For general laboratory use.
This product is not available in all territories due to different national regulations.
This document is not intended for use in the USA.

RNA Process Control Kit

Version: 11
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Includes RNA Process Control, Control Assay, and multiplex one-step RT-qPCR master mix for monitoring the whole workflow, from nucleic acid purification to RT-qPCR.

Cat. No. 07 099 622 001	1 kit 2 × 96 control reactions and 200 RT-qPCR reactions of 20 µL final volume each *Not available in US*
Cat. No. 07 099 592 001	1 kit 6 × 96 control reactions and 600 RT-qPCR reactions of 20 µL final volume each *Not available in US*
Cat. No. 07 099 606 001	1 refill kit for the RNA Process Control detection only. 400 RT-qPCRs with 20 µL reaction volume each *Not available in US*

Store the kits at −15 to −25°C.

1. General Information

1.1. Contents

RNA Process Control Kit

Vial / Bottle	Cap	Label	Function / Description	Catalog Number	Content
1	purple	RNA Process Control Kit, RNA Process Control, conc.	Nuclease-resistant RNA concentrate in storage buffer.	07 099 592 001	6 vials, 10 µL each
				07 099 622 001	2 vials, 10 µL each
2	colorless	RNA Process Control Kit, RNA Process Control Diluent	RNA Process Control Diluent	07 099 592 001	3 bottles, 17 mL each
				07 099 622 001	1 bottle, 17 mL
3	blue	RNA Process Control Kit, RT Enzyme Solution, 200x conc.	LightCycler® Multiplex RNA Virus Master	07 099 592 001	3 vials, 28 µL each
				07 099 622 001	1 vial, 28 µL
4	red	RNA Process Control Kit, RT-qPCR Reaction Mix, 5x conc.	LightCycler® Multiplex RNA Virus Master	07 099 592 001	3 vials, 880 µL each
				07 099 622 001	1 vial, 880 µL
5	yellow	RNA Process Control Kit, RNA Process Control Detection Assay, 20x conc.	Primer/Probe Mix for detection of the RNA Process Control.	07 099 592 001	6 vials, 120 µL each
				07 099 622 001	2 vials, 120 µL each
6	white	RNA Process Control Kit, Water, PCR Grade	To adjust the final reaction volume.	07 099 592 001	12 vials, 1 mL each
				07 099 622 001	4 vials, 1 mL each

RNA Process Control Detection Kit

Vial / Bottle	Cap	Label	Function / Description	Catalog Number	Content
3	blue	RNA Process Control Detection Kit, RT Enzyme Solution, 200x conc.	LightCycler® Multiplex RNA Virus Master	07 099 606 001	2 vials, 28 µL each
4	red	RNA Process Control Detection Kit, RT-qPCR Reaction Mix, 5x conc.	LightCycler® Multiplex RNA Virus Master	07 099 606 001	2 vials, 880 µL each
5	yellow	RNA Process Control Detection Kit, RNA Process Control Detection Assay, 20x conc.	Primer/Probe Mix for detection of the RNA Process Control.	07 099 606 001	4 vials, 120 µL each
6	white	RNA Process Control Detection Kit, Water, PCR Grade	To adjust the final reaction volume.	07 099 606 001	8 vials, 1 mL each

The RNA Process Control Detection Kit can only be used in combination with the RNA Process Control that is contained in the RNA Process Control Kit.

1.2. Storage and Stability

Storage Conditions (Product)

The kits are shipped on dry ice.
When stored at −15 to −25°C, the kits are stable through the expiry date printed on the label.
⚠ ***Store the kits protected from light.***

Vial / Bottle	Cap	Label	Storage
1	purple	RNA Process Control, conc.	Store at −15 to −25°C for up to 12 months. ⚠ ***Avoid repeated freezing and thawing.***
2	colorless	RNA Process Control Diluent	Store at −15 to −25°C for up to 12 months or +2 to +8°C for a maximum of 4 weeks.
3	blue	RT Enzyme Solution, 200x conc.	Store at −15 to −25°C for up to 12 months or +2 to +8°C for a maximum of 4 weeks. ⚠ ***Close lid immediately after use.***
4	red	RT-qPCR Reaction Mix, 5x conc.	Store at −15 to −25°C for up to 12 months. ⚠ ***Avoid repeated freezing and thawing (more than 5 times).*** Aliquot Vial 4 and store at −15 to −25°C or store Vial 4 at +2 to +8°C for a maximum of 4 weeks.
5	yellow	RNA Process Control Detection Assay, 20x conc.	Store at −15 to −25°C for up to 12 months. ⚠ ***Store protected from light!*** ⚠ ***Avoid repeated freezing and thawing.***
6	white	Water, PCR Grade	Store at +2 to +8°C for up to 12 months.

Storage Conditions (Working Solution)

Working solution	Storage
RNA Process Control working solution	▪ Store at +2 to +8°C for up to 1 week. ▪ See Section **Preparation of the RNA Process Control working solution**.
RNA Process Control Detection Assay working solution	▪ Store protected from light at +2 to +8°C for up to 1 week (optional for automated PCR setup workflows). ▪ See Section **Preparation of RT-qPCR: Preparation of the master mix for automated RT-qPCR setup** on how to prepare the Detection Assay working solution. ▪ Prepare the RT-qPCR reagents right before use, the Detection Assay working solution (everything combined except RNA template) is stable at +15 to +25°C for up to 4 hours, and is therefore ideal for use in automated workflows.

1.3. Additional Equipment and Reagent required

Standard laboratory equipment

- Nuclease-free pipette tips
- 1.5 mL RNase-free microcentrifuge tubes to prepare master mixes and dilutions
- To minimize risk of RNase contamination, autoclave all vessels

For automated viral RNA purification

- MagNA Pure 96 Instrument*
- MagNA Pure 96 Internal Control Tube*, optional
- MagNA Pure 96 DNA and Viral NA Kit, Large Volume* or
- MagNA Pure 96 DNA and Viral NA Kit, Small Volume*

For manual viral RNA purification

- High Pure Viral Nucleic Acid Kit*

For RT-qPCR

- Real-Time PCR systems such as the LightCycler® PRO, LightCycler® 480, LightCycler® 96 Systems*, or other Real-Time PCR Systems
- LightCycler® 480 Multiwell Plate 96, white*
- LightCycler® 480 Multiwell Plate 384, white*
- LightCycler® 480 Multiwell Plate 96, white, 4 bar codes*
- LightCycler® 480 Multiwell Plate 384, white, 4 bar codes*
- LightCycler® 480 Sealing Foil*
- Sealing Foil Applicator*
- LightCycler® 8-Tube Strips (white)*
- LightCycler® 8-Tube Strip Adapter Plate*
- Centrifuge with swinging-bucket rotor

1.4. Application

The RNA Process Control Kit is a tool to control for potential failures of sample preparation, amplification, detection, and handling errors. The product is intended for use with a variety of sample materials, such as blood, serum, stool, and urine, and a variety of viral RNA targets.
The RNA Process Control Kit is intended for general laboratory use. Any use of the product for *in vitro* diagnostic tests is the sole responsibility of the operator and must be validated by the operator, following the relevant national regulations.

Product Description

The RNA Process Control Kit contains a nuclease-resistant RNA that can be added at various steps during the nucleic acid purification of viral RNA detection workflows. Successful detection of this control RNA serves as a positive control, verifying the functionality of the RNA purification as well as the detection using RT-qPCR.

1.5. Preparation Time

Typical Run Time

The RNA Process Control purification step has various run times from 20 minutes (High Pure) up to 50 to 60 minutes for a typical run on the MagNA Pure 96 Instrument with 96 samples, depending on the protocol. The RNA Process Control detection can be used with a fast RT-qPCR protocol with run times of less than 75 minutes using the LightCycler® Multiplex RNA Virus Master on the LightCycler® PRO Instrument.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Use any template RNA suitable for RT-qPCR in terms of purity, concentration, and absence of RT-PCR inhibitors.
For reproducible isolation of nucleic acids, use:
- Either a MagNA Pure System together with a dedicated reagent kit (for automated isolation),
- or a High Pure Nucleic Acid Isolation Kit (for manual isolation).

Options for nucleic acid isolation and RT-qPCR using the RNA Process Control Kit	
Nucleic acid isolation	RT-qPCR
MagNA Pure 96 Instrument or High Pure Viral Nucleic Acid Kit	LightCycler® PRO Instrument or LightCycler® 480 Instrument II or LightCycler® 96 Instrument

Control Reactions

Control samples and no template controls (NTCs)

Always run appropriate negative and positive controls for each of your target parameters.
- In addition to the RNA Process Control, test known negative and known positive samples as controls in each run to check the entire procedure, including sample preparation and RT-qPCR.
- If the RNA Process Control is added manually to the sample material, include one known positive and one known negative sample without the RNA Process Control.
- If the RNA Process Control is added automatically to all samples, check for the presence of contamination by performing a no template control (NTC) RT-qPCR using Water, PCR Grade (Vial 6) instead of sample eluate in the RT-qPCR. The negative control is necessary for determining potential contamination issues.

Primers

Suitable concentrations of PCR primers for the target range from 0.2 to 0.5 µM (final concentration in RT-qPCR). The recommended starting concentration is 0.5 µM each.

Probe

Suitable concentrations of hydrolysis probes range from 0.05 to 0.5 µM (final concentration in RT-qPCR). The recommended starting concentration is 0.25 µM each.

The optimal probe concentration is the lowest concentration that results in the lowest quantification cycle value (Cq) and adequate fluorescence dynamics for a given target concentration.

For a hydrolysis probe hybridization complex, the Tm of the hydrolysis probe has to be higher than the Tm of the primers.

General Considerations

Precautions

Use RNase-free techniques. RNase-contaminated reagents and reaction vessels will degrade template RNA. Follow these guidelines to minimize risk of contamination:

- Wear disposable gloves and change them frequently.
- Avoid touching surfaces or materials that could cause RNase carryover.
- Use only reagents provided in this kit. Substitutions may introduce RNases.
- Clean and decontaminate work areas and instruments, including pipettes with commercially available decontamination reagents.
- Use only new RNase-free aerosol-blocking pipette tips and microcentrifuge tubes.
- Use a work area specifically designated for RNA work, and if possible, use reaction vessels and pipettes dedicated only for work with template RNA.

This product is for use by experienced personnel who have training in standardized molecular testing procedures and expertise in viral research, in laboratories with appropriate biosafety equipment and containment procedures.

Safety Information

Laboratory procedures

All sample material and resulting waste should be considered potentially infectious. Thoroughly clean and disinfect all work surfaces with disinfectants recommended by the local authorities.

- Do not eat, drink, or smoke in the laboratory work area.
- Do not pipette by mouth.
- Wear protective disposable gloves, laboratory coats, and eye protection when handling samples and kit reagents.
- Clean and decontaminate work areas and instruments, including pipettes with commercially available decontamination reagents.
- Avoid microbial and nuclease contamination of reagents when removing aliquots from reagent bottles. Use sterile disposable pipette tips.
- Wash hands thoroughly after handling samples and kit reagents.

In addition, to minimize the risk of carryover contamination which may result in false-positive results, follow the guidelines listed below.

- Use a PCR hood.
- Wipe and UV-illuminate PCR workstations and biosafety cabinets before use.
- Have separate areas for sample preparation, PCR reaction setup, and PCR amplification.
- Discard pipette tips in sealed containers to prevent airborne contamination.
- The RNA Process Control concentrates and the working solution must be handled with care; open and prepare the solutions in a separate location.
- Avoid opening LightCycler® 480 Multiwell Plates/8-tube strips containing amplification products.

For customers in the European Economic Area

Contains SVHC: octyl/nonylphenol ethoxylates. For use in research and under controlled conditions only – acc. to Art. 56.3 and 3.23 REACH Regulation.

Working Solution

Preparation of the RNA Process Control working solution

The procedure below will result in a consistent RNA Process Control concentration that can be added to the sample material. The process control Cq values may vary depending on the type of sample material, the applied purification protocol, and workflow. It is recommended to use the protocol as outlined below and add 20 µL of the working solution to the sample material. If lower or higher Cqs of the RNA Process Control assay are required, the dilutions can be adjusted accordingly.

1. Thaw one aliquot of the RNA Process Control, conc. (Vial 1) and one bottle of RNA Process Control Diluent (Bottle 2).

2. Add 100 µL RNA Process Control Diluent (Bottle 2) to the aliquot in Vial 1 (preliminary mixture).

3. Mix briefly by vortexing.

4. Pipette 100 µL from the preliminary mixture from Vial 1 and add to 3.9 mL RNA Process Control Diluent (Bottle 2). This will serve as the RNA Process Control working solution.

5. Mix briefly by vortexing.

2.2. Protocols

The RNA Process Control is added to the sample material directly or with the lysis buffer at the beginning of the procedure. Purification can be done using a variety of automated systems as indicated above. To control the workflow for a particular sample, the RNA Process Control can be used with viral target-specific assays using either a single (one well) multiplex, multicolor format, or in two independent monoplex reactions.
For very low-abundant templates, it is best to perform monoplex assays, as they generally produce higher sensitivity. In combination with appropriate positive target samples and valid NTCs, a positive RNA Process Control assay ensures correct sample processing during the nucleic acid purification and verifies the functionality of the RT-qPCR detection reaction. This RNA Process Control assay is best used with the one-step RT-qPCR procedure described below.
The workflow for the MagNA Pure 96 System nucleic acid purification and subsequent one-step detection is described in detail below.
For automated primary sample handling and automated RT-qPCR setup workflows, it can be beneficial to increase the pipetting volume of the individual reaction components. For this purpose, a 4x RNA Process Control detection assay working solution can be prepared.

Isolation of viral RNA

Example of a MagNA Pure 96 Instrument run using an internal control position

1. Prepare the MagNA Pure 96 Instrument according to the Operator's Guide.

2. Depending on the kit used, select one of the following protocols:
   For large volume kit with 500 µL sample volume:
   - Pathogen Universal 500
   - Viral NA Universal LV
   - Viral NA Plasma LV

   For small volume kit with 200 µL sample volume:
   - Pathogen Universal 200
   - Viral NA Universal SV
   - Viral NA Plasma SV

3 Select elution volume:
– 50 µL or 100 µL

4 Add the required volume of **RNA Process Control working solution** to a MagNA Pure 96 Internal Control Tube and proceed according to the MagNA Pure 96 System User Training Guide.

Manual nucleic acid purification method

Add the **RNA Process Control working solution** volume shown in the table below, directly into the sample material just prior to starting the purification process.

Method	Volume of RNA Process Control working solution [µL]
High Pure Viral Nucleic Acid Kit	20.0

Follow the procedure below, see Section **Preparation of the RT-qPCR** to prepare 96, 20 µL standard reactions with the LightCycler® Multiplex RNA Virus Master, using either the LightCycler® PRO System, LightCycler® 480 System, or the LightCycler® 96 System.
Always wear gloves during handling.

Preparation of the RT-qPCR

Preparation of the master mix for manual RT-qPCR setup

1 Thaw the solutions and, to ensure recovery of all the contents, briefly spin vials in a microcentrifuge before opening.
– Mix carefully by pipetting up and down or vortex briefly.

2 In a 1.5 mL reaction tube, prepare master mix as shown in the table below for 95 samples plus 1 NTC (20 µL each, 10 µL amplifications to be used for 384-well plate setups).

Vial	Reagent[1]	Reagent conc.	Master mix for RT-qPCR			
			1 reaction [µL]		100 reactions [µL]	
			96-well plate	384-well plate	96-well plate	384-well plate
3	RT Enzyme Solution	200x	0.1	0.05	10	5
4	RT-qPCR Reaction Mix	5x	4.0	2.00	400	200
5	RNA Process Control Detection Assay	20x	1.0	0.50	100	50
X	Gene-specific primers	–	2.0	1.00	200	100
6	Water, PCR Grade	–	7.9	3.95	790	395
	Master mix volume		**15.0**	**7.50**	**1,500**	**750**

[1] For eluates derived from stool samples, it is recommended to add 0.2 µg/µL (final) of molecular biology-grade Bovine Serum Albumin. Adjust for BSA volume by subtracting from PCR Water volume.

Preparation of the master mix for automated RT-qPCR setup with a RNA Process Control Detection Assay working solution

For automated PCR setup environments that require larger pipetting volumes, the RNA Process Control Detection Assay, 20x conc. can be used to create a 4x working solution.

1. Thaw one vial of RNA Process Control Detection Assay, 20x conc. (Vial 5) and one vial Water, PCR Grade (Vial 6).

2. Add 480 µL water to Vial 5 (RNA Process Control Detection assay, 20x conc.) from Step 1.

3. Mix briefly by vortexing.

4. Add 5 µL of this 4x working solution to a 20 µL final one-step RT-qPCR reaction volume to achieve a final concentration of 1x.

5. Prepare master mix as shown below for 95 samples plus 1 NTC.

Vial	Reagent[1]	Reagent conc.	Master mix for RT-qPCR	
			1 reaction [µL]	100 reactions [µL]
3	RT Enzyme Solution	200x	0.1	10
4	RT-qPCR Reaction Mix	5x	4.0	400
5	Detection Assay Working Solution, 4x conc.	4x	5.0	500
X	Gene-specific primers	–	2.0	200
6	Water, PCR Grade	–	3.9	390
	Master mix volume		**15.0**	**1,500**

[1] For eluates derived from stool samples, it is recommended to add 0.2 µg/µL (final) of molecular biology-grade Bovine Serum Albumin. Adjust for BSA volume by subtracting from PCR Water volume.

Setup of the RT-qPCR reaction for the LightCycler® PRO, LightCycler® 480, and LightCycler® 96 Instruments

1. Place your samples on ice. Prepare the RT-qPCR master mix as described in the pipetting protocol above (**Preparation of the master mix for manual RT-qPCR setup** or **Preparation of the master mix for automated RT-qPCR setup with a RNA Process Control Detection Assay working solution**).

2. Dispense 15 µL of the reaction mixture RT-qPCR master mix (Step 1) and the sample eluates to the respective wells of the LightCycler® 480 Multiwell Plate 96. Always start with the negative control, followed by the prepared samples. Positive controls are pipetted last.
   – Add 5 µL Water, PCR Grade (Vial 6) into the NTC position.
   – Add 5 µL sample eluate to all sample positions.

3. Seal the LightCycler® 480 Multiwell Plate 96, white with a LightCycler® 480 Sealing Foil.

4. Place the multiwell plate into the centrifuge with a swinging-bucket rotor and suitable adapter, and balance it with a suitable counterweight, such as another multiwell plate.
   – Centrifuge at 1,500 × *g* for 2 minutes.

5. Proceed immediately with the RT-qPCR.

LightCycler® PRO, LightCycler® 480, and LightCycler® 96 System protocols

The following procedure is optimized for use with the corresponding LightCycler® System you are using.

⚠ ***Program the LightCycler® Instrument before preparing the reaction mixes.***

ⓘ *For details on how to program the experimental protocol, see the LightCycler® PRO System User Assistance, LightCycler® 480 Instrument Operator's Manual, or LightCycler® 96 System Operator's Guide.*

Protocol for use with the LightCycler® PRO System (Multiwell Plate 96 or 384)

The LightCycler® PRO System protocol contains the following programs:

- **Reverse transcription** of the viral template RNA
- **Pre-incubation** for denaturation of cDNA/RNA hybrid
- **Amplification** of the cDNA
- **Cooling** of the thermal cycler

Setup[1]				
Thermal cycler type		Reaction volume [µL]		
96 (384)		10 – 100 (5 – 20)		
Detection format				
Select dyes used in your assays. If the dyes are not available, select appropriate filters in the user-defined detection format.				
Programs				
Program name		Cycles		
Reverse transcription		1		
Pre-incubation		1		
Amplification		45[2]		
Cooling		1		
Temperature targets				
	Target [°C]	Acquisition mode	Duration [s]	Ramp rate [°C/s]
Reverse transcription	50[3]	None	600[4]	4.4 (4.8)
Pre-incubation	95	None	30	4.4 (4.8)
2-step amplification	95	None	5	4.4 (4.8)
	60[5]	Single	30	2.2 (2.5)
Cooling (automatically added)	40	None	30	2.2 (2.5)

[1] The LightCycler® Multiplex RNA Virus Master includes an aptamer for hot start. Anneal/Extend temperatures significantly below 60°C may not be well tolerated because aptamer will inhibit DNA polymerase activity at low temperatures.

[2] Forty-five cycles is suitable for most assays. If the assay shows steep amplification curves and early crossing points, 40 cycles should be sufficient. Reducing the number of cycles will improve uniformity of the product and reduce the time required for the assay.

[3] We recommend running the reverse transcription at +50°C. If necessary, it is also possible to run it at +55°C.

[4] Ten minutes is the recommended default protocol. Depending on your assay, it should be possible to reduce the time for reverse transcription down to 4 minutes. For best results, run a test with 10, 8, 6, 4 minutes, etc.

[5] Most available assays are designed for an annealing temperature of +60°C. If the Tm of the primers and probe indicate using different settings, select an annealing temperature +5°C below the calculated primer Tm for initial experiments. For assay optimization, choose the highest annealing temperature which still results in a high yield of amplicon with a low Cq and adequate fluorescence dynamics.

Color Compensation protocol for the LightCycler® PRO Instrument

The LightCycler® PRO Instrument does not require the creation of a color compensation object.

Protocol for use with the LightCycler® 480 Instrument II (Multiwell Plate 96 or 384)

A LightCycler® 480 Instrument II protocol that uses the LightCycler® Multiplex RNA Virus Master contains the following programs:

- **Reverse transcription** of the viral template RNA
- **Pre-incubation** for denaturation of cDNA/RNA hybrid
- **Amplification** of the cDNA
- **Cooling** of the thermal cycler

Programming a customized detection format for the LightCycler® System filter combination selection

The detection format in the LightCycler® 480 Instrument II Software, Version 1.5 setup needs to be customized for the applied dual-color or triple-color-hydrolysis format used in the RT-qPCR detection.

- Dual-color measurement and the simultaneous analysis of one additional parameter (e.g., FAM, Yellow 555, or Red 610 channel), and the RNA Process Control (Cy5).
- Triple-color measurement and simultaneous analysis of multiple parameters (e.g., FAM and Yellow 555 or FAM and Red 610) and the RNA Process Control (Cy5).

In the **Tool** module, the **Detection Formats** option allows creating new detection formats specified by the user, including a **Detection Format** list, a **Filter Combination** selection area, and **Selected Filter Combination List**. Different filter settings for the LightCycler® 480 Instrument II are defined.

The following table shows the parameters that must be programmed for an RT-qPCR run using the LightCycler® Multiplex RNA Virus Master(1) on the LightCycler® 480 Instrument II (Multiwell Plate 96 or 384).

Setup					
Thermal cycler type			Reaction volume [µL]		
96 (384)			20 (10)		
Detection format	Excitation filter		Emission filter		
For example: 3 Color Hydrolysis Probe					
FAM Yellow 555 (or Red 610) Cy5	465 533 (533) 618		510 580 (610) 660		
For new customized hydrolysis probes detection formats, set for all selected filters in the “Selected Filter Combination List” (under Tools), the following values:					
Melt factor	1				
Quant factor	10				
Max integration time (sec)	2				
Programs					
Program name	Cycles		Analysis mode		
Reverse transcription	1		None		
Pre-incubation	1		None		
Amplification	45(2)		Quantification		
Cooling	1		None		
Temperature targets					
	Target [°C]	Acquisition mode	Hold [hh:mm:ss]	Ramp rate [°C/s]	Acquisitions [per °C]
Reverse transcription	50(3)	None	00:10:00(4)	4.4 (4.8)	–
Pre-incubation	95	None	00:00:30	4.4 (4.8)	–
Amplification	95	None	00:00:05	4.4 (4.8)	–
	60(5)	Single	00:00:30	2.2 (2.5)	–
Cooling	40	None	00:00:30	2.2 (2.5)	–

(1) The LightCycler® Multiplex RNA Virus Master includes an aptamer for hot start. Anneal/Extend temperatures significantly below +60°C may not be well tolerated because aptamers will inhibit DNA polymerase activity at low temperatures.
(2) Forty-five cycles is suitable for most assays. If the assay shows steep amplification curves and early crossing points, 40 cycles should be sufficient. Reducing the number of cycles will improve uniformity of the product and reduce the time required for the assay.
(3) We recommend running the reverse transcription at +50°C. If necessary, it is also possible to run it at +55°C.
(4) Ten minutes is the recommended default protocol. Depending on your assay, it should be possible to reduce the time for reverse transcription down to 4 minutes. For best results, run a test with 10, 8, 6, 4 minutes, etc.
(5) Most available assays are designed for an annealing temperature of +60°C. If the Tm of the primers and probe indicate using different settings, select an annealing temperature +5°C below the calculated primer Tm for initial experiments. For assay optimization, choose the highest annealing temperature which still results in a high yield of amplicon with a low Cq and adequate fluorescence dynamics.

Color Compensation protocol for the LightCycler® 480 Instrument II

The application of a color compensation file is necessary to compensate for optical crosstalk between two or three detection channels.
For the LightCycler® 480 Instrument II, an instrument-specific color compensation file is mandatory for most multicolor experiments, and a color compensation object can be generated by performing the following experiment.
The LightCycler® 480 Instrument II Color Compensation protocol contains the following programs:

- **Reverse transcription** of the viral template RNA
- **Pre-incubation** for denaturation of cDNA/RNA hybrid
- **Amplification** of the cDNA
- **Temperature gradient step** to create the Color Compensation file
- **Cooling** of the thermal cycler

For details on how to program the experimental protocol, see the LightCycler® 480 Operator's Manual.

The following table shows the RT-qPCR parameters that must be programmed for a LightCycler® 480 System Color Compensation file run with a LightCycler® 480 Multiwell Plate 96, white.

Setup					
Thermal cycler type			Reaction volume [µL]		
96			20		
Detection format					
Customized (see section above)					
Programs					
Program name		Cycles		Analysis mode	
Reverse transcription		1		None	
Pre-incubation		1		None	
Amplification		45		Quantification	
Temperature gradient step		1		Color Compensation	
Cooling		1		None	
Temperature targets					
	Target [°C]	Acquisition mode	Hold [hh:mm:ss]	Ramp rate [°C/s]	Acquisitions [per °C]
Reverse transcription	50	None	00:10:00	4.4	–
Pre-incubation	95	None	00:00:30	4.4	–
Amplification	95	None	00:00:05	4.4	–
	60	Single	00:00:30	2.2	–
Temperature gradient step	95	None	00:00:10	4.4	–
	40	None	00:00:10	2.2	–
	95	Continuous	–	–	5
Cooling	40	None	00:00:30	2.2	–

Preparation of the Color Compensation run

Prepare the calibrator RT-qPCR mix for more than one reaction; multiply the amount in the columns below by the number of reactions (minimum of 3 to 5 replicates). Note that there will be a slight loss of liquid during the pipetting steps. Please calculate extra volume of RT-qPCR mix by adding at least one additional reaction volume. In order to ensure accuracy, we do not recommend pipetting volumes below 1 µL when adding the individual reagents.

Reagent	1x buffer [µL]	1x for each dye [µL]	1x RNA Process Control [µL]
RT Enzyme Solution, 200x conc. (Vial 3)	0.1	0.1	0.1
RT-qPCR Reaction Mix, 5x conc. (Vial 4)	4.0	4.0	4.0
RNA Process Control Detection Assay, 20x conc. (Vial 5)	–	–	1.0
Detection mix for each dye (one dye per well)	–	X	–
Water, PCR Grade (Vial 6)	15.9	Y	9.9
Template such as viral RNA or positive samples eluates	–	5.0	–
RNA Process Control eluate	–	–	5.0
Total volume	**20.0**	**20.0**	**20.0**

1. Pipette the replicates of each different calibrator mix into a LightCycler® 480 Multiwell Plate 96.

2. Seal the LightCycler® 480 Multiwell Plate using a sealing foil.

3. Place the multiwell plate into the centrifuge with a swinging-bucket rotor and suitable adapter, and balance it with a suitable counterweight, such as another multiwell plate.
   – Centrifuge at 1,500 × *g* for 2 minutes.

4. Load the multiwell plate into the LightCycler® 480 Instrument II and start the program.

Create Color Compensation object

When the experiment is finished, open the **Sample Editor**. Select workflow **Color Comp** and designate the used positions of the multiwell plate as “Water” for buffer replicates, and the appropriate dyes (FAM, Yellow 555, Red 610, or Cy5), respectively.
Open the **Analysis** module “Color Compensation”; click on **Calculate**, and save the color compensation object in the database.
From this point on, you can apply this “CC Object” to each dual- or triple-color experiment performed with FAM, Yellow 555, Red 610, and Cy5 on the same instrument.

Protocol for use with the LightCycler® 96 Instrument protocol

The LightCycler® 96 System protocol contains the following programs:

- **Reverse transcription** of the viral template RNA
- **Pre-incubation** for denaturation of cDNA/RNA hybrid
- **Amplification** of the cDNA

Run editor				
Detection format			Reaction volume (µL)	
Select Cy5 and any other dye used in your assays.			20	
Programs[1]				
	Temp. [°C]	Ramp [°C/s]	Duration [s]	Acquisition mode
Reverse transcription	50[2]	4.4	600[3]	None
Pre-incubation	95	4.4	30	None
2-step amplification	No. of cycles: 45[4]			
	95	4.4	5	None
	60[5]	2.2	30	Single

[1] The LightCycler® Multiplex RNA Virus Master includes an aptamer for hot start. Anneal/Extend temperatures significantly below 60°C may not be well tolerated because aptamers will inhibit DNA polymerase activity at low temperatures.

[2] We recommend running the reverse transcription at +50°C. If necessary, it is also possible to run it at +55°C.

[3] Ten minutes is the recommended default protocol. Depending on your assay, it should be possible to reduce the time for reverse transcription down to 4 minutes. For best results, run a test with 10, 8, 6, 4 minutes, etc.

[4] Forty-five cycles is suitable for most assays. If the assay shows steep amplification curves and early crossing points, 40 cycles should be sufficient. Reducing the number of cycles will improve uniformity of the product and reduce the time required for the assay.

[5] Most available assays are designed for an annealing temperature of +60°C. If the Tm of the primers and probe indicate using different settings, select an annealing temperature +5°C below the calculated primer Tm for initial experiments. For assay optimization, choose the highest annealing temperature which still results in a high yield of amplicon with a low Cq and adequate fluorescence dynamics.

Color Compensation protocol for the LightCycler® 96 Instrument

The LightCycler® 96 Instrument does not require the creation of a color compensation object.

3. Results

Stability in sample material

The RNA Process Control Kit was used according to the protocols described above. 20 µL of the RNA Process Control working solution was added to 200 µL serum and a PBS-based mouth rinse solution. The samples were incubated at either +15 to +25°C for 2 or 18 hours or directly subjected to a MagNA Pure 96 System purification (MagNA Pure 96 DNA and Viral NA Small Volume Kit using the Pathogen Universal 200 protocol with 100 µL eluate). 5 µL of the eluates were used in a 20 µL RT-qPCR detection reaction using the above described protocols. The amplification curves below show the results for two independently treated samples of each sample type and time point.

- The RNA Process Control detection assay shows no Cq shift and hence, no degradation is detectable even after 18 hours of incubation in the sample materials.

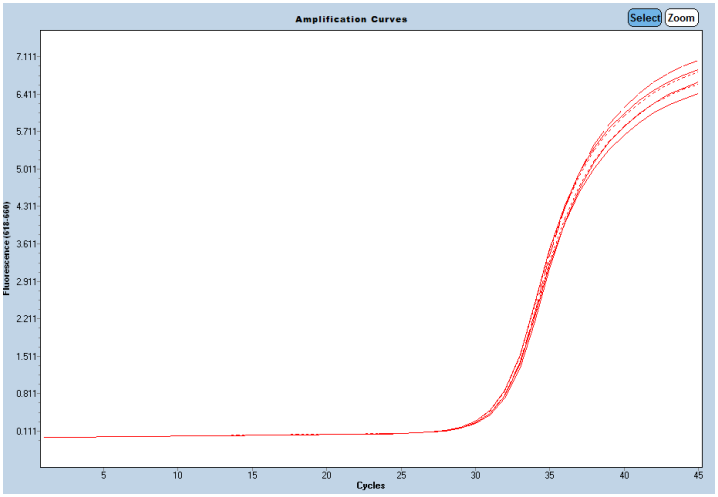

Fig. 1: Amplification curves of the RNA Process Control assay. The RNA Process Control was added to serum samples and processed after 0, 2, or 18 hours incubation at +15 to +25°C. Monoplex RT-qPCR was performed in a 20 µL reaction volume using the LightCycler® Multiplex RNA Virus Master on a LightCycler® 480 System II (0 h = solid line, 2 h = dashed line, 18 h = dotted line).

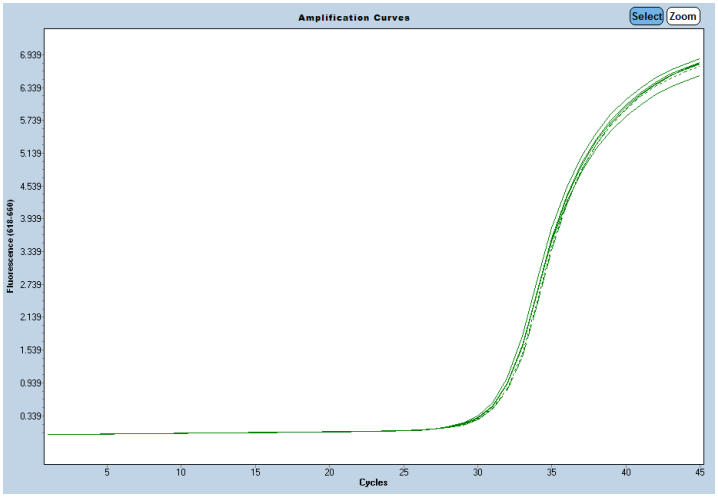

Fig. 2: Amplification curves of the RNA Process Control assay. The RNA Process Control was added to mouth rinse solution samples and processed after 0, 2, or 18 hours incubation at +15 to +25°C. Monoplex RT-qPCR was performed in a 20 µL reaction volume using the LightCycler® Multiplex RNA Virus Master on a LightCycler® 480 System II (0 h = solid line, 2 h = dashed line, 18 h = dotted line).

Use with different sample materials

The RNA Process Control Kit was used in a panel of six different sample materials (whole blood, EDTA-plasma, citrate-plasma, serum, PBS-based mouth rinse solution, and urine) according to the same standard protocol as described above (MagNA Pure 96 DNA and Viral NA Small Volume Kit using the Pathogen Universal 200 protocol with 100 µL eluate). Each sample material shows an individual specific mean Cq with the standard protocols from above, and the Cq variation from sample to sample is typically within 1 - 2 Cq values.

- All NTCs are clean and the RNA Process Control assay does not produce false positives in any of the sample materials.
- RNA Process Control assay performs comparably in all different sample materials.

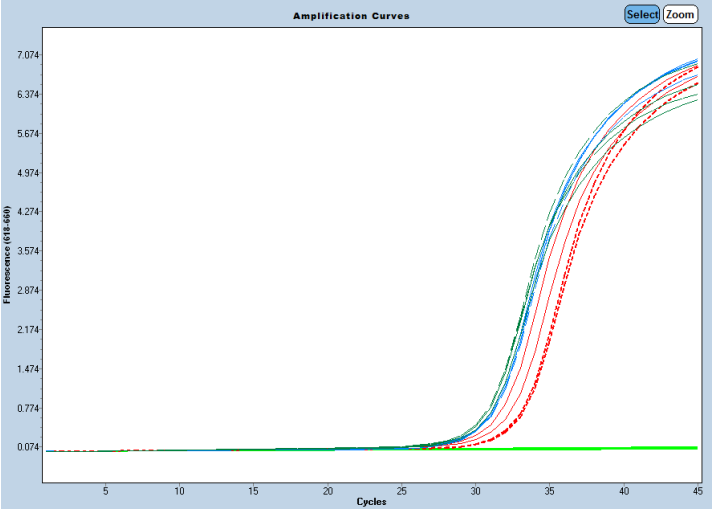

Fig. 3: RNA Process Control amplification curves in 6 different sample materials using the LightCycler® Multiplex RNA Virus Master (red solid = citrate plasma; red dashed = whole blood; blue solid = EDTA-plasma; blue dashed = serum; green solid = urine; green dashed = mouth rinse; light green = NTCs) showing Cq values from 30.1 to 32.7.

Interpretation of data

The robust workflow described above ensures that a constant amount of RNA Process Control is added to the sample material. However, the detection of Cq values may vary depending on the overall workflow setup (used sample material, purification instrument and protocol, detection instrument, multiplexing parameters, experimenter, etc.). In general, expect the Cq value to vary in a range of approximately ± 2 - 3 Cqs for one particular setup.
Using the standard procedures with different nucleic acid purification protocols on different instruments with different sample materials, the RNA Process Control resulted in Cq values of ~ 30 - 35 (100 µL eluate and 5 µL eluate in a 20 µL RT-qPCR detection reaction with the LightCycler® Multiplex RNA Virus Master on a LightCycler® System. For one such particular workflow, the following table shows an exemplary interpretation guideline.

Target (Channel 465 - 510)	RNA Process Control (Channel 618 - 660)	Results/target status
No Cq	Valid Cq (~ 30 - 35)	Valid/target negative
Cq < 40	Valid Cq or no Cq	Valid/target positive
No Cq	No valid Cq ▪ For example, outside of ~ 30 - 35 ▪ No sigmoid curve	Invalid/target unknown

⚠ ***For any result assessments, do not solely rely on Cq callings. Ensure that the amplification curves are sigmoid in shape and that the Cq values are reasonable. The RT-qPCR should be repeated if Cq values show an uncertain Character Code and Description of the status in the result screen (LightCycler® 480 Software, Version 1.5).***

⚠ ***For a valid test batch (including negative controls with a negative result and positive controls with a positive result), verify each individual sample for its result, including the RNA Process Control result.***

Negative control/no template control (NTC)

The assay result for a negative control, measured in the appropriate channel, always needs to be negative. If the result is positive, all sample results controlled by the corresponding negative control are invalid because of potential contamination of the reagents. In the case of a positive result for the negative control, sample preparation and RT-qPCR with the appropriate detector must be repeated.

Positive controls

The assay result for the positive controls, measured in the appropriate channel, always needs to be positive. If the result is negative, all samples results controlled by the corresponding positive control are invalid, and the respective RT-qPCR with the appropriate detector must be repeated.

Sample result

Check if the results of the negative and positive controls in the run are valid, and interpret the sample results for each target and RNA Process Control according to the table above (Interpretation of data).

RNA Process Control/internal control

If samples results are negative for a target, the simultaneous RNA Process Control measured in channel 618 - 660 always needs to be positive to prove that there actually was sample material in the reaction and that the RT-qPCR was not inhibited. For positive samples with a high amount of target RNA, the extraction control may be negative because of the competition of the two reactions.

4. Troubleshooting

Observation	Possible cause	Recommendation
Fluorescence intensity varies.	Some of the reagent is still in the upper part of the microwell or an air bubble is trapped in the microwell.	Repeat centrifugation, but allow sufficient centrifugation time so that all reagent is at the bottom of the microwell and air bubbles are expelled.
	Skin oils or dirt are present on the lid or sealing foil.	Always wear gloves when handling the multiwell plate/8-tube strip.
Fluorescence intensity is very low.	Low concentration or deterioration of dyes in the reaction mixtures because dye was not stored properly.	Keep dye-labeled reagents protected from light.
		Store the reagents at −15 to −25°C and avoid repeated freezing and thawing.
	Poor RT-qPCR efficiency (reaction conditions not optimized).	Check annealing temperature of primers and probes.
		Check experimental protocol.
		Always run a positive control along with your samples.
	RNA is degraded during isolation or improper storage.	If possible, check RNA quality.
		Check RNA with an established RT-qPCR primer when available.
	Pipetting errors and omitted reagents.	Check for missing reagents.
		Check the pipetting procedure.
	Impure sample material inhibits reaction.	Dilute sample 1:10 and repeat the analysis.
		Repurify the nucleic acids to ensure removal of inhibitory agents.
Negative control sample gives a positive signal.	Contamination	Remake all critical reaction mixes. Use separate RT-qPCR setup working areas.

5. Additional Information on this Product

5.1. Test Principle

Background information

Two major outcomes can occur during a typical detection workflow for viral RNA targets. The sample can be either positive or negative. Whereas a positive detection also verifies the functionality of workflow components, a negative test outcome could be due either to a true negative sample or to a failure of critical workflow components and thus be a false negative. To rule out the possibility of workflow failures, a positive control that is similar to the target material but inert to the target detection is supplied in this kit and can be used in the workflow.

In order to prevent any cross-reaction with sample-derived nucleic acids or target-specific detection systems, the RNA Process Control Assay amplicon has been designed to have no significant homologies to any other known sequence. The RNA Process Control Detection Assay primers and probe are added in a low concentration to further lower any possible competition effects in multiplex reactions.
The RNA Process Control concentrate is adjusted carefully to achieve a robust Cq value within one specified workflow. Different sample materials and workflows may require adjusted dilution of the RNA Process Control concentrate.

The RNA Process Control Kit is designed for the detection of an endogenous heterologous control (RNA Process Control) during a sample purification procedure when using:
– either a MagNA Pure System together with a dedicated reagent kit (for automated isolation),
– or a High Pure Nucleic Acid Isolation Kit (for manual isolation).

How this product works

The RNA Process Control Kit provides a non-competitive internal control to monitor nucleic acid purification and detection processes in order to prevent false-negative results. The RNA Process Control working solution contains a constant amount of nuclease-resistant RNA[(1)] that can be added to a large variety of sample materials without impairing the purification of other sample intrinsic nucleic acid[(2)], such as from RNA viruses.

(1) The strictly monitored production and quality processes in conjunction with the easy-to-use workflow ensure that constant amounts of the control RNA are added to each and every sample.

(2) The RNA Process Control has a unique and completely artificial sequence that makes primer competition with any other target parameters less likely. The primer and the probe of the RNA Process Control are designed to only amplify and detect the unique sequence. The Cy5-labeled probe ensures that other target-specific assays with common dyes such as FAM, Yellow 555, HEX, or Red 610 can be combined in multiplex setups.

Test concept

The control is added to the sample material and co-purified with all other sample endogenous nucleic acids. After purification, the RNA Process Control as well as any target-specific parameter is detected in a RT-qPCR reaction.
- In monoplex reactions in single wells, the RNA Process Control must be positive.
- In multiplex reactions, the RNA Process Control detection must be positive in all target-negative samples. In target-positive samples, the RNA Process Control may be out-competed by the target-specific assay and a negative control result is allowed.

Any failure of the RNA Process Control Detection Assay in target-negative samples indicates an erroneous purification/detection workflow and the sample must be retested.

The key steps in the process are:

1. RNA Process Control working solution is added to the sample material.

2. The protective coat of the internal control is lysed during the extraction process, thus enabling co-purification of the control RNA with other sample endogenous nucleic acids.

3. The sample eluates are used for RT-qPCR reactions.
   – The RNA Process Control Detection Assay specifically detects the internal control.
   – Lab-developed assays assess the status of other targets in the sample material.
   Since the RNA Process Control is added to all samples, its successful detection proves the correct sample processing as well as the functionality of the generic detection reagents.

5.2. Quality Control

The RNA Process Control Kit is function tested using the MagNA Pure System for the nucleic acid purification and the LightCycler® System for RT-qPCR.

6. Supplementary Information

6.1. Conventions

To make information consistent and easier to read, the following text conventions and symbols are used in this document to highlight important information:

Text convention and symbols	
Information Note: Additional information about the current topic or procedure.	
Important Note: Information critical to the success of the current procedure or use of the product.	
① ② ③ etc.	Stages in a process that usually occur in the order listed.
1 2 3 etc.	Steps in a procedure that must be performed in the order listed.
* (Asterisk)	The Asterisk denotes a product available from Roche Diagnostics.

6.2. Changes to previous version

Editorial changes.
Information about the LightCycler® PRO System has been added.
List of additional reagents and equipment has been updated.
Quality Control has been changed to MagNA Pure System and LightCycler® System.
Specific information on MagNA Pure LC System and MagNA Pure Compact has been removed due to their phase out.

6.3. Ordering Information

Roche offers a large selection of reagents and systems for life science research. For a full overview of related products and manuals, please visit and bookmark our homepage lifescience.roche.com.

Product	Pack Size	Cat. No.
Accessories general (hardware)		
LightCycler® 480 Block Kit 384 Silver	1 block kit	05 015 197 001
LightCycler® 480 Block Kit 96 Silver	1 block kit	05 015 219 001
Sealing Foil Applicator	1 piece	10 018 607 001
Thermal Cycler Assembly 96	1 piece	09 742 565 001
Thermal Cycler Assembly 384	1 piece	09 742 581 001
LightCycler® 8-Tube Strip Adapter Plate	1 piece, The adapter plate can be used multiple times.	06 612 598 001
Consumables		
LightCycler® 480 Multiwell Plate 96, white	5 x 10 plates	04 729 692 001
LightCycler® 480 Multiwell Plate 384, white	5 x 10 plates	04 729 749 001
MagNA Pure 96 Internal Control Tube	150 tubes	06 374 905 001
LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
LightCycler® 480 Multiwell Plate 96	5 x 10 plates without sealing foils	05 220 319 001
LightCycler® 480 Multiwell Plate 384	5 x 10 plates without sealing foils	05 217 555 001
LightCycler® 8-Tube Strips (white)	10 x 12 white strips and clear caps	06 612 601 001
Instruments		
LightCycler® 480 Instrument II	1 instrument, 96-well version	05 015 278 001
	1 instrument, 384-well version	05 015 243 001
MagNA Pure 96 Instrument	1 instrument	06 541 089 001
LightCycler® 96 Instrument	1 instrument	05 815 916 001
LightCycler® PRO Instrument	1 instrument, 96-well version	09 541 713 001
	1 instrument, 384-well version	09 582 487 001
MagNA Pure 24 Instrument	Instrument with built-in control unit, software and accessories	07 290 519 001
Reagents, kits		
Bovine Serum Albumin	20 mg, 1 ml	10 711 454 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	For up to 3 x 192 isolations	06 543 588 001
High Pure Viral Nucleic Acid Kit	1 kit, up to 100 isolations	11 858 874 001
MagNA Pure 96 DNA and Viral NA Large Volume Kit	Kit for up to 3 x 96 isolations	06 374 891 001
MagNA Pure 24 Total NA Isolation Kit	Kit for up to 96 isolations (200 µL)	07 658 036 001

6.4. Trademarks

MAGNA PURE and LIGHTCYCLER are trademarks of Roche.
All other product names and trademarks are the property of their respective owners.

6.5. License Disclaimer

For additional documentation such as certificates and safety data sheets, please visit:
documentation.roche.com.

6.6. Regulatory Disclaimer

For general laboratory use.

6.7. Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS).

6.8. Contact and Support

If you have questions or experience problems with this or any Roche product for Life Science, please contact our Technical Support staff. Our scientists are committed to providing rapid and effective help.
Please also contact us if you have suggestions for enhancing Roche product performance or using our products in new or specialized ways. Such customer information has repeatedly proven invaluable to the research community worldwide.
To ask questions, solve problems, suggest enhancements or report new applications,
please visit our **Online Technical Support** Site.

Visit **documentation.roche.com**, to download or request copies of the following Materials:
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- Certificates of Analysis
- Information Material

To call, write, fax, or email us, visit **lifescience.roche.com** and select your home country to display country-specific contact information.

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