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Easy® SARS-CoV-2

User manual – version 2020/05

The "**Easy® SARS-CoV-2**" kit allows the qualitative detection of **SARS-CoV-2** virus by One Step Real-Time RT-PCR.

For *in vitro* diagnostic use

REF	RT020
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INTENDED USE

The *in vitro* diagnostic "**Easy® SARS-CoV-2**" kit is intended for the qualitative detection of *Severe Acute Respiratory Syndrome Coronavirus 2* (SARS-CoV-2) RNA by One Step Real-Time RT-PCR in nasopharyngeal swabs, oropharyngeal swabs, sputum, bronchoalveolar lavage (BAL).

The "**Easy® SARS-CoV-2**" kit is validated for the use in combination with the following instruments:

- **EasyPGX® qPCR instrument 96** - Diatech Pharmacogenetics (96 positions)
- **CFX96** - Bio-Rad (software v.3.1)
- **ABI 7500, 7500 Fast** - Applied Biosystems (software v. 2.0.5)
- **RotorGene Q** – Qiagen (software v. 2.3.5)

PRINCIPLE OF THE ASSAY

The "**Easy® SARS-CoV-2**" kit amplifies two specific SARS-CoV-2 genes, N and RdRp, and a human endogenous control gene by One-step Real-Time RT-PCR.

The detection is achieved using fluorescent probes labelled with FAM and ROX for viral targets and with HEX for endogenous control gene.
The amplification of the endogenous control gene enables to verify the amplification procedure, the amount of input RNA and the possible presence of inhibitors, which may cause false negative results.

The "**Easy® SARS-CoV-2**" kit is composed of one multiplex assay for the detection of both viral targets and human endogenous control gene.

Distribution of target detection per single channel:

FAM	ROX	HEX
N	RdRp	IC

CLINICAL RELEVANCE

Coronaviridae is a family of RNA virus that causes different diseases from common cold to more serious diseases like *Middle East respiratory syndrome* (MERS) and *Severe Acute Respiratory Syndrome* (SARS) responsible for the outbreak of 2003.

In December 2019 in Wuhan, China, a new *Coronavirus,* named SARS-CoV-2 (genus *Betacoronavirus*, sub-genus *Sarbecovirus*), responsible for an outbreak of *Severe Acute Respiratory Syndrome* (COVID-19) has been isolated. This virus has spead efficiently between humans globally and, in March 2020, the World health Organization classified COVID-19 as pandemic.

SARS-CoV-2 RNA detection is essential both for COVID-19 diagnosis and the containment of pandemic outbreak.

References:
- C.-C. Lai, T.-P. Shih and W.-C. Ko et al., Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): The epidemic and the challenges, International Journal of Antimicrobial Agents, 2020.
- Corman Victor M, et al.. Detection of 2019 novel coronavirus (2019-nCoV) by real-timeRT-PCR. Euro Surveill. 2020;25(3)

KIT CONTENTS

The kit contains sufficient reagents to carry out 192 tests in maximum 4 runs (46 samples and two reaction controls each).

Destination **EXTRACTION AREA**
Storage temperature **+2/+8°C**

Easy® RNA Extraction reagents

COMP	QUAN	
Lysis Buffer (1)	1 x 10 ml	Lysis buffer for viral RNA extraction
RNA purification beads	1 x 5 ml	Magnetic beads for RNA purification
Elution Buffer	1 x 8 ml	Buffer for eluiton of RNA from beads
SQ reagent tray	2 x 5 units	Reagent reservoir for multichannel pipette transfer of solutions into strips.
Sample preparation foil	1 x 4 units	Adhesive foil for 96-well plate sealing
Adhesive pre-cutted foil	1 x 2 units	Pre-cutted adhesive foil for 96-well plate sealing
RNA Storage Plate	2 x 12 strip	8-well strips **WHITE** for RNA extraction

Destination **AMPLIFICATION AREA**
Storage temperature **-35/-20°C**

Easy® Amplification reagents

COMP		QUAN	Cap color	
SARS-CoV-2 mix		2 x 200 µl	BROWN	Primers and probes mix specific for viral targets N, RdRp and for internal control
Enzyme 1 (2)		2 x 1000 µl	BLUE	Solution for amplification reaction
Enzyme 2 (3)		2 x 20 µl	GREEN	Solution for retrotranscription reaction
Enzyme 3		2 x 40 µl	ORANGE	Solution for retrotranscription reaction
EasyPGX SARS-CoV-2 pos ctrl	CONTROL +	2 vials	PURPLE	Positive control in a dry format containing a mixture of synthetic RNA sequences that corresponds to viral targets and total human RNA. Every aliquot must be resuspended with 50 µl of **WATER** before the use.
WATER	CONTROL -	2 x 1.5 ml		DNase-, RNase-free water, to be used exclusively to resuspend the dry positive controls, and as negative control in the PCR reaction.

(1) **Lysis Buffer**:

(Warning – GHS07) / H302; H315; H319; P280; P301+P312; P302+P352; P305+P351+P338

(2) **Enzyme 1**:

(Warning – GHS07, GHS09) / H302; H410; P301+P312; P330

(3) **Enzyme 2:**

(Warning - GHS09) / H410

Description:
H302 - Harmful if swallowed.
H315 - Causes skin irritation.
H319 - Causes serious eye irritation.
H410 - Very toxic to aquatic life with long-lasting effects

P280 - Wear protective gloves/protective clothing/eye protection/face protection.
P301+P312 - IF SWALLOWED: Call a POISON CENTER/doctor if you feel unwell.
P302+P352 - IF ON SKIN: Wash with plenty of water
P305+P351+P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing.
P330 - Rinse mouth.

DOCUMENTS AVAILABLE ON-LINE

The following documents are available at www.diatechpharmacogenetics.com/area-riservata:

- "**Easy® SARS-CoV-2**" - User Manual
- Template for amplification (xxxx it refers to current version):
  - **EasyPGX® qPCR instrument 96**: "RT800-96_RT020_template_xxxx.adxt"
  - **ABI7500/7500fast**: "ABI7500_RT020_template_xxxx.edt"
  - **CFX96**: "CFX96_RT020.prcl"
  - **Rotor-Gene**: "RG_RT020_template_xxxx.ret"
- Safety Data Sheets (SDSs)

ⓘ For further details please contact the Diatech Pharmacogenetics technical support:
email: support@diatechpharmacogenetics.com, tel. +39 0731 213243

MATERIALS REQUIRED BUT NOT PROVIDED

General materials	▪ 1.5 ml polypropylene twist-lock tubes (DNase-, RNase-, DNA-, PCR inhibitor-free) ▪ Micropipettes (volumes from 1 to 1.000 µl) ▪ Sterile filter tips DNase-, RNase-free (volumes from 1 to 1.000 µl) ▪ Powder-free disposable gloves ▪ Vortex ▪ Centrifuge/spinner ▪ Decontamination solution for nucleic acids, example: "**DNA Cleaner**" cod. DC001, Diatech Pharmacogenetics.
RNA extraction	ⓘ The "**Easy® SARS-CoV-2**" kit contains reagents for RNA extraction from nasopharyngeal swabs or oropharyngeal swabs. Instruments and materials required for RNA extraction with **EasyPGX® RNA extraction reagents**: ▪ **EasyPGX® dry block 96-well plate** (cod. RT804, Diatech Pharmacogenetics) or equivalent thermoblock compatible with 96-well plate low profile – Temperature 98°C. ▪ Centrifuge_Mini Plate Spinner and Adapter PCR strips_C1000 (cod. C1000-230V e C1000-ADAPT, Labnet International, Inc.) or equivalent vertical centrifuge compatible with 96-well plate low profile. ▪ DynaMag-96 Side Magnet (cod. 12331D, Thermo Fisher), or equivalent magnet compatible with 96-well plate low profile. ▪ 8-channel pipette (volume 5 – 50 µl) ▪ 8-channel pipette (volume 20 – 200 µl) ▪ Ultrapure water molecular biology grade, example: "**WATER**" cod. FR0001, Diatech Pharmacogenetics. ▪ Ethanol (analytical grade). Other recommended option for RNA extraction and purification: ▪ "MagCore® Viral Nucleic Acid Extraction Kit (Low PCR Inhibition)" (cod. MVN400-04, RBC); to be used in combination with MagCore Automated Nucleic Acid Extractor (RBC Bioscience). ⓘ RNA extraction and purification must be done following the related user manual indications and prescriptions. ⓘ In case you employ kits which are different from those recommended, it is the user's responsability to use standardized samples (e.g: VEQ – EQAS quality schemes) to verify that this does not imply a reduction of the performance of the system under analysis.
Amplification (RT-PCR)	*Real-Time PCR instruments (options)*: ▪ **EasyPGX® qPCR instrument 96** cod. RT800-96, Diatech Pharmacogenetics (Agilent Aria Sofware v1.4) ▪ **CFX96 - Bio-Rad** (software v.3.1) ▪ **ABI 7500, 7500 Fast - Applied Biosystems** (software v. 2.0.5) ▪ **Rotor-Gene Q** – Qiagen (software v. 2.3.5) Detection channels for FAM, ROX and HEX fluorescence. Range of environmental temperature: 15-30°C ▪ AriaDx ROX Optical Cartridge (cod. G8830-67002, Agilent), only for **EasyPGX® qPCR instrument 96**. ▪ Centrifuge_Mini Plate Spinner and Adapter PCR strips_C1000 (cod. C1000-230V e C1000-ADAPT, Labnet International, Inc.) or vertical centrifuge compatible with 96-well plate low profile. ▪ 8-channel pipette (volume 5 – 50 µl). ▪ 96 well plates and foil or caps compatibles with the thermal-cycler used (check the compatibility in the user manual of the instrument) for instance: -Bio-Rad CFX96: Hard Shell PCR plates 96-well WHT/CLR cod. HSP 9601; MICROSEAL B SEALS cod. MSB 1001 -ABI 7300/ABI 7500: MICROAMP OPTICAL 96 WELL RNX PLATE cod. N8010560; OPTICAL

	ADHESIVE COVERS cod.4360954 -ABI 7500 Fast: MICROAMP FAST OPTICAL 96 WELL RNX PLATE cod. 4346907; OPTICAL ADHESIVE COVERS cod. 4360954 -EasyPGX® qPCR instrument 96: "96-well plate, non-skirted, low profile" cod. 401494 and "Optical Strip Caps", 60 strip cod. 401427, Agilent or "FrameStar® Break-A-Way PCR Plate, Low Profile" cod. 4ti-1200 and "Strip of 8 flat optical caps" cod. 4ti-0751, Brooks life science. ▪ DNase-and RNase-free, thin-wall, PCR tubes with flat cap or 0.1 ml tubes in strip, suitable for use on Rotor-Gene, for instance: -PCR tubes 0.1 (1000) - cod. DIA-PL1, Diatech Pharmacogenetics -Strip tubes and caps, 0.1 ml (250) – cod. 981103, Qiagen
Sample Grid creation and data analysis	▪ **EasyPGX® Analysis software** cod. RT800-SW vers.4.0.2 or above, Diatech Pharmacogenetics

STABILITY AND STORAGE

Store all the reagents according to the instructions on the packages, in particular:

- Store all the amplification reagents at -35/-20°C in the original package immediately upon receipt.
- Store all the extraction reagents at +2/+8°C in the original package immediately upon receipt.
- Once resuspended, store the **EasyPGX SARS-CoV-2 pos ctrl** at -35/-20°C and use it within the expiration date. Avoid thawing and re-freezing more than twice, as this could lead to poor performance.
- Avoid thawing and re-freezing the reagents more than twice, as this could lead to poor performance.
- Protect all mixes containing probes from light to avoid degradation of the fluorescent dyes.
- If properly stored, the reagents remain stable until the expiration date displayed on the individual label.

SYMBOLS

REF	Catalogue number (product code)	CONTROL +	Positive control
GTIN	Global Trade Item Number	CONTROL –	Negative control
LOT	Batch code		Consult the instruction for use
Σ	Content sufficient for <n> tests	H B	User manual (handbook)
IVD	For *in vitro* diagnostic use		Use by date
CONT	Contents		Temperature limits
COMP	Components		Manifacturer
NUM	Number of aliquots		Important Note
QUAN	Quantity per aliquot		

PRODUCT USE LIMITATIONS

- The "**Easy® SARS-CoV-2**" kit can only be used by specialized personnel, properly instructed and trained.
- It is necessary to operate in compliance with the general guidelines of Good Laboratory Practice (GLP) and the instructions contained in this manual.
- Do not use expired or incorrectly stored reagents.
- The "**Easy® SARS-CoV-2**" kit has been designed and validated for the use with the real-time qPCR instrument **EasyPGX® qPCR instrument 96** (code RT800-96) CFX96 (Biorad), ABI7500/7500fast (Applied Biosystems), RotorGene Q (Qiagen).
- Diatech Pharmacogenetics can't respond of results obtained using instruments or accessories other than those recommended in this user manual.
- The reliability of the results also depends on the procedures carried out in the pre-amplification stages, including the selection of starting biological specimens, the preservation of the samples and the RNA extraction.
- Any diagnostic results generated by this procedure must be interpreted with reference to other clinical or laboratory findings.
- A positive results indicates the presence of SARS-CoV-2 RNA, but is not indicative of a transmissible virus. Negative results do not preclude infection with the SARS-CoV-2 virus and must be interpreted with reference to other clinical or epidemiological findings of the patient.
- Mutations whitin the target regions may compromise SARS-CoV-2 virus detection.
- The "**Easy® SARS-CoV-2**" kit is covered by the CE Mark in compliance with the European directive 98/79/EC on the in vitro diagnostic (IVD) medical devices, only in those countries that accept the user manual translated in the languages available on the website www.diatechpharmacogenetics.com/area-riservata.

QUALITY ASSURANCE

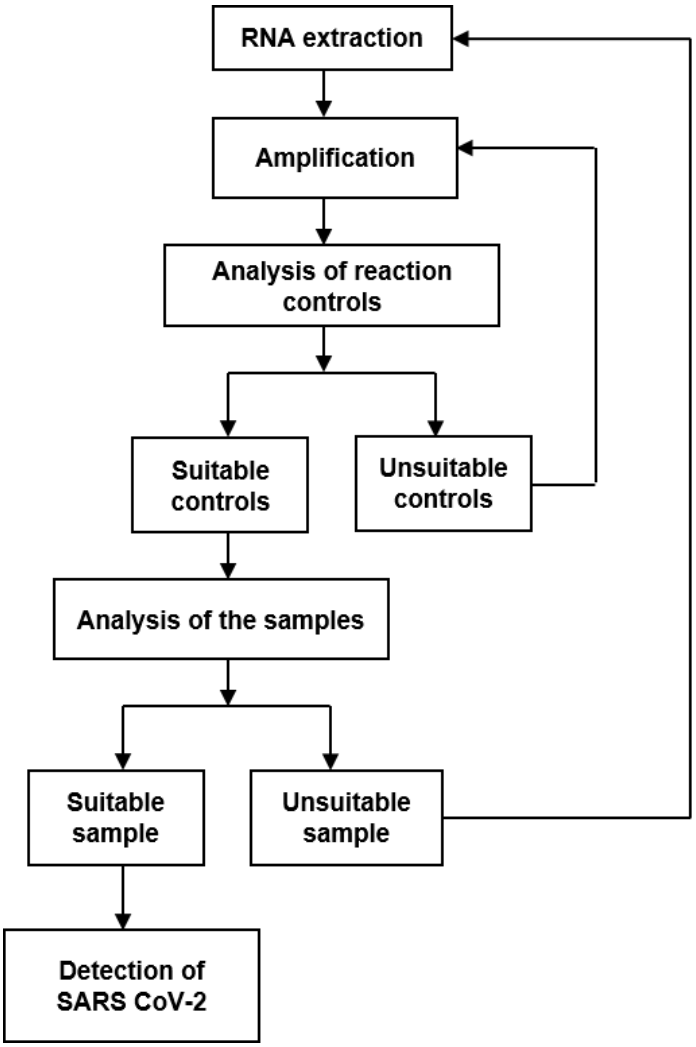
- The "**Easy® SARS-CoV-2**" kit has been designed, developed and validated in compliance with the Directive 98/79/EC on in vitro diagnostic (IVD) medical devices, transposed in Italy in the D.Lgs No 332/2000 and subsequent legislative changes, and in accordance with the procedures of the Company's Quality System certified for conformance to the European regulatory standards EN ISO 9001 and ISO 13485.
- The consistent quality of the "**Easy® SARS-CoV-2**" kit is guaranteed by the application of a tight process control on materials and on operative procedures for product realization and its management till the Customer. The quality of each lot is attested in the related Certificate of Analysis available upon request to the Customer Service (support@diatechpharmacogenetics.com).

WARNINGS AND PRECAUTIONS

- The kit may only be used by specialist personnel, properly instructed and trained to perform in vitro laboratory techniques.
- Carefully read this User Manual.
- Check that the version of the User Manual in use corresponds to the one described on the "**Easy® SARS-CoV-2**" kit box label.
- Handle all samples as potentially infectious material inside a laminar flow hood (class II biological safety cabinet or higher).
- Follow the laboratory safety procedures described in "Biosafety in Microbiological and Biomedical Laboratories" (Richmond, JY and McKinney, RW (eds) - 5th edition (2009) and in the NCCLS (National Committee for Clinical Laboratory Standards) Document M29-T. Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids and Tissue. Tentative guidelines. – Villanova, PA:NCCLS, 1989).
- Follow the laboratory safety procedures described in "Laboratory biosafety guidance related to the novel coronavirus (2019-nCoV)" Interim guidance 12 February 2020, WHO; "Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)", CDC.
- Do not eat, drink or smoke in the laboratory. When handling biological samples, disposable gloves, gowns and goggles or face masks should be worn to protect against biological agents.
- Constantly check that the gloves are free from contamination by the biological material being treated. If not, replace them immediately to avoid the possibility of cross-contamination between samples and contamination of the workplace. Wash hands thoroughly after handling samples and reagents.
- The Safety Data Sheet (SDS) is available in the reserved area of the web-site Diatech Pharmacogenetics www.diatechpharmacogenetics.com, or can be requested to the the Diatech Pharmacogenetics technical support (e-mail: support@diatechpharmacogenetics.com; tel. +39 0731-213243).
- Perform the procedure in accordance with Good Laboratory Practice (GLP) general guidelines.
- It is recommended to ensure that the laboratory work flow proceeds in a unidirectional manner, setting up two separate working areas for:
  - extraction of nucleic acids
  - amplification reaction
- Organize the laboratory so that dedicated pipettes, tips and materials are used for each activity.
- Use sterile filter tips. Avoid aerosols.
- Use tubes with twist-lock caps during the extraction of nucleic acids in order to avoid the leakage of the samples and potential contamination.
- During the procedures for nucleic acid extraction and amplification, avoid contamination of reagents with airborne microbes by opening the reagents only within the hood.
- Change the pipette tip before each pick up of reagents and every time you move from one sample to another in any stage of the procedure.
- The precision pipettes used should have an accuracy of within 3% of the set volume.

- Periodically check the calibration status of the dispensing instruments.
- Do not use reagents after the expiration date shown on each container.
- All reagents supplied in the "**Easy® SARS-CoV-2**" kit are intended to be used solely with the other reagents included in the same kit. Do not substitute or mix reagents from different batches, in order to maintain optimal performance.
- Discard unused reagents and the expired kit and waste in accordance with current national laws and local regulations.
- Extraction area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which work has been carried out, by cleaning with appropriate products (e.g. FD 322, Dürr Dental, Germany) and UV irradiate the work surface of the biological cabinet where the pipettes should be carefully placed after decontamination.
- Amplification area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which work has been carried out, by cleaning with appropriate products to eliminate nucleic acids and amplicons (e.g. "DNA Cleaner" - code DC001, Diatech Pharmacogenetics) and subsequent UV irradiation, if available.
- Avoid contamination of samples and reagents.
- Store reagents and samples separately.
- In order to avoid possible contamination from carry-over, do not open the reaction tubes after amplification.
- Before use all reagents need to be mixed by inverting 10 times and centrifuged briefly.
- All reagents contained in the kit are ready-to-use and don't need to be diluted. The reagent dilution may result in a loss of performance.
- Include in each run at least 1 negative control (**WATER**) and 1 positive control (**EasyPGX SARS-CoV-2 pos ctrl**).
- In order to avoid any mixing up of samples pay particular attention to samples dispensation, placement of strips into the instrument, editing the sample name in the software.
- The right to contest the kit before the expiration date becomes void if the product is used in violation of GLP guidelines and the manufacturer's recommendations.
- The registered names and trademarks indicated in this document are to be considered protected by law, even when not explicitly stated.

ANALYTICAL PROCEDURE



RNA EXTRACTION

ⓘ Perform this step in the area dedicated to RNA isolation and dilution, using dedicated materials and instruments.

ⓘ Refer to https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html for clinical specimens collection and storage.
ⓘ Use only synthetic fiber swabs with plastic shafts. Do not use calcium alginate swabs or swabs with wooden shafts, as they may contain substances that inhibit nucleic acid amplification.
ⓘ After the extraction, proceed immediately with the test, or store the extracted RNA at ≤-70°C, divided into aliquots in order to maintain the experimental conditions constant in case of repetition.

Use of Easy® RNA Extraction reagents

ⓘ **Easy® RNA Extraction reagents** are validated only for RNA extraction from nasopharyngeal swabs or oropharyngeal swabs.

BEFORE TO START:

- Use "**EasyPGX® Analysis Software**" for plate set-up. Refer to the software user manual for detailed procedure.
- Enter the number of clinical samples to be tested, to generate the Sample Grid.
- Enter unique pseudonymised or, if possible, anonymized sample names and export the Sample Grid.

ⓘ In order to perform data anlysis with "**EasyPGX® Analysis Software**", positive control **EasyPGX SARS-CoV-2 pos ctrl** and negative control **WATER** must be dispensed in the positions specified in the Sample Grid.

- Turn-on the thermoblock and set the temperature at 98°C.
- Bring reagents to room temperature.
- Mix **RNA purification beads** in order to have an homogeneous solution.
- Mark **SQ reagent tray**, with a permanent marker, with the following letters: **L** (Lysis), **B** (Binding), **W** (Washing), **E** (Elution).

  ⓘ Each **SQ reagent tray**, can be used twice if correctly identified and free from residual reagents. After the first use, wash carefully the **SQ reagent tray** with deionized water and dry on absorbent paper.

- Prepare a fresh solution of **Ethanol 70%**, considering that are necessary:
  - 20 ml for a session of 46 clinical samples
  - 40 ml for a session of 94 clinical samples

Lysis

- Add **20 µl** of sample in the **RNA storage plate.**
- Add in the **SQ reagent tray (L)** the **Lysis buffer**:
  - **2.5 ml** for a session of **46** samples
  - **5 ml** for a session of **94** samples
- Add to each well **40 µl** of **Lysis buffer**, mix by pipetting 10 times.
- Cover the plate with an **Adhesive pre-cutted foil**, centrifuge briefly, then incubate at **98°C** for **5 minutes**.
- Place the plate at **+2°C/ +8°C** for **5 minutes**, then centrifuge for 15 seconds.
- Gently remove the **Adhesive pre-cutted foil** from the plate (one column at a time).

Binding

ⓘ Do not mix together **RNA Purification beads** and **100% Ethanol**

- Add in the **SQ reagent tray (B)** the **RNA Purification beads**:
  - **1.25 ml** for a session of **46** samples
  - **2.5 ml** for a session of **94** samples
- Add to each well of the plate **20 µl** of **RNA Purification beads**.
- Remove the remaining beads and add in the **SQ reagent tray (B)** the **100% Ethanol**:
  - **4 ml** for a session of **46** samples
  - **8 ml** for a session of **94** samples
- Add to each well of the plate **60 µl** of **100% Ethanol**, then mix by pipetting 10 times
- Incubate for **5 minutes** at room temperature outside the magnet.
- Transfer the strips to the magnetic plate and wait for a well-defined pellet on the wall and the solution to become clear (about 5 minutes).
- When the solution appears clear, leaving the strips on the magnetic plate, remove the supernatant without disturbing the beads, taking 130 ul.

ⓘ When removing the supernatant, make sure that no beads have been removed by observing the tip and making sure that the aspirated liquid is clear. If the liquid withdrawn is clear, discard the supernatant, otherwise repeat the entire step by resuspending the beads and transferring them back to the magnetic rack until complete separation.

ⓘ The residual supernatant in the strip does not affect purification.

Washing

- Leave the strips on the magnetic separator.
- Add in the **SQ reagent tray (W) the 70% Ethanol**:
  - **20 ml** for a session of **46** samples
  - **40 ml*** for a session of **94** samples

  *Add the **70% Ethanol** twice in the tray.
- Add **160 µl** of freshly prepared **70% Ethanol** using a multichannel pipette to each well without touching the pellet.
- Incubate for **30 seconds**.
- Remove the Ethanol by setting the multichannel to 180 µl
- Carry out a second wash by adding an additional **160 µl** of **70% Ethanol**.
- Incubate for **30 seconds**, then remove the Ethanol by setting the multichannel to 180 µl
- Remove any Ethanol residues still present in the bottom of the wells with the multichannel set at 200 µl.
- Check for the presence of residual Ethanol and eventually eliminate it using a P10 pipette.

Elution

- Remove the strips from the magnetic rack.
- Allow the pellet to air dry, avoiding it from drying out excessively (about 3 minutes).
- ⓘ Do not excessively dry the pellets because this could invalidate the elution of RNA from the beads.
- Add in the **SQ reagent tray (E)** the **Elution buffer**:
  - **2 ml** for a session of **46** samples
  - **4 ml** for a session of **94** samples
- Add to each well **20 µl** of **Elution buffer**.
- Resuspend the pellet completely and mix thoroughly by pipetting up and down **10 times** with multichannel pipette set to 20 µl.
- Incubate for **5 minutes** at room temperature outside the magnetic rack.
- Transfer the strips to the magnetic rack.
- Wait for a well-defined pellet on the wall and the solution to become clear (about 5 minutes).
- When the solution appears clear proceed immediately with the amplification reaction by taking **8 µl** of sample.

Use of the other recommended kits (see "Materials Required but Not Provided")

- The quantity of biological material required for the RNA extraction depends on protocols.
- Refer also to the extraction kit manual for treatment of samples.
- Perform the RNA extraction following the instructions of the extraction kit in use.
- If the extraction protocol involves the use of wash buffers containing ethanol, it is advisable to perform a further centrifugation before final elution to remove any possible traces of ethanol. This will prevent inhibition of the reaction by the ethanol.

INSTRUMENT SETUP

- Follow the instructions indicated in the instrument user manual to import the correct template, available in the reserved area of Diatech Pharmacogenetics web-site, with the following plate setup and thermal profile:
- Reaction volume 20 µl.

Step	Temperature/Time
RT (1 Cycle)	50°C per 10 minutes
Hot Start (1 Cycle)	95°C per 5 minutes
Amplification (40 cycles)	95°C per 5 seconds
	58°C per 30 seconds (Data collection FAM/Green, ROX/Orange, HEX/JOE/Yellow)#
# CFX96: select "All Channels" to acquire signals in all channels.	

Plate set-up

- Use "**EasyPGX® Analysis Software**" for plate set-up. Refer to the software user manual for detailed procedure.
- Enter the number of clinical samples to be tested, to generate the Sample Grid.
- Enter unique pseudonymised or, if possible, anonymized sample names and export the Sample Grid.

ⓘ In order to perform data analysis with "**EasyPGX® Analysis Software**", positive control **EasyPGX SARS-CoV-2 pos ctrl** and negative control **WATER** must be dispensed in the positions specified in the Sample Grid.

		1	2	3	4	5	6	7	8	9	10	11	12
SARS-CoV-2 Mix	A	sample 1	sample 9	sample 17	sample 25	sample 33	sample 41	sample 50	sample 58	sample 65	sample 73	sample 81	sample 89
SARS-CoV-2 Mix	B	sample 2	sample 10	sample 18	sample 26	sample 34	sample 42	sample 51	sample 59	sample 66	sample 74	sample 82	sample 90
SARS-CoV-2 Mix	C	sample 3	sqample 11	sample 19	sample 27	sample 35	sample 43	sample 52	sample 60	sample 67	sample 75	sample 83	sample 91
SARS-CoV-2 Mix	D	sample 4	sample 12	sample 20	sample 28	sample 36	sample 44	sample 53	sample 61	sample 68	sample 76	sample 84	sample 92
SARS-CoV-2 Mix	E	sample 5	sample 13	sample 21	sample 29	sample 37	sample 45	sample 54	sample 62	sample 69	sample 77	sample 85	sample 93
SARS-CoV-2 Mix	F	sample 6	sample 14	sample 22	sample 30	sample 38	sample 46	sample 55	sample 62	sample 70	sample 78	sample 86	sample 94
SARS-CoV-2 Mix	G	sample 7	sample 15	sample 23	sample 31	sample 39	sample 47	sample 56	sample 63	sample 71	sample 79	sample 87	Positive
SARS-CoV-2 Mix	H	sample 8	sample 16	sample 24	sample 32	sample 40	sample 48	sample 57	sample 64	sample 72	sample 80	sample 88	Negative

AMPLIFICATION

ⓘ Perform this step in the area dedicated to PCR mixes preparation, using dedicated materials and instruments. Before starting decontaminate pipettes, benches and hood in order to degrade any trace of RNA and possibly radiate with UV light for at least 30 minutes.

ⓘ The kit content is optimised to analyse 46 clinical samples and 2 controls (**EasyPGX SARS-CoV-2 pos ctrl** e **WATER**) in maximum 4 sessions.

BEFORE TO START:

- Switch on the instrument and the software at least 20-30 minutes before starting the reaction to allow the heating of the lamps, if necessary.
- Thaw **SARS-CoV-2 mix,** mix in a vortex, or inverting each tube ten times, and spin briefly before use.
- Mix **Enzyme 1, Enzyme 2, Enzyme 3**. Do not use vortex.
- During reaction set-up put **Enzyme 1, Enzyme 2, Enzyme 3** on ice or in a PCR-cooler. Store the reagents at -35/-20°C immediately after use.
- Centrifuge for 10 seconds the **EasyPGX SARS-CoV-2 pos ctrl** and resuspend it by adding 50 µl of the provided **WATER**. Vortex carefully for 10 seconds and then centrifuge for 10 seconds (perform this step in the area dedicated to RNA isolation and dilution, using dedicated materials and instruments). To achieve a complete resuspension of the dry cake, after adding **WATER**, store the liquid positive control at +2/+8°C for 15 minutes before use.

- Prepare and mark an appropriate number of tubes or wells of the plate to use.
- For each sample and control prepare an amplification mixture, according to the following table:

	Reagent volume for 1 reaction (µl)	Reagent volume for 48 reaction (µl)	Reagent volume for 96 reaction (µl)
Enzyme 1	10	500	1000
Enzyme 2	0.2	10	20
Enzyme 3	0.4	20	40
SARS-CoV-2 mix	2	100	200
Total Volume	12.6	630	1260

- Mix the amplification mix thoroughly by repeated pipetting or rapid vortexing, then centrifuge briefly.
- Pipette **12 µl** of mix in all the tubes/wells previously marked.
  Add to the respective tubes/wells:

Negative control	8 µl **WATER**	CONTROL –
samples	8 µl RNA	
Positive control	8 µl of resuspended **EasyPGX SARS-CoV-2 pos ctrl**	CONTROL +

- Reaction volume: 20 µl.
- Briefly centrifuge the plate.
- Check that the thermal profile is setted up correctly and start the run.

ⓘ It is strongly recommended to use as negative control the **WATER** provided with the kit.

ⓘ Before starting the run, please pay attention to the plate orientation (well A1 on the upper left position) or to the Rotor-Gene 0.1ml strips of tubes orientation (mark the first tube of each strip).

DATA ANALYSIS

ⓘ Data analysis must be performed automatically using the **EasyPGX® analysis software** version 4.0.2 or above (code RT800-SW, Diatech Pharmacogenetics).

ⓘ For **EasyPGX® qPCR instrument 96** refer to the **EasyPGX® analysis software** user manual for the export of the raw data in excel format and its import in the analysis software.

ⓘ For **CFX96, Rotor-Gene** and **ABI7500/7500fast** instruments, refer, respectively, to **Appendix A, B, C** for set-up of analysis parameters, export of the raw data in excel format and its import in the **EasyPGX® Analysis Software**.

- Launch **EasyPGX® Analysis Software** version 4.0.2 or above and import raw data in excel format. Refer to software user manual for details.
- The "Summary" tab in the "Results" section allows to view a synthetic visualization of the obtained results for each sample.

		Gene N-SARS-CoV-2	Gene RdRP SARS-CoV-2	Internal Control	
		FAM	ROX	HEX	
N.	Name	Result	Result	Result	Interpretation
1	1_C	Neg	Pos	Ok	SARS-CoV-2 Positive
2	2_C	Neg	Neg	Ok	SARS-CoV-2 Not Positive (1)
3	3_C	Pos	Pos	Ok	SARS-CoV-2 Positive
4	4_C	Neg	Pos	Ok	SARS-CoV-2 Positive
5	5_C	Pos	Pos	Ok	SARS-CoV-2 Positive
6	6_C	Neg	Pos	Ok	SARS-CoV-2 Positive
7	7_C	Neg	Neg	Ok	SARS-CoV-2 Not Positive (1)
8	8_C	Neg	Neg	Ok	SARS-CoV-2 Not Positive (1)
9	9_C	Neg	Neg	Ok	SARS-CoV-2 Not Positive (1)
10	10_C	Neg	Neg	Ok	SARS-CoV-2 Not Positive (1)

- The "Details" tab in the "Results" section allows to view in detail the obtained results for each sample. In this representation is possible to visualize the Cq values, the ΔR values and the results for each target.

Reaction Controls Analysis

		Gene N-SARS-CoV-2			Gene RdRP SARS-CoV-2			Internal Control		
		FAM			ROX			HEX		
N.	Name	Cq	ΔR	Result	Cq	ΔR	Result	Cq	ΔR	Result
1	Positive	21.3	3301	OK	21.2	1132	OK	24.5	236	OK
2	Negative	No Cq	9	OK	No Cq	0	OK	No Cq	6	OK

Analysis of the Viral Target and Internal Control

		Gene N-SARS-CoV-2			Gene RdRP SARS-CoV-2			Internal Control		
		FAM			ROX			HEX		
N.	Name	Cq	ΔR	Result	Cq	ΔR	Result	Cq	ΔR	Result
1	1_C	38.1	234	Neg	36.0	142	Pos	28.4	339	Ok
2	2_C	No Cq	5	Neg	No Cq	1	Neg	28.8	427	Ok
3	3_C	27.3	2804	Pos	25.7	1462	Pos	29.7	220	Ok
4	4_C	No Cq	4	Neg	37.0	106	Pos	27.0	472	Ok
5	5_C	34.3	1067	Pos	32.3	614	Pos	29.4	381	Ok
6	6_C	36.4	555	Neg	34.1	481	Pos	29.1	452	Ok
7	7_C	No Cq	3	Neg	No Cq	0	Neg	29.8	419	Ok
8	8_C	No Cq	6	Neg	No Cq	7	Neg	33.0	302	Ok
9	9_C	No Cq	0	Neg	No Cq	-8	Neg	29.0	388	Ok
10	10_C	No Cq	0	Neg	No Cq	-3	Neg	33.2	327	Ok

ⓘ In presence of errors, refer to the Troubleshooting section of this user manual.

ⓘ In case of a SARS-CoV-2 positive sample, a one negative viral target could be caused by a concentration close to the sensitivity limit ofthe test (LoD, Limit of Detection) or by a mutation in one of the target regions.

ⓘ In order to verify that the signal come from a real amplification reaction (sigmoidal curve) and not from an artifact (linear curve), inspect visually the amplification plot.

TROUBLESHOOTING

Problem	Possible reason	Recommendation
Low or absent amplification signal in all channels both for positive control **EasyPGX SARS-CoV-2 pos ctrl** and samples **Possible error in the set up of the reaction /run: it is not possible to analyze the samples (E01)**	Incorrect selection of the fluorescence acquisition channels.	▪ Check the fluorescence acquisition channels and repeat amplification with the settings described in this manual.
	Incorrect set-up of the thermal profile	▪ Check the temperature profile and repeat amplification with the settings described in this manual.
	Incorrect resuspension/storage of the **EasyPGX SARS-CoV-2 pos ctrl**.	▪ Add 50 µl of the provided **WATER** to a new aliquot. Vortex and centrifuge for 10 seconds. ▪ Store the resuspended **EasyPGX SARS-CoV-2 pos ctrl** at -35/-20°C and avoid thawing and refreezing more than two times.
	Reagents improperly stored or expired.	▪ Protect **SARS-CoV-2 mix** from light. ▪ Store all amplification reagents at -35/-20°C. ▪ Do not use expired reagents.
Low or absent amplification signal in all channels only for positive control **EasyPGX SARS-CoV-2 pos ctrl** **Possible error in the set up of the reaction /run: it is not possible to analyze the samples (E01)**	Incorrect resuspension/storage of the **EasyPGX SARS-CoV-2 pos ctrl**.	▪ Add 50 µl of the provided **WATER** to a new aliquot. Vortex and centrifuge for 10 seconds. ▪ Store the resuspended **EasyPGX SARS-CoV-2 pos ctrl** at -35/-20°C and avoid thawing and refreezing more than two times.
	Incorrect or no dispensation of **EasyPGX SARS-CoV-2 pos ctrl**.	▪ Repeat amplification pipetting the correct volume of **EasyPGX SARS-CoV-2 pos ctrl**. ▪ Dispense **EasyPGX SARS-CoV-2 pos ctrl** in the positions indicated in the Sample Grid 96 generated by **EasyPGX® Analysis Software**.
	Inversion during dispensation of **EasyPGX SARS-CoV-2 pos ctrl** and negative control **WATER**.	▪ Dispense samples acoording to Sample Grid 96 generated by **EasyPGX® Data Analysis Software**.
The negative control **WATER**, shows an amplification signal in one or more than one channel. **Possible contamination: it is not possible to analyze the samples (E02)** **Possible error in the set up of the reaction /run: it is not possible to analyze the samples (E01)**	Contamination	▪ The results shall be rejected and samples must be reamplified using new reagents. ▪ Prepare the PCR reaction in a dedicated area. Carefully decontaminate benches, pipettes and instruments.
	Clinical sample or **EasyPGX SARS-CoV-2 pos crtrl** dispensed instead of negative control **WATER**.	▪ Dispense samples and controls in the positions indicated in the Sample Grid 96 generated by **EasyPGX® Analysis Software**.
Absent amplification signal in all channels for one or more samples. Positive control **EasyPGX SARS-CoV-2 pos ctrl** fits in the acceptability ranges. **Suboptimal amount of starting template / PCR inhibition / possible dispensing error of the sample (E03)**	Insufficient RNA amount and/or PCR inhibition.	Use of **EasyPGX® RNA Extraction reagents**: ▪ Perform extraction according to the procedure described in this user manual. ▪ Bring extraction reagents at room temperature before to start. ▪ Mix **RNA purification beads** in order to have an homogeneous solution before use. ▪ Do not freeze **RNA purification beads**. ▪ After washing with ethanol 70%, do not over-dry beads pellet as this can affect elution. ▪ If you suspect the presence of inhibitors, repeat the amplification diluting the sample 1:10 with **WATER** Use of other recommended extraction and purification reagents not included in the kit: ▪ If the extraction protocol involves the use of wash buffers containing ethanol, it is advisable to perform a further centrifugation before final elution to remove any possible traces of ethanol. This will prevent inhibition of the reaction by the ethanol. ▪ Repeat extraction reducing elution volume or dilution factor. ▪ If you suspect the presence of inhibitors, repeat the amplification diluting the sample 1:5 or 1:10 with **WATER**
	Incorrect or no dispensation of samples	▪ Repeat amplification dispencing the correct volume of RNA and including positive and negative controls.
Cq values for "HEX" channel ouside the acceptability range for internal control; **EasyPGX SARS-COV-2 pos ctrl** is within the expected values. **Excess of template (E04)**	Excess of RNA	▪ Dilute sample in **WATER**. Consider that the dilution 1:2 of the RNA increases the Cq of 1 unit.
If the problems persist despite the implementation of the given recommendations and for any further questions or problems, please contact the Diatech Pharmacogenetics technical support: ▪ e-mail: support@diatechpharmacogenetics.com ▪ telephone: +39 0731 213243 ▪ fax: +39 0731 213239		

PERFORMANCE VALIDATION

Performance validation has been carried out using all the reagents included in the "**Easy® SARS-CoV-2**" kit.
The experiments have been performed according to the instructions reported in this user manual on the following instruments:

- **EasyPGX® qPCR instrument 96** - Diatech Pharmacogenetics (96 posizioni)
- **CFX96** - Bio-Rad
- **Rotor-Gene Q** - Qiagen
- **ABI 7500, 7500 Fast** - Applied Biosystems

Data analysis has been performed using "**EasyPGX® Analysis Software**" cod. RT800-SW v. 4.0.2 (Diatech Pharmacogenetics).

Analitical specificity

An *in silico* analysis was run through alignment of "**Easy® SARS-CoV-2**" primers' and probes' sequences against all SARS-CoV-2 genome sequences available in the NCBI database on 4 May 2020 (1560 sequences). The results demonstrated that the oligos have >99% homology with all available SARS-CoV-2 genome sequences.

Primers and probes specificity has beeen evaluated *in silico*; alignment results against all the sequences of NCBI database do not show aspecific pairing.

Moreover, specificity has been evaluated experimentally for the assay that detects N gene, testing two plasmids (MERS-CoV Control cod. 10006623 e SARS-CoV Control cod. 10006624, IDT) containinig N genes of SARS and MERS; no cross-reactivity has been detected.

Analitical sensibility

The Limit of Detection (LoD) was determined testing serial dilutions of two synthetic quantified RNA standards ("Synthetic SARS-CoV-2 RNA Control 1 MT007544.1" Twist cod.102019; "Synthetic SARS-CoV-2 RNA Control 2 MN908947.3" Twist cod.102024) containing 50, 10, 5 copies/reaction.

The LOD of the kit is defined as the lowest amount of RNA at which sample will provide a positive SARS-CoV-2 result in at least 95% of tests.

According to the above criteria, the LoD is:

- **EasyPGX® qPCR instrument 96**: 10 copies/reaction
- **CFX96:** 5 copies/reaction
- **Rotor-Gene**: 5 copies/reaction
- **ABI7500/7500fast**: 5 copies/reaction

	% Detection			
SARS-CoV-2 RNA Copies/rxn	**Easy PGX qPCR instrument**	**ABI-7500**	**CFX96**	**RotorGeneQ**
50	100%	100%	100%	100%
10	96%	100%	100%	100%
5	93%	100%	100%	100%

Clinical specificity, sensibility, accuracy

Clinical specificity has been evaluated testing 41 negative SARS-CoV-2 samples, already characterized by an alternative RT-PCR assay.
Clinical sensibility has been evaluated testing 10 positive SARS-CoV-2 samples, already characterized by an alternative RT-PCR assay.
Moreover, 10 additional samples, classified as indetermined by the alternative RT-PCR assay have been tested. Five samples have been classified as SARS-CoV-2 negative and five as SARS-CoV-2 positive by "**Easy® SARS-CoV-2**" kit.
RNA has been extracted from nasopharingeal swabs using both the **Easy® RNA Extraction reagents** and other commercial RNA extraction kit based on magnetic beads.

Considering all tested samples results are:

Samples	N° tested samples	N° samples correctly detected
SARS-CoV-2 positive	10	10
SARS-CoV-2 negative	41	41
TOTAL	51	

SPECIFICITY[1]	100%
SENSIBILITY[2]	100%
ACCURACY[3]	100%

Notes:
1. Specificity is defined as: n.samples true negative / (n.samples true negative + n.samples false positive) x 100.
2. Sensibility is defined as: n.samples true positive / (n.samples true positive + n.samples false negative) x 100.
3. Accuracy is defined as: (n.samples correctly detected/ n.total samples tested) x 100.

Comparison of RNA extraction methods

The equivalence between RNA extraction performed with "**Easy® RNA extraction reagents**", supplied with the kit, and other commercial extraction methods, that include RNA purification, has been evaluated testing 43 nasopharingeal swabs.
Each swab has been extracted both with "**Easy® RNA extraction reagents**" and MagCore Automated Nucleic Acid Extractor using "MagCore® Viral Nucleic Acid Extraction Kit (Low PCR Inhibition)" cod. MVN400-04, (RBC Bioscience). Both RNA have been tested with "**Easy® SARS-CoV-2"** kit in the same run.
43/43 samples show a comparable results, in terms of Cq of the internal control assay (HEX channel), with both extraction methods.

Repeatability, Reproducibility

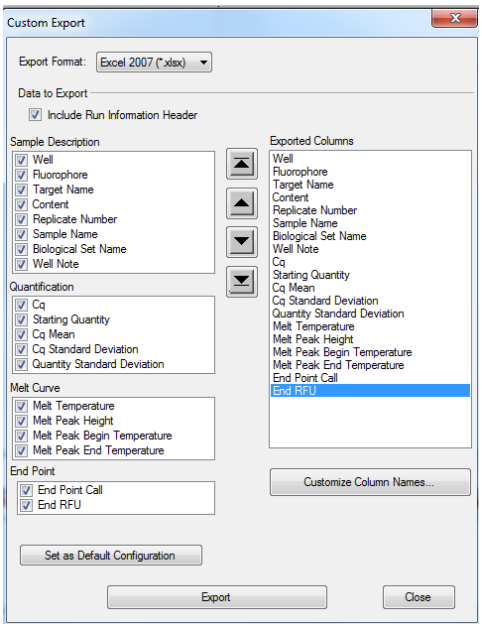
Reproducibility of the test (*inter-assay* variabily) has been evaluated analyzing a synthetic RNA standard ("Synthetic SARS-CoV-2 RNA Control 1 MT007544.1" Twist cod.102019) at 50, 10, 5 copies/reaction in at least two independent experimental sessions.

Repeatability of the test (*intra-assay* variability) has been evaluated analyzing the same synthetic RNA standard at 50, 10, 5 copies/reaction. Each concentration has been tested in triplicates in two independent experimental sessions.
Results are repeatable and reproducible, in terms of SARS-CoV-2 detection, for all samples analyzed.

	Easy PGX qPCR instrument		ABI-7500		CFX96		RotorGeneQ	
Copies/reaction	**% detected**	**Mean Cq ± SD**	**% detected**	**Mean Cq ± SD**	**% detected**	**Mean Cq ± SD**	**% detected**	**Mean Cq ± SD**
50	100%	31.9 ± 0.4	100%	32.2 ± 0.6	100%	32.7 ± 0.4	100%	30.5 ± 1.0
10	96%	34.3 ± 1.4	100%	34.6 ± 1.0	100%	35.4 ± 0.7	100%	33.4 ± 0.9
5	93%	35.3 ± 1.6	100%	35.6 ± 0.3	100%	35.9 ± 0.8	100%	34.2 ± 0.9

Appendix A – CFX96 ANALYSIS PARAMETERS

- At the end of the run, click Plate Setup – View/Edit Plate, select the wells used, pick the FAM, HEX and ROX fluorophores, enter samples name.
- In the section Settings set up the following analysis criteria:
  - Cq determination mode: Single Threshold
  - Baseline settings: Baseline Subtracted Curve fit e Apply fluorescence Drift correction
  - Analysis Mode: Fluorophore
  - Baseline Threshold: User Defined (FAM 200; ROX 100; HEX 100)
- In the page Quantification data, the results are displayed for all channels with the corresponding values of Cq.
- Click Save.
- In the section Export, select Custom Export, select Export Format: Excel 2007(*xlsx), tick all the boxes and click Export.
- Import data into "**EasyPGX® Analysis Software**" cod. RT800-SW.



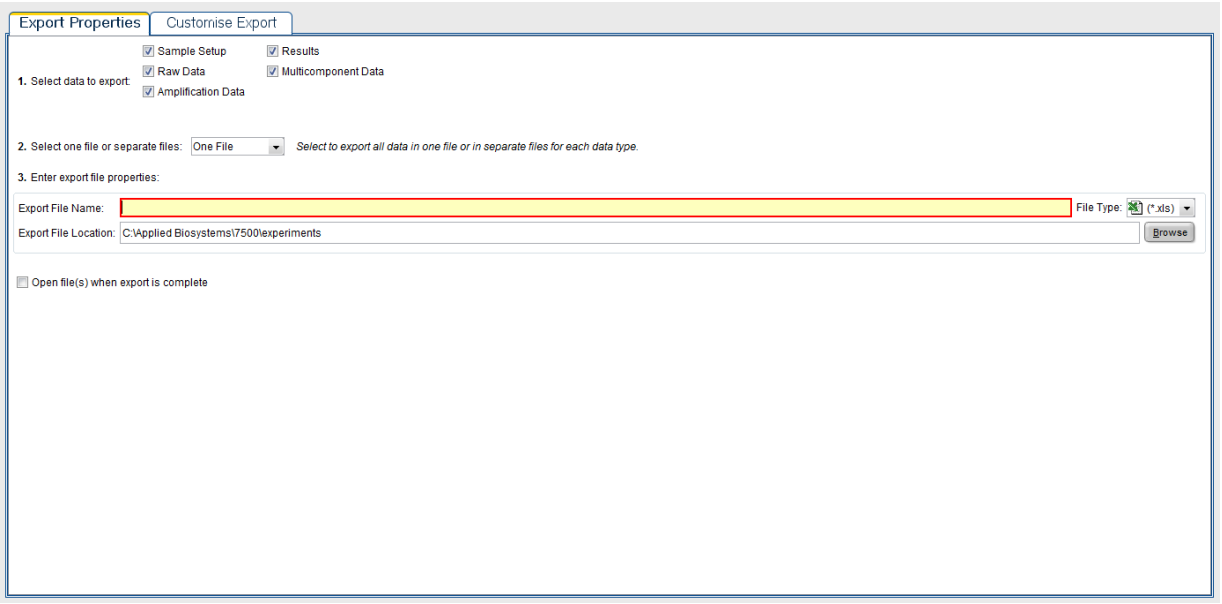
Appendix B – Rotor-Gene ANALYSIS PARAMETERS

At the end of the run, enter sample name, click Analysis and select Quantitation.

- Select the options Dynamic tube and Slope correct for all channels.
- Select the options Outlier Removal – NTC Threshold 5%, for all channels.
- Set Threshold Green 0.04; Yellow 0.04; Rox 0.02.
- In File, Save As, select Excel analysis sheet.
- Import data into "**EasyPGX® Analysis Software**" cod. RT800-SW.

Appendix C – ABI7500/7500fast ANALYSIS PARAMETERS

- At the end of the run, click Setup – Plate setup – Assign Targets and Samples and enter samples name.
- In the section Analysis – Amplification plot in the page View Plate layout select all the samples and controls and omit all empty wells.
- Select Reanalyse.
- In Analysis settings, for each channel, deselect Use Default Settings, Automatic Threshold and Automatic Baseline.
- Set the following threshold: FAM 100000; HEX 20000; ROX 20000
- Click on Apply Analysis Settings and then Reanalyse.
- In the page View Well Table Ct values are visualized for both channels.
- Click Save.
- In the section File, Export, tick all the boxes and click Start Export.



OPERATOR NOTES

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