_t values are mutually comparable per target. Standard deviation varied between targets but overall allowed for conclusion that the RealAccurate® Quadruplex Respiratory PCR Kits generate reproducible results.

16. Troubleshooting

Problem	Possible cause	Recommendations
Controls remains negative (IC or Positive Control)	The sample is inhibited, one of the assay steps has failed or a manual error has occurred	Ensure that all components have been added
	The Internal Control or Positive Control was not stored properly	Store all components according to the manufacturer's instructions
	Wrong PCR profile	Check programming of real-time cycler
Negative Control gives a fluorescence signal	Carry over / contamination	Repeat the entire experiment with fresh reagents Handle samples, kit components and consumables as prescribed
Very weak fluorescence signals also for controls	Incorrect instrument settings	Check channel settings
	Incorrect real-time PCR mix	Check the preparation of the PCR mix(es)
Very weak fluorescence signal in a detection channel	Remaining cross-talk	Check results with and without cross-talk correction. When the signal significantly decreases with the use of cross-talk correction the weak signal is most probably caused by some remaining cross-talk. Check also signals in neighboring channels.

17. References

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18. Notice to the purchaser

RealAccurate® products are manufactured by PathoFinder BV in Maastricht, The Netherlands within quality systems accredited to ISO 13485:2016. The products are sold for use by the end-user only and may not be re-sold, distributed or re-packaged.
It is not recommended to combine RealAccurate® Quadruplex Respiratory PCR Kits reagents of different lots.
If a RealAccurate® Quadruplex Respiratory PCR Kit is received in a damaged packaging, please contact PathoFinder or your local PathoFinder distributor.

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19. Related products

- **RealAccurate® Quadruplex Color Compensation v2:** kit containing Color Compensation reagents for RealAccurate® Quadruplex assays for LightCycler® 480 instruments.

  Catalog number: PFCC-R

- **RespiFinder® 2SMART:** SmartFinder assay for the detection of 22 respiratory pathogens (16 RNA viruses, 2 DNA viruses and 4 bacteria)
  - Influenza A
  - Influenza B
  - Influenza A(H1N1)pdm09
  - Respiratory syncytial virus A
  - Respiratory syncytial virus B
  - Parainfluenza virus 1
  - Parainfluenza virus 2
  - Parainfluenza virus 3
  - Parainfluenza virus 4
  - Coronavirus OC43
  - Coronavirus 229E
  - Coronavirus NL63(no differentiation from HKU1)
  - Coronavirus HKU1(no differentiation from NL63)
  - Rhinovirus (no differentiation from Enterovirus)
  - Enterovirus (no differentiation from Rhinovirus)
  - Adenovirus
  - Human Metapneumovirus
  - Bocavirus
  - *Chlamydophila pneumoniae*
  - *Mycoplasma pneumoniae*
  - *Legionella pneumophila*
  - *Bordetella pertussis*

  Catalog number: PF2600-2S, 50 reactions

RealAccurate® Quadruplex Respiratory PCR Kits

Product no.: PF0970-R/0971-R/0971B-R/0971C-R/0972-R/0973-R/0974-R

50 reactions

Store at –30 °C to –15 °C

Keep away from sunlight

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