SARS-CoV-2 Real Time PCR LAB-KIT™

Technical instruction

Kit for the qualitative detection of SARS-CoV-2 coronavirus nucleic acid
using Reverse Transcriptase real-time PCR.
In vitro diagnostic medical device

Packaging:
8 - well strips - 96 tests – catalog number: PCR 5008
96-well plate – catalog number: PCR 5096

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CE
IVD

1. Intended use

SARS-CoV-2 Real Time PCR LAB-KIT™ is designed for the specific identification and differentiation of 2019 Novel Coronavirus (SARS-CoV-2) in respiratory samples from patients with signs and symptoms of COVID-19 infection. This test is intended for use as an aid in the diagnosis of SARS-CoV-2 in combination with clinical and epidemiological risk factors. RNA is extracted from respiratory specimens, amplified using RT-PCR and detected using fluorescent reporter dye probes specific for SARS-CoV-2.

For in vitro diagnostic medical device

Keywords: COVID-19, SARS-CoV-2, marker genes *Orf1ab* and *N*, RT - PCR, sputum, tracheal aspirate or bronchoalveolar lavage, nasopharyngeal aspirate, nasopharyngeal and pharyngeal swabs, blood urine and stool

2. Summary and Explanation

Coronavirus are enveloped non-segmented positive-sense RNA viruses and belong to *Coronaviridae* family. There are six coronavirus species known to cause human diseases. Four viruses (229E, OC43, NL63 and HKU1) cause common cold symptoms and the other two (severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV)) are zoonotic and producing more severe complications. SARS-CoV and MERS-CoV have caused more than 10,000 cumulative cases in the past two decades, with mortality rates of 34% MERS-CoV and 10% SARS-CoV.

In December 2019, some people that worked at or lived around the Huanan seafood market in Wuhan, Hubei Province, China, have presented pneumonia of unknown cause. Deep sequencing analysis of the respiratory samples indicated a novel coronavirus, which was named firstly 2019 novel coronavirus (2019-nCoV) and lately SARS-CoV-2.

Human-to-human transmission of the SARS-CoV-2 has been confirmed, even in the incubation period without symptoms, and the virus causes severe respiratory illness like those SARS-CoV produced. Although the pneumonia is the principal illness associated, a few patients have developed severe pneumonia, pulmonary edema, acute respiratory distress syndrome, or multiple organ failure and death. Centers of Disease Control and Prevention (CDC) believes that symptoms of SARS-CoV-2 may appear in as few as 2 days or as long as 14 days after exposure, being the most common fever, cough, myalgia and dyspnea. Less common symptoms are sore throat, headache, diarrhea and vomiting. It seems that older males with comorbidities have been more affected.

Diagnosis of SARS-CoV-2 is performed detecting conventional causes of pneumonia early and detected by next-generation sequencing or real-time RT-PCR methods. Several assays that detect the SARS-CoV-2 have been are currently available, such as China CDC (gene targets, ORF1ab and N), Charité – Germany (gene targets, RdRP, E, N) or US CDC (gene targets, three N primers, RdRP).

WHO recommends lower respiratory specimens (sputum, endotracheal aspirate, or bronchoalveolar lavage) for the identification of SARS-CoV-2. However, if the collection is not possible, upper respiratory tract specimens such as a nasopharyngeal aspirate or combined nasopharyngeal and oropharyngeal swabs should be collected. In addition, other clinical specimens as blood, urine and stool may be collected to monitor the presence of the virus.

3. Principle of the procedure

SARS-CoV-2 Real Time PCR LAB-KIT™ is designed for the diagnosis of SARS-CoV-2 in respiratory samples. The detection is done in one step real time RT - PCR format where the reverse transcription and the subsequent amplification of specific target sequence occur in the same reaction well. The isolated RNA target is transcribed generating complementary DNA by reverse transcriptase which is followed by the amplification of a conserved region of *ORF1ab and N genes* for SARS-CoV-2 using specific primers and a fluorescent-labeled probe.

SARS-CoV-2 Real Time PCR LAB-KIT™ is based on the 5´ exonuclease activity of DNA polymerase. During DNA amplification, this enzyme cleaves the probe bounded to the complementary DNA sequence, separating the quencher dye from the reporter. This reaction generates an increase in the fluorescent signal which is proportional to the quantity of target template. This fluorescence can be measured on Real Time PCR platforms.

SARS-CoV-2 Real Time PCR LAB-KIT™ contains in each well all the components necessary for real time PCR assay (specific primers/probes, dNTPS, buffer, polymerase and retrotranscriptase) in an stabilized format, as well as an internal control to monitor PCR inhibition. *ORF1ab* gene is amplified and detected in FAM channel, *N* gene is amplified and detected in ROX channel and the internal control (IC) in HEX channel, VIC or JOE channel (depending on the equipment used select the proper detection channel, see Annex 2).

4. Reagents provided

SARS-CoV-2 Real Time PCR LAB-KIT™ includes the following materials and reagents detailed in Tables 1 and 2. Based on the commercial presentation and the Real Time PCR platform used, the stabilized PCR reaction mix could be placed inside different wells and could be marketed on multiple formats. Table 1 includes materials and reagents to be used with 8-well strips compatible devices (See Annex 1). Table 2 includes materials and reagents to be used with 96-well plate compatible devices (See Annex 1).

Table 1.- Catalog number: PCR 5008

Reagent/Material	**Description**	**Colour**	**Amount**
SARS-CoV-2 8-well strips	A mix of enzymes, primers probes, buffer, dNTPs, stabilizers and Internal control in stabilized format	White	12 x 8-well strip
Rehydration Buffer	Solution to reconstitute the stabilized product	Blue	1 vial x 1.8 mL
SARS-CoV-2 Positive Control	Non-infectious synthetic lyophilized cDNA	Red	1 vial
Negative control	Non template control	Amber	1 vial x 1 mL
Water RNAse/DNAse free	RNAse/DNAse free water	White	1 vial x 1 mL
Tear-off 8-cap strips	Optical caps for sealing wells during thermal cycling	Transparent	12 x 8-cap strip

Table 2.- Catalog number: PCR 5096

Reagent/Material	**Description**	**Color**	**Amount**
SARS-CoV-2 96-well plate	A mix of enzymes, primers probes, buffer, dNTPs, stabilizers and Internal control in stabilized format	White	1 plate
Rehydration Buffer	Solution to reconstitute the stabilized product	Blue	1 vial x 1.8 mL
SARS-CoV-2 Positive Control	Non-infectious synthetic lyophilized cDNA	Red	1 vial
Negative control	Non template control	Amber	1 vial x 1 mL
Water RNAse/DNAse free	RNAse/DNAse free water	White	1 vial x 1 mL
Tear-off 8-cap strips strip	Optical caps for sealing plate during thermal cycling	Transparent	12 x 8-cap

5. Reagents and equipment to be supplied by the user

The following list includes the materials that are required for use but not included in the SARS-CoV-2 Real Time PCR LAB-KIT™.

- Real Time PCR instrument (thermocycler).
- RNA extraction kit.
- Centrifuge for 1.5 mL tubes and PCR-well strips or 96-well plate (if available).
- Vortex.
- Micropipettes (0.5-20 µL, 20-200 µL).
- Filter tips.
- Powder-free disposable gloves.

SARS-CoV-2 Real Time PCR LAB-KIT™ has been validated on the following equipments: Applied Biosystems 7500 Fast Real-Time PCR System, Applied Biosystems StepOne™ Real-Time PCR System, Bio-Rad CFX96™ Real-Time PCR Detection System, Agilent Technologies AriaMx Real-Time PCR System, DNA-Technology DTprime Real-time Detection Thermal Cycler, DNA-Technology DTlite Real-Time PCR System, Rotor-Gene® Q (Qiagen), SmartCycler® (Cepheid), Roche Molecular Diagnostics Cobas z480 Analyzer, VIASURE 48 Real Time PCR System and VIASURE 96 Real Time PCR System. When using the Applied Biosystems 7500 Fast with strips it is recommend to place a plate holder to reduce the risk of crushed tube (Ref. PN 4388506).
To check thermocycler compatibility, see Annex 1

6. Transport and storage conditions

- The kits can be shipped and stored at (2-40ºC) until the expiration date which is stated on the label.
- Once the positive control has been re-suspended, store it at (-20ºC). We recommend to separate it in aliquots to minimize freeze and thaw cycles. Positive control has been validated as still being stable after 6 freeze-thaw cycles.
- Keep components away from sunlight.

7. Precautions for users

- The product is indented for use by professional users only, such as laboratory or health professionals and technicians, trained in molecular biological techniques.
- The test meets the requirements of the WHO Recommendation "Laboratory testing for coronavirus disease 2019 (COVID-19) in suspected human cases. Interim guidance. March 2, 2020.
- Do not use past expiration date.
- Do not use reagents if the protective pouches are open or broken upon arrival.
- Do not use reagents if desiccant is not present or broken inside reagent pouches.
- Do not remove desiccant from reagent pouches once is open.
- Close protective pouches of reagents promptly with the zip seal after each use. Remove any excess air in the pouches prior to sealing.
- Do not use reagents if the foil has been broken or damaged.
- Do not mix reagents from different envelopes and / or kits and / or lots and / or another supplier.
- Protect reagents against from humidity. Prolonged exposure to humidity may affect product performance.
- Design a unidirectional workflow. It should begin in the Extraction Area and then move to the Amplification and Detection Area. Do not return samples, equipment and reagents to the area in which the previous step was performed.

- Follow Good Laboratory Practices. Wear protective clothing, use disposable gloves, goggles and mask. Do not eat, drink or smoke in the working area. Once you finish the test wash your hands.
- Specimens must be treated as potentially infectious, as well as all the reagents and materials that have been exposed to the samples and they must be handled according to the national safety regulations. Take necessary precautions during the collection, storage, treatment and disposal of samples.
- Regular decontamination of commonly used equipment is recommended, especially micropipettes and work surfaces.
- Consult safety data sheets, upon request.
- Consult each Real Time PCR instrument's reference manual for additional warnings, precautions and procedures.

8. Test procedure

8.1.RNA extraction

Perform the sample preparation according to the recommendations appearing in the instructions for use of the extraction kit used.

For RNA extraction from respiratory samples you can use your manual or automatic routine optimized system. Also, you can use any commercially available RNA extraction kit and follow the manufacturer´s instructions. We have validated the following extraction kits:

- Maxwell® 16 Viral Total Nucleic Acid Purification Kit, using the Maxwell® 16 instrument (Promega).
- Total Nucleic Acid Isolation (TNAI) Kit, using COBAS® AmpliPrep (ROCHE).
- MagDEA Dx SV kit, using the magLEAD® 12gC instrument (Precision System Science Co.).
- MagCore® Viral Nucleic Acid Extraction kit, using the MagCore® HF16 automated Nucleic Acid Extractor System.

8.2 Lyophilized positive control

SARS-CoV-2 Positive Control contains high copies of the template, the recommendation is to open and manipulate it in a separate laboratory area away from the other components. Reconstitute the lyophilized *SARS-CoV-2* Positive Control (red vial) by adding 100 µL of the supplied Water RNAse/DNAse free (white vial) and vortex thoroughly.

Once the positive control has been re-suspended, store it at (-20ºC). We recommend to separate it in aliquots to minimize freeze and thaw cycles.

8.3. PCR protocol

Determine and separate the number of required reactions including samples and controls. One positive and negative control must be included in each run for each assay. Peel off protective aluminium seal from plates or strips.

1) Reconstitute the number of wells you need.

Add 15 µL of Rehydration Buffer (blue vial) into each well.

2) Adding samples and controls.

Add 5 µL of RNA sample, reconstituted *SARS-CoV-2* Positive Control (red vial) or Negative Control (amber vial) in different wells and close them with the provided caps. It is recommended to briefly centrifuge the 8-well strips or 96-well plate. Load the plate or the strips in the thermocycler.

3) Set up the thermocycler (to check compatibility see Annex 1).

4) Program the thermocycler following the conditions listed below and start the run:

Cycles	Step	Time	Temperature
1	Reverse transcription	15 min	45ºC
1	Initial denaturation	2 min	95ºC
45	Denaturation	10 sec	95ºC
	Annealing/Extension (Data collection*)	50 sec	60ºC

Fluorogenic data should be collected during the extension step (*) through the **FAM (*ORF1ab* gene)**, **ROX (*N* gene)** and HEX, JOE or VIC (Internal Control (IC)). Depending on the equipment used select the proper detection channel. In Applied Biosystems 7500 Fast Real-Time PCR System, Applied Biosystems StepOne™ Real-Time PCR System and Stratagene Mx3005P™ Real Time PCR System check that passive reference option ROX is none. In the Applied Biosystems 7500 Fast Real-Time PCR System select Ramp Speed Standard in Select New Experiment/Advanced Setup/Experiment Properties.

PERFORMANCE DIAGRAM

Sample:
sputum, endotracheal aspirate, bronchoalveolar lavage, a nasopharyngeal aspirate, nasopharyngeal and oropharyngeal swabs, blood, urine and stool

Isolation of virus genetic material

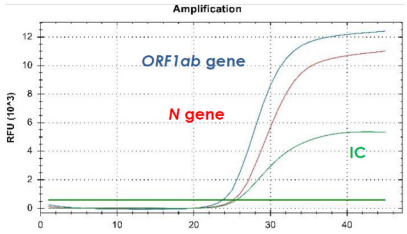
RNA SARS-CoV-2

15 ∝L Rehydration Buffer + **5∝L Negative control**	15 ∝L Rehydration Buffer + **5∝L RNA SARS-CoV-2**	15 ∝L Rehydration Buffer + **5 ∝L Positive control**

Each PCR microtube contains a lyophilized reaction mixture

Negative control	**Examinated sample**	**Positive control**

Close all PCR microtubes tightly and place in thermocycler



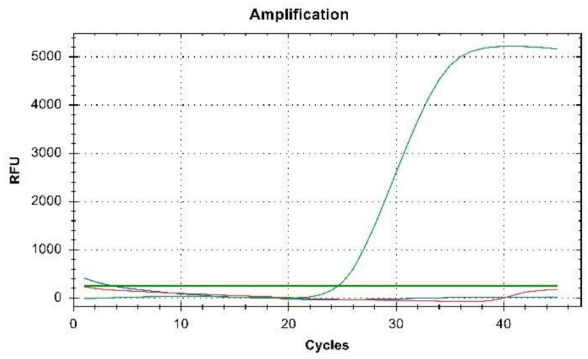
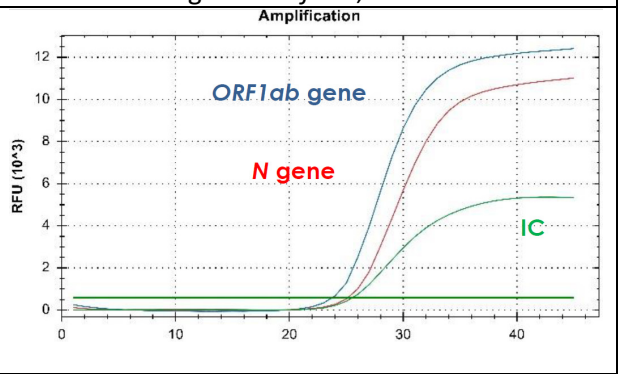
9. Result interpretation

The use of positive and negative controls in each run, validate the reaction by checking the absence of signal in the negative control well and the presence of signal for SARS-CoV-2 in the positive control well. Check Internal Control signal to verify the correct functioning of the amplification mix. The analysis of the samples is done by the software of the used real time PCR equipment itself according to manufacturer´s instructions. Using the following table read and analyze the results:

***ORF1ab* gene (FAM)**	***N* gene (ROX)**	**Internal control (HEX)**	**Negative control**	**Positive control**	**Interpretation**
+	+	+/-	-	+	**SARS-CoV-2 Positive**
-	-	+	-	+	**SARS-CoV-2 Negative**
+	-*	+/-	-	+	**SARS-CoV-2 Positive***
-**	+	+/-	-	+	**SARS-CoV-2 Negative****
+	+	+	+	+	**Experiment fail**
-	-	-	-	-	**Experiment fail**

(+): Amplification curve present, "S" shaped,
(-): No amplification curve
* Repeat the extraction and test again, in case of *N* gene is still negative the interpretation is SARS-CoV-2 Positive.
** Repeat the extraction and test again, if *ORF1ab* gene is still negative the interpretation is SARS-CoV-2 Negative (Possible infections of other coronavirus).

A sample is considered positive if the Ct value obtained is less than 38 and the internal control shows or not an amplification signal. Sometimes, the detection of internal control is not necessary because a high copy number of target can cause preferential amplification of target-specific nucleic acids.
A sample is considered negative, if the sample shows no amplification signal in the detection system but the internal control is positive. An inhibition of the PCR reaction can be excluded by the amplification of internal control.

Standard "S" curve for internal control (IC)	Standard "S" curves for: the genes: *Orf1ab*, *N* and IC
	

The result is considered invalid if there is signal of amplification in negative control or absence of signal in the positive well. We recommend to repeat the assay again.

In case of absence of internal control signal in sample wells we recommend to repeat the assay diluting the sample 1:10 or to repeat the extraction to check for possible problems of inhibition.
In case of a doubtful interpretation result, it is recommended to verify the correct performance of each of the steps and review the parameters and the sigmoid shape of the curve. If the situation is not solved, it is recommended to repeat the assay, preferably in duplicate. The results of the test should be evaluated by a health care professional in the context of medical history, clinical symptoms and other diagnostic tests.

10. Limitations of the test

- The results of the test should be evaluated by a health care professional in the context of medical history, clinical symptoms and other diagnostic tests.
- Although this assay can be used with other types of samples it has been validated only with RNA extracted from respiratory samples (nasopharyngeal swab and oropharyngeal swab).
- The quality of the test depends on the quality of the sample; proper extracted nucleic acid from clinical samples must be extracted. Unsuitable collection, storage and/or transport of specimens may give false negative results.
- Extremely low levels of target below the limit of detection might be detected, but results may not be reproducible.
- There is a possibility of false positive results due to cross-contamination by SARS-CoV-2, either samples containing high concentrations of target RNA or contamination due to PCR products from previous reactions.

11. Quality control

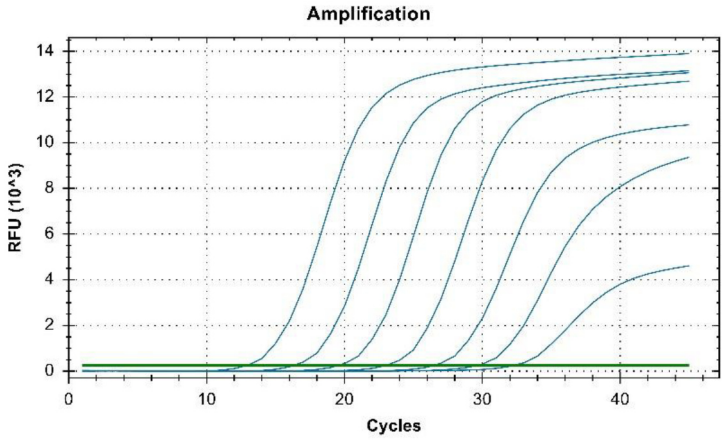
SARS-CoV-2 Real Time PCR LAB-KIT™ contains a positive and a negative control that must be included in each run to correctly interpret the results. Also, the internal control (IC) in each well confirms the correct performance of the technique. technique.

12. Performance characteristics

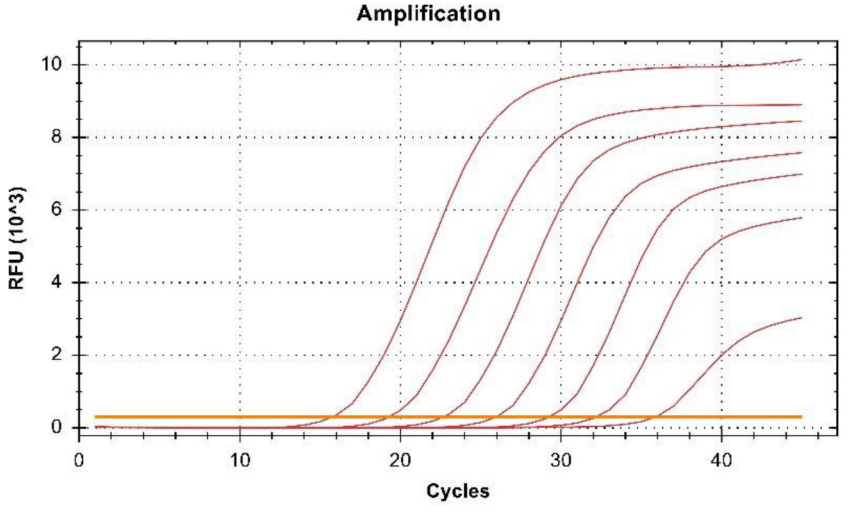
Sensitivity: > 99%
Specificity: > 99%

12.1 Test sensitivity

SARS-CoV-2 Real Time PCR LAB-KIT™ has a detection limit of ≥10 RNA copies per reaction for *ORF1ab* and *N* genes
Dilution series of *ORF1ab* gene (10^{7}-10^{1} copies/rxn) template run on the Bio-Rad CFX96™ Real-Time PCR Detection System (FAM channel).



Dilution series of *N* gene (10^{7}-10^{0} copies/rxn) template run on the Bio-Rad CFX96™ Real-Time PCR Detection System (ROX channel).



12.2 Analytical specificity

The specificity of the SARS-CoV-2 assay was confirmed by testing a panel consisting of different microorganisms representing the most common respiratory pathogens. No cross-reactivity was detected between any of the following microorganisms tested.

Cross-reactivity testing:

Bordetella pertussis	*Streptococcus pneumoniae* Z022	Influenza A/Anhui/1/2013 (H7N9) virus
Bordetella parapertussis	*Staphylococcus aureus subsp. aureus*	Influenza B/Brisbane/60/2008 virus
Bordetella holmesii	*Moraxella catarrhalis*	Influenza B/Florida/04/06 virus
Bordetella bronchiseptica	*Mycobacterium tuberculosis* not rifampin resistant	Influenza B/Phuket/3073/2013 virus
Haemophilus influenzae MinnA	*Pneumocytis jirovecii*	Human parainfluenza 1, 2, 3 and 4 viruses
Chlamydia caviae	Influenza A/New Caledonia/20/99(H1N1) virus	Human metapneumovirus A and B
Chlamydia psittaci genotypes A and C	Influenza A/California/7/2009(H1N1) virus	Human rhinovirus type C
Chlamydophila pneumoniae CM-1	Influenza A/Michigian/45/2015 (H1N1)pdm09 virus	Human Adenovirus Types 1-5, 8, 15, 31, 40 and 41
Legionella bozemanii	Influenza A/Singapore/GP1908/2015 (H1N1)pdm09 virus	Human Bocavirus
Legionella micdadei	Influenza A/Perth/16/2009(H3N2) virus	Respiratory Syncytial virus (RSV) A and B
Legionella dumoffii	Influenza A/Thüringen/5/2017 (H3N2) virus	Human coronavirus 229E, OC43, NL63 and HKU1
Legionella pneumophila	Influenza A/Switzerland/9715293/2013 (H3N2) virus	MERS Coronavirus

Legionella longbeache	Influenza A/Hong Kong/4801/2014(H3N2) virus	SARS Coronavirus Strain Frankfurt 1
Mycoplasma pneumoniae	Influenza A/DE-SH/Reiherente/AR8444/ 2016 (H5N8) virus	

12.3 Analytical reactivity

The reactivity of SARS-CoV-2 Real Time PCR LAB-KIT™ was evaluated against RNA from Human 2019-nCoV strain BetaCoV/Germany/BavPat1/2020 p.1 showing positive results.

Literature:

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Aneks 1.

COMPATIBILITY WITH THE MOST COMMON REAL TIME PCR EQUIPMENT

Low profile strips can be used in all PCR thermocyclers equipped with a low profile block, like the systems listed in table A.1. High profile strips can be used in all PCR thermocyclers equipped with a high or regular profile block, like the systems listed in table A.2. If you do not find your thermocycler in the list below, please contact with your supplier.

Table A.1 LOW PROFILE BLOCK THERMOCYCLERS		**Table A.2 HIGH PROFILE BLOCK THERMOCYCLERS**	
Agilent Technologies	AriaMx/AriaDx Real-Time PCR System	Abbott	Abbott m2000 RealTime System
Applied Biosystems	7500 Fast Real-Time PCR System (1)	Applied Biosystems	7300 Real-Time PCR System (5)
Applied Biosystems	7500 Fast Dx Real-Time PCR System (1)	Applied Biosystems	7500 Real-Time PCR System
Applied Biosystems	QuantStudio™ 12K Flex 96-well Fast	Applied Biosystems	7900 HT Real-Time PCR System (5)
Applied Biosystems	QuantStudio™ 6 Flex 96-well Fast	Applied Biosystems	ABI PRISM 7000 (6)
Applied Biosystems	QuantStudio™ 7 Flex 96-well Fast	Applied Biosystems	ABI PRISM 7700 (5)
Applied Biosystems	QuantStudio™ 3 Fast Real-Time PCR System (5)	Applied Biosystems	QuantStudio™ 12K Flex 96-well
Applied Biosystems	QuantStudio™ 5 Fast Real-Time PCR System	Applied Biosystems	QuantStudio™ 6 Flex 96-well
Applied Biosystems	StepOne Plus™ Real-Time PCR System (5)	Applied Biosystems	QuantStudio™ 7 Flex 96-well
Applied Biosystems	StepOne™ Real-Time PCR System (5)	Applied Biosystems	QuantStudio™ 3 Real-Time PCR System (5)
Applied Biosystems	ViiA™ 7 Fast Real-Time PCR System	Applied Biosystems	QuantStudio™ 5 Real-Time PCR System
BIONEER	Exicycler™ 96	Applied Biosystems	ViiA™ 7 Real-Time PCR System
Bio-Rad	CFX96TM / CFX96TM IVD Real-Time PCR Detection System	Analytik Jena Biometra	TOptical
Bio-Rad	Mini OpticonTM Real-Time PCR Detection System (6)	Analytik Jena Biometra	qTOWER 2.0
Cepheid	SmartCycler® (3)	BIONEER	Exicycler™ 96
Qiagen	Rotor-Gene® Q(3)	Bio-Rad	CFX96TM Deep Well / CFX96TM Deep Well IVD Real-Time PCR Detection System
Roche	LightCycler ®480 Real-Time PCR System (4)	Bio-Rad	iCycler iQTM Real-Time PCR Detection System

Roche	LightCycler ®96 Real-Time PCR System (4)	Bio-Rad	iCycler iQTM5 Real-Time PCR Detection System
Roche	Cobas z480 Analyzer (4)	Bio-Rad	MyiQTM Real-Time PCR Detection System (6)
Bio-Rad		MyiQTM2 Real-Time PCR Detection System (6)	
Cepheid		SmartCycler®(3)	
DNA-Technology		DTprime Real-time Detection Thermal Cycler (2)	
DNA-Technology		DTlite Real-Time PCR System (2)	
Eppendorf		MastercyclerTMep *realplex*	
Qiagen		Rotor-Gene® Q(3)	
Stratagene / Agilent Technologies		Mx3000P™ Real Time PCR System	
Stratagene / Agilent Technologies		Mx3005P™ Real Time PCR System	