

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.



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# RNA Process Control Kit

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 **Version 04**

Content version: January 2015

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. 07 099 592 001**      1 kit  
6 × 96 control reactions and 600 RT-qPCR  
reactions of 20µl final volume each.

**Cat. No. 07 099 622 001**      1 kit  
2 × 96 control reactions and 200 RT-qPCR  
reactions of 20µl final volume each.

Refill Kit for the RNA Process Control detection only.

## **RNA Process Control Detection Kit**

**Cat. No. 07 099 606 001**      1 kit  
400 RT-qPCRs with 20 µl reaction volume each.

**Store the kits at – 15 to –25°C!**

**Store protected from light!**

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# 1. What this Product Does

## Introduction

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.

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.

In order to prevent any cross-reaction with sample-derived nucleic acids or target-specific detection systems, the RNA Process Control assay amplicon has no significant homologies to any other known sequence. The RNA Process Control Detection Assay primer and probes 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 further dilution of the RNA concentrate.

## Number of Tests

- The RNA Process Control Kit is designed for the detection of an endogenous heterologous control (RNA Process Control) during a sample purification procedure and is compatible with either the MagNA Pure 96 Instrument, MagNA Pure LC 2.0 Instrument, MagNA Pure Compact Instrument, or the High Pure Purification Kit (for  $\geq 6 \times 96$  standard nucleic acid isolations on the MagNA Pure 96 Instrument and 600 RT-qPCRs with 20  $\mu$ l reactions volume each using the LightCycler<sup>®</sup> 480 System or the LightCycler<sup>®</sup> 96 System).
- The RNA Process Control Kit Cat. No. 07 099 622 001 contains the same reagents but only for 192 samples and 200 detection reactions.
- The RNA Process Control Detection Kit contains the LightCycler<sup>®</sup> Multiplex RNA Virus Master and the RNA Process Control Detection Assay for the RNA Process Control for 400 reactions. Please note that 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 (Cat. No. 07 099 592 001 or 07 099 622 001).

**Kit Contents RNA Process Control Kit**

<b>Vial/ Cap/ Bottle</b>	<b>Label</b>	<b>Content/Function</b>	<b>Catalog Number/ Quantity</b>
1 purple	RNA Process Control, conc.	Nuclease-resistant RNA concentrate in storage buffer	Cat. No. 07 099 592 001, 6 vials, 10 µl each Cat. No. 07 099 622 001, 2 vials, 10 µl each
2 colorless	RNA Process Control Diluent	RNA Process Control Diluent	Cat. No. 07 099 592 001, 3 bottles, 17 ml each Cat. No. 07 099 622 001, 1 bottle, 17 ml each
3 blue	RT-Enzyme Solution (200×)	LightCycler® Multiplex RNA Virus Master	Cat. No. 07 099 592 001, 3 vials, 28 µl each Cat. No. 07 099 622 001, 1 vial, 28 µl each
4 red	RT-qPCR Reaction Mix (5×)	LightCycler® Multiplex RNA Virus Master	Cat. No. 07 099 592 001, 3 vials, 880 µl each Cat. No. 07 099 622 001, 1 vial, 880 µl each
5 yellow	RNA Process Control Detection Assay (20×)	Primer/Probe Mix for detection of the RNA Process Control	Cat. No. 07 099 592 001, 6 vials, 120 µl each Cat. No. 07 099 622 001, 2 vials, 120 µl each
6 white	Water, PCR grade		Cat. No. 07 099 592 001, 12 vials, 1 ml each Cat. No. 07 099 622 001, 4 vials, 1 ml each

**RNA Process Control Detection Kit**

<b>Vial/ Cap/ Bottle</b>	<b>Label</b>	<b>Contents/Function</b>	<b>Catalog Number/ Quantity</b>
3 blue	RT-Enzyme Solution (200×)	LightCycler® Multiplex RNA Virus Master	Cat. No. 07 099 606 001, 2 vials, 28 µl each
4 red	RT-qPCR Reaction Mix (5×)	LightCycler® Multiplex RNA Virus Master	Cat. No. 07 099 606 001, 2 vials, 880 µl each
5 yellow	RNA Process Control Detection Assay (20×)	Primer/Probe mix for detection of the RNA Process Control	Cat. No. 07 099 606 001, 4 vials, 120 µl each
6 white	Water, PCR grade		Cat. No. 07 099 606 001, 8 vials, 1 ml each

**Storage and Stability**

The kits are shipped on dry ice. Store the kits at  $-15$  to  $-25^{\circ}\text{C}$  in the dark. Kit components are stable at  $-15$  to  $-25^{\circ}\text{C}$  until the expiration date printed on the label.

Store the RNA Process Control, conc. (Vial 1) at  $-15$  to  $-25^{\circ}\text{C}$  for up to 12 months.

- Avoid freeze thawing.

RNA Process Control working solution

- See Chapter **RNA Isolation**: How to prepare the working solution.
- Store the RNA process control working solution at  $+2$  to  $+8^{\circ}\text{C}$  for up to 1 week.

Store the RNA Process Control Diluent (Vial/Bottle 2) at  $-15$  to  $-25^{\circ}\text{C}$  for up to 12 months.

- Store the RNA Process Control Diluent at  $+2$  to  $8^{\circ}\text{C}$  for a maximum of 4 weeks.

Store the RT-Enzyme Solution (200 $\times$ ) (Vial 3) at  $-15$  to  $-25^{\circ}\text{C}$  for up to 12 months or at  $+2$  to  $+8^{\circ}\text{C}$  for 4 weeks. Close lid immediately after use.

Store the RT-qPCR Reaction Mix (5 $\times$ ) (Vial 4) at  $-15$  to  $-25^{\circ}\text{C}$  for up to 12 months. For vial 4, avoid repeated freeze/thaw cycles ( $> 5\times$ ). To avoid repeated freeze/thaw cycles either aliquot vial 4 or store at  $+2$  to  $+8^{\circ}\text{C}$ . Vial 4 is stable at  $+2$  to  $+8^{\circ}\text{C}$  for 4 weeks.

Store the RNA Process Control Detection Assay (Vial 5) at  $-15$  to  $-25^{\circ}\text{C}$  for up to 12 months.

- Avoid freeze thawing.
- See Chapter **Preparation of the master mix for automated RT-qPCR setup**: How to prepare the detection assay working solution.
- Store the detection assay working solution in the dark at  $+2$  to  $+8^{\circ}\text{C}$  for up to 1 week (optional for automated PCR setup workflows).

Store the Water, PCR grade (Vial 6) at  $+2$  to  $+8^{\circ}\text{C}$  for up to 12 months.

Although we recommend working on ice and preparing the RT-qPCR reagents right before use, the detection assay working solution (everything combined except RNA template) is stable at  $+15$  to  $+25^{\circ}\text{C}$  up to 4 hours, and is therefore ideal for use in automated workflows.

### **Additional Equipment and Reagents Required**

- Standard laboratory equipment
- Nuclease-free pipet tips
- 1.5 ml RNase-free microcentrifuge tubes to prepare master mixes and dilutions.
- To minimize risk of RNase contamination, autoclave all vessels.
- Wear gloves at all times.

#### **For RT-qPCR:**

- LightCycler® 480 Instrument II\* or LightCycler® 96 Instrument\*
- LightCycler® 480 Multiwell Plate 96, white\*
- Standard swing-bucket centrifuge with rotor for multiwell plates

#### **For Viral RNA Purification:**

- MagNA Pure 96 Instrument\* including disposables
- 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\*

Alternatively, use other nucleic acid purification instruments and manual sample preparation products such as:

- MagNA Pure LC 2.0 Instrument\* with MagNA Pure LC Total Nucleic Acid Isolation Kit - High Performance\*
- MagNA Pure Compact Instrument\* with MagNA Pure Compact Nucleic Acid Isolation Kit I\*
- High Pure Viral Nucleic Acid Kit\*
- FLOW Solution\*

#### **For Color Compensation with the LightCycler® 480 Instrument II:**

- LightCycler® Multiplex RNA Virus Master\*

### **Application**

The RNA Process Control Kit is a tool to be used as control for potential failures of sample preparation, amplification, detection and handling errors. The product is intended for use with a variety of sample materials (*e.g.* blood, serum, stool, 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.

### **Assay Time**

The RNA Process Control purification step has various run times from 20 minutes (High Pure) up to 50 - 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 65 minutes using the LightCycler® Multiplex RNA Virus Master on the LightCycler® 480 Instrument II or LightCycler® 96 Instrument.

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## 2. How to Use this Product

### 2.1 Before You Begin

#### Precautions

Use RNase-free techniques.

RNase contaminated reagents and reaction vessels will degrade template RNA. Please follow these guidelines to minimize risk of contamination:

- Wear disposable gloves and change them frequently.
- Avoid touching surfaces or materials that could cause RNase carry-over.
- 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 pipet tips and microcentrifuge tubes.
- Use a work area specifically designated for RNA work, and if possible use reaction vessels and pipettors 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.

#### 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 specimens and kit reagents.
- Avoid microbial and nuclease contamination of reagents when removing aliquots from reagent bottles. Use sterile disposable pipette tips.
- Wash hands thoroughly after handling specimens 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, white, containing amplification products.

**Sample Material**

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, several options are possible.

For example:

- MagNA Pure 96 Instrument using the MagNA Pure 96 DNA and Viral NA Small Volume Kit, see section 2.2.1.
- MagNA Pure 96 Instrument using the MagNA Pure 96 DNA and Viral NA Large Volume Kit, see section 2.2.1.
- MagNA Pure LC 2.0 Instrument using the MagNA Pure LC Total Nucleic Acid Kit - High Performance in combination with the "Total NA HS 200" Protocol. Elution is performed using 100 µl elution buffer.
- MagNA Pure Compact Instrument using the MagNA Pure Compact Nucleic Acid Isolation Kit I in combination with the "Total\_NA\_Plasma\_100\_400" Protocol with 100 µl elution volume.
- High Pure Viral Nucleic Acid Kit (for manual isolation) with 200 µl specimen and 100 µl elution volume.

**Options for Nucleic Acid Isolation and One-Step RT-qPCR using the RNA Process Control Kit**

<b>Nucleic Acid Isolation</b>	<b>One Step RT-qPCR</b>
<ul style="list-style-type: none"> <li>• MagNA Pure 96 Instrument</li> <li>or</li> <li>• MagNA Pure LC Instrument</li> <li>or</li> <li>• MagNA Pure Compact Instrument</li> <li>or</li> <li>• High Pure Viral Nucleic Acid Kit</li> </ul>	<ul style="list-style-type: none"> <li>• LightCycler® 480 Instrument II</li> <li>or</li> <li>• LightCycler® 96 Instrument</li> </ul>

Always run appropriate negative and positive controls for each of your target parameters.

## Control Samples and No Template Controls (NTCs)

- In addition to the RNA Process Control, we highly recommend testing known negative and known positive specimens as controls in each run to check the complete procedure, including sample preparation and RT-qPCR.
- If the RNA Process Control is added manually to the sample material, we recommend having one known positive and one known negative specimen without the RNA Process Control.
- If the RNA Process Control is added automatically to all samples, we recommend performing a no template control (NTC) RT-PCR using Water, PCR grade (Vial 6) instead of specimen 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  $\mu\text{M}$  (final concentration in RT-qPCR). The recommended starting concentration is 0.5  $\mu\text{M}$  each.

### Probes

Suitable concentrations of hydrolysis probes range from 0.05 to 0.5  $\mu\text{M}$  (final concentration in PCR). The recommended starting concentration is 0.25  $\mu\text{M}$  each.

- ④ 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.
- ④ For a hydrolysis probe hybridization complex, the  $T_m$  of the hydrolysis probe has to be higher than the  $T_m$  of the primers.

## 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 either using 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 used best with the one-step RT-qPCR procedure described below.

The workflow for the MagNA Pure 96 nucleic acid purification and subsequent one-step detection is described in detail in the following chapter.

In case of 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 4 $\times$  RNA Process Control detection assay working solution can be prepared.

### 2.2.1 Isolation of viral RNA

#### A) 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.

Step	Action
1	Thaw one aliquot of the RNA Process Control, conc. (Vial 1) and one bottle of RNA Process Control Diluent (Vial 2).
2	Add 100 µl RNA Process Control Diluent (Vial 2) to the aliquot in Vial 1 (preliminary mixture).
3	Mix briefly by vortexing.
4	Take 100 µl from the preliminary mixture from Vial 1 and add to 3.9 ml RNA Process Control Diluent (Vial 2). This will serve as the RNA Process Control working solution.
5	Mix briefly by vortexing.

#### B) Example of a MagNA Pure 96 Instrument run using an Internal Control position

Step	Action
1	Prepare the MagNA Pure 96 Instrument according to the Operator's Manual.
2	Depending on the used kit, select one of the following protocols:  For large volume kit with 500 µl sample volume: <ul style="list-style-type: none"> <li>• Pathogen Universal 500</li> <li>• Viral NA Universal LV</li> <li>• Viral NA Plasma LV</li> </ul> For small volume kit with 200 µl sample volume: <ul style="list-style-type: none"> <li>• Pathogen Universal 200</li> <li>• Viral NA Universal SV</li> <li>• Viral NA Plasma SV</li> </ul>
3	Select elution volume: <ul style="list-style-type: none"> <li>• 50 µl or 100 µl</li> </ul>
4	Add the required volume of RNA Process control working solution (A) to an MagNA Pure 96 Internal Control Tube and proceed according to the MagNA Pure 96 User Training Guide (software version 2.0).

**C) Other Nucleic Acid purification methods**

- For the MagNA Pure LC, 20  $\mu\text{l}$  of the RNA Process Control working solution is added directly into the sample material shortly before starting the purification process.
- For the MagNA Pure Compact, 20  $\mu\text{l}$  of the RNA Process Control working solution is added directly into the sample material shortly before starting the purification process.
- For the High Pure Viral Nucleic Acid Kit, 20  $\mu\text{l}$  of the RNA Process Control working solution is added directly into the sample material shortly before starting the purification process.

Follow the procedure below to prepare 96, 20  $\mu\text{l}$  standard reactions with the LightCycler<sup>®</sup> Multiplex RNA Virus Master, using either the LightCycler<sup>®</sup> 480 System or the LightCycler<sup>®</sup> 96 System and the LightCycler<sup>®</sup> 480 Multiwell Plate 96, white.

Do not touch the surface of the LightCycler<sup>®</sup> 480 Multiwell Plate 96, white.

**2.2.2 Preparation of the RT-qPCR****A1) Preparation of the master mix for manual RT-qPCR setup**

- Keep all reagents on ice

Prepare master mix on ice as shown below for 95 samples plus 1 NTC (20  $\mu\text{l}$  each, 10  $\mu\text{l}$  amplifications included in brackets to be used for 384 well plate set-ups).

Vial	Component	Reagent Concentration	Master Mix for RT-qPCR	
			1 Reaction	100 Reactions
3	RT-Enzyme Solution	200 $\times$	0.1 $\mu\text{l}$ (0.05 $\mu\text{l}$ )	10 $\mu\text{l}$ (5 $\mu\text{l}$ )
4	RT-qPCR Reaction Mix	5 $\times$	4 $\mu\text{l}$ (2 $\mu\text{l}$ )	400 $\mu\text{l}$ (200 $\mu\text{l}$ )
5	RNA Process Control Detection Assay	20 $\times$	1 $\mu\text{l}$ (0.5 $\mu\text{l}$ )	100 $\mu\text{l}$ (50 $\mu\text{l}$ )
x	Gene-Specific Primers		2 $\mu\text{l}$ (1 $\mu\text{l}$ )	200 $\mu\text{l}$ (100 $\mu\text{l}$ )
6	Water, PCR grade		7.9 $\mu\text{l}$ (3.95 $\mu\text{l}$ )	790 $\mu\text{l}$ (395 $\mu\text{l}$ )
<b>Master Mix Volume</b>			<b>15 <math>\mu\text{l}</math> (7.5 <math>\mu\text{l}</math>)</b>	<b>1500 <math>\mu\text{l}</math> (750 <math>\mu\text{l}</math>)</b>

**A2) Preparation of the master mix for automated RT-qPCR setup**Preparation of the RNA Process Control Detection Assay working solution

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

Step	Action
①	Thaw one vial of RNA Process Control Detection Assay, 20× (Vial 5) and one vial Water, PCR grade (Vial 6).
②	Add 480 µl water to the vial 5 (RNA Process Control Detection assay, 20×) from step 1.
③	Mix briefly by vortexing.
④	Add 5 µl of this 4× working solution to a 20 µl final one-step RT-qPCR reaction volume to achieve a final concentration of 1×.

- Keep all reagents on ice.

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


Vial	Component	Reagent Concentration	Master Mix for RT-qPCR 1 Reaction	100 Reactions
3	RT-Enzyme Solution	200 ×	0.1 µl	10 µl
4	RT-qPCR Reaction Mix	5 ×	4 µl	400 µl
5	Detection Assay Working Solution (4×)	4 ×	5 µl	500 µl
6	Water, PCR grade		4.9 µl	490 µl
	<b>Master Mix Volume</b>		<b>15 µl</b>	<b>1500 µl</b>

## B) Setting up the RT-qPCR reaction

Step	Action
①	Place your samples on ice. Prepare the RT-qPCR master mix, as described in the pipetting protocol above (A1 or A2), and place on ice.
②	<p>Dispense 15 <math>\mu</math>l of the reaction mixture RT-qPCR Mastermix from <b>A1</b> or <b>A2</b> and the sample eluates to the respective wells of the precooled LightCycler<sup>®</sup> 480 Multiwell Plate 96. Always start with the negative control, followed by the prepared specimen. Positive controls are pipetted last.</p> <ul style="list-style-type: none"> <li>• Add 5 <math>\mu</math>l Water, PCR grade (Vial 6) into the NTC position.</li> <li>• Add 5 <math>\mu</math>l sample eluate to all sample positions.</li> </ul>
③	Seal the LightCycler <sup>®</sup> 480 Multiwell Plate 96, white, with a LightCycler <sup>®</sup> 480 Sealing Foil.
④	Place the multiwell plate in a standard swing-bucket centrifuge containing a rotor for multiwell plates with suitable adapters. Balance it with a suitable counterweight (e.g., another multiwell plate), and centrifuge for 2 minutes at 1,500 $\times$ g.
⑤	Proceed immediately with the RT-qPCR. Avoid leaving the plate at room temperature (+15 to +25 $^{\circ}$ ).

### 2.2.3 LightCycler<sup>®</sup> 480 Instrument II Protocol

The RNA Process Control can be used with the LightCycler<sup>®</sup> 480 Instrument II, 96 well or 384 well, as well as with the LightCycler<sup>®</sup> 96 Instrument. The following procedure is optimized for use with the corresponding LightCycler<sup>®</sup> System you are using.

 Program the LightCycler<sup>®</sup> Instrument before preparing the reaction mixes. A LightCycler<sup>®</sup> Instrument protocol that uses the LightCycler<sup>®</sup> Multiplex RNA Virus Master contains the following programs:

- **Reverse Transcription** of viral template RNA
- **Denaturation**: of cDNA/RNA hybrid
- **Amplification** of the cDNA
- **Cooling** of the thermal block

For details on how to program the experimental protocol, see the current LightCycler<sup>®</sup> 480 Instrument Operator's Manual or the LightCycler<sup>®</sup> 96 System Operator's Guide.

### 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, Yellow555, or Red610 channel), and the RNA Process Control (Cy5).
- Triple-color measurement and simultaneous analysis of multiple parameters (e.g., FAM and Yellow555 or FAM and Red610) 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. Use the following filter combination:

#### Example of a (multi) 3-color Hydrolysis Probes filter combination:

Detection Formats	Excitation Filter	Emission Filter
FAM	465	510
Yellow555 or Red610	533	580  610
Cy5	618	660

For the new customized detection format, set for all selected filters in the "Selected Filter Combination List", the following values:

Melting Factor	1
Quantification Factor	10
Integration Time	2

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

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

<b>Setup</b>					
<b>Detection Format</b>		<b>Reaction Volume</b>	<b>Block Type</b>		
For example: Detection format 3 color Hydrolysis probe [FAM/Yellow555/Cy5]		20 µl (10 µl)	96 (384)		
<b>Programs</b>					
<b>Program Name</b>		<b>Cycles</b>	<b>Analysis Mode</b>		
Reverse Transcription		1	None		
Initial Denaturation		1	None		
Amplification		45 <sup>2)</sup>	Quantification		
Cooling		1	None		
<b>Temperature Targets</b>					
<b>Target [°C]</b>	<b>Acquisition Mode</b>	<b>Hold [hh:mm:ss]</b>	<b>Ramp Rate [°C/s]</b>	<b>Acquisitions [n/°C]</b>	
<b>Reverse Transcription</b>					
50 <sup>3)</sup>	None	00:10:00 <sup>4)</sup>	4.4 (4.8)	-	
<b>Initial Denaturation</b>					
95	None	00:00:30	4.4 (4.8)	-	
<b>Amplification</b>					
95	None	00:00:05	4.4 (4.8)	-	
60 <sup>5)</sup>	Single	00:00:30	2.2 (2.5)	-	
<b>Cooling</b>					
40	None	00:00:30	2.2 (2.5)	-	

<sup>1)</sup> 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) 45 cycles are 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 T<sub>m</sub> of the primers and probe indicate using different settings, select an annealing temperature +5°C below the calculated primer T<sub>m</sub> for initial experiments. For assay optimization, choose the highest annealing temperature which still results in a high yield of amplicon with a low C<sub>q</sub> 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 program:

- **Reverse Transcription** of viral template RNA
- **Initial Denaturation** of cDNA/RNA hybrid
- **Amplification** of the cDNA
- **Temperature Gradient Step** to create the Color Compensation file
- **Cooling** of the thermal block

For details on how to program the experimental protocol, see the LightCycler® 480 Software Operator's Manual, version 1.5.

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.

<b>Setup</b>			
<b>Detection Format</b>		<b>Block Type</b>	
Customized (see section above)		96	
<b>Programs</b>			
<b>Program Name</b>	<b>Cycles</b>	<b>Analysis Mode</b>	
Reverse Transcription	1	None	
Initial Denaturation	1	None	
Amplification	45	Quantification	
Temperature Gradient Step	1	Color Compensation	
Cooling	1	None	
<b>Temperature Targets</b>			
<b>Target [°C]</b>	<b>Acquisition Mode</b>	<b>Hold [hh:mm:ss]</b>	<b>Ramp Rate [°C/s]</b>
<b>Reverse Transcription</b>			
50	None	00:10:00	4.4
<b>Initial Denaturation</b>			
95	None	00:00:30	4.4
<b>Amplification</b>			
95	None	00:00:05	4.4
60	Single	00:00:30	2.2
<b>Temperature Gradient Step</b>			
95	None	00:00:10	4.4
40	None	00:00:10	2.2
95	Continuous	-	5 Acq./°C
<b>Cooling</b>			
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 "Volume" column 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  $\mu\text{l}$  when adding the individual reagents.

<b>Component</b>	<b>1 × Buffer</b>	<b>1 × for each dye</b>	<b>1 × RNA Process Control</b>
RT-Enzyme Solution 200× (vial 3)	0.1 $\mu\text{l}$	0.1 $\mu\text{l}$	0.1 $\mu\text{l}$
RT-qPCR Reaction Mix, 5× (vial 4)	4.0 $\mu\text{l}$	4.0 $\mu\text{l}$	4.0 $\mu\text{l}$
RNA Process Control Detection Assay, 20× (vial 5)	-	-	1 $\mu\text{l}$
Detection mix for each dye (one dye per well)	-	X $\mu\text{l}$	-
Water, PCR grade (vial 6)	15.9 $\mu\text{l}$	Y $\mu\text{l}$	9.9 $\mu\text{l}$
Template, such as viral RNA or positive samples eluates	-	5 $\mu\text{l}$	-
RNA Process Control eluate	-	-	5.0 $\mu\text{l}$
<b>Total Volume</b>	<b>20 <math>\mu\text{l}</math></b>	<b>20 <math>\mu\text{l}</math></b>	<b>20 <math>\mu\text{l}</math></b>

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Step	Action
①	Pipette the replicates of each different calibrator mix into a precooled LightCycler® 480 Multiwell Plate 96.
②	Seal the LightCycler® 480 Multiwell Plate using a sealing foil.
③	Place the multiwell plate in a standard swing-bucket centrifuge containing a rotor for multiwell plates with suitable adaptors. Balance it with a suitable counterweight ( <i>e.g.</i> , another plate), and centrifuge for 2 minutes at 1,500 × g.
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, Y555, Red610, 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, Yellow555, Red610, and Cy5 on the same instrument.

## 2.2.4 LightCycler® 96 Instrument Protocol<sup>1)</sup>

The following procedure is optimized for use with the LightCycler® 96 System. Program the LightCycler® 96 Instrument before preparing the reaction mixes. For details on how to program the experimental protocol, see the LightCycler® 96 Operator's Manual.

<b>Run Editor</b>			
<b>Detection Format</b>		<b>Reaction Volume</b>	
Select Cy5 in Channel 4 and any other Dye used in your assays		20 µl	
<b>Programs</b>			
<b>Temp [°C]</b>	<b>Ramp Rate [°C/s]</b>	<b>Hold [s]</b>	<b>Acquisition Mode</b>
<b>Preincubation (Reverse Transcription)</b>			
50 <sup>2)</sup>	4.4	600 <sup>3)</sup>	None
<b>Preincubation (Initial Denaturation)</b>			
95	4.4	30	None
<b>2-Step Amplification</b>			
No. of Cycles: 45 <sup>4)</sup>			
95	4.4	5	None
60 <sup>5)</sup>	2.2	30	Single

<sup>1)</sup> 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.

<sup>2)</sup> We recommend running the reverse transcription at +50°C. If necessary, it is also possible to run it at +55°C.

<sup>3)</sup> 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.

<sup>4)</sup> 45 cycles are 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.

<sup>5)</sup> Most available assays are designed for an annealing temperature of +60°C. If the T<sub>m</sub> of the primers and probe indicate using different settings, select an annealing temperature +5°C below the calculated primer T<sub>m</sub> for initial experiments. For assay optimization, choose the highest annealing temperature which still results in a high yield of amplicon with a low C<sub>q</sub> 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.

#### **2.3 Quality Control**

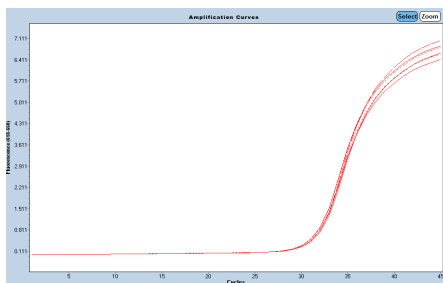
Each component of the three RNA Process Control Kits is function tested using the MagNA Pure 96 Instrument for the nucleic acid purification and the LightCycler480® Instrument II for one-step RT-qPCR.

### 3. Results

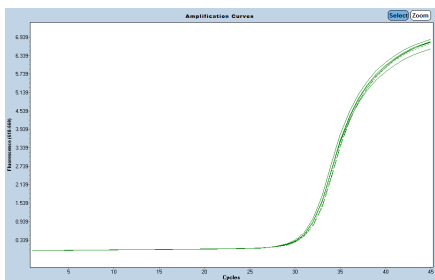
#### Stability in sample material

The RNA Process Control Kit was used according to the protocols described above. 20  $\mu\text{l}$  of the RNA process control working solution was added to 200  $\mu\text{l}$  serum and a PBS based mouth rinse solution. The samples were either incubated at room temperature for 2 or 18 h or directly subjected to a MagNA Pure 96 purification (MagNA Pure 96 DNA and Viral NA Small Volume Kit using the Pathogen Universal 200 protocol with 100  $\mu\text{l}$  eluate). 5  $\mu\text{l}$  of the eluates were used in a 20  $\mu\text{l}$  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 h 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 h incubation at room temperature. Monoplex RT-qPCR was performed in a 20  $\mu\text{l}$  reaction volume using the LightCycler<sup>®</sup> Multiplex RNA Virus Master on a LightCycler<sup>®</sup> 480 System II (0 h = solid line, 2 h dashed line, 18 h dotted line).

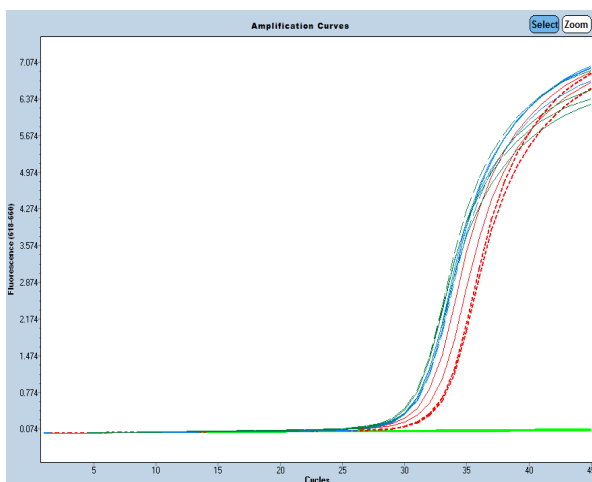


**Fig. 2: Amplification curves of the RNA Process Control assay.** The RNA Process Control was added to mouth rinse solution samples and processed after 0, 2, or 18 h incubation at room temperature. Monoplex RT-qPCR was performed in a 20  $\mu\text{l}$  reaction volume using the LightCycler<sup>®</sup> Multiplex RNA Virus Master on a LightCycler<sup>®</sup> 480 System II (0 h = solid line, 2 h dashed line, 18 h dotted line).

## Usage with different sample materials

The RNA Process Control Kit was used in a panel of 6 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  $\mu$ l 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.
- The 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 & protocol, detection instrument, multiplexing parameters, experimenter, etc.). In general, expect the Cq value to vary in a range of  $\sim \pm 2$ -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  $\sim 30$  - 35 (100  $\mu$ l eluate and 5  $\mu$ l eluate in a 20  $\mu$ l RT-qPCR detection reaction with the LightCycler<sup>®</sup> Multiplex RNA Virus Master on a LightCycler<sup>®</sup> 480 II System). For one such particular workflow, the following table shows an exemplary interpretation guideline.

Target (e.g., Channel 465-510)	RNA Process Control (Channel 618-660)	Results/Target Status
No Cq	Valid Cq ( $\sim 30$ - 35)	Valid/target negative
Cq < 40	Valid Cq or No Cq	Valid/target positive
no Cq	No valid Cq • e.g., outside of $\sim 30$ - 35 • no sig. curve	Invalid/target unknown

- 1. 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<sup>®</sup> 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 specimen 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 need to be negative. If the result is "positive", all specimen 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 need to be positive. If the result is "negative", all specimen results controlled by the corresponding positive control are invalid, and the respective RT-qPCR with the appropriate detector must be repeated.

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**Specimen Results** 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 table above (Interpretation of data).

**RNA Process Control / Internal Control** If specimen results are "negative" for a target, the simultaneous RNA Process Control measured in channel 618-660 always need to be positive to prove that there actually was specimen 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

	Possible Cause	Recommendation
<b>Fluorescence intensity varies</b>	Some of the reagent is still in the upper part of the microwell or an air bubble is trapped in microwell.	Repeat centrifugation, but allow sufficient centrifugation time so all reagent is at the bottom of the microwell and air bubbles are expelled.
	Skin oils or dirt on the surface of the microwell	Always wear gloves when handling the multiwell plate.
<b>Fluorescence intensity is very low</b>	Low concentration or deterioration of dyes in the reaction mixtures because dye was not stored properly	<ul style="list-style-type: none"> <li>• Keep dye-labeled reagents away from light.</li> <li>• Store the reagents at <math>-15</math> to <math>-25^{\circ}\text{C}</math> and avoid repeated freezing and thawing.</li> </ul>
	Poor PCR efficiency (reaction conditions not optimized)	<ul style="list-style-type: none"> <li>• Check annealing temperature of primers and probes.</li> <li>• Check experimental protocol.</li> <li>• Always run a positive control along with your samples.</li> </ul>
	RNA is degraded during isolation or improper storage.	<ul style="list-style-type: none"> <li>• If possible check RNA quality.</li> <li>• Check RNA with an established RT-qPCR primer when available.</li> </ul>
	Pipetting errors and omitted reagents	<ul style="list-style-type: none"> <li>• Check for missing reagents.</li> <li>• Check the pipetting procedure.</li> </ul>
	Impure sample material inhibits reaction.	<ul style="list-style-type: none"> <li>• Dilute sample 1:10 and repeat the analysis.</li> <li>• Repurify the nucleic acids to ensure removal of inhibitory agents.</li> </ul>
<b>Negative control sample gives a positive signal</b>	Contamination	Remake all critical reaction mixes. Use separate RT-qPCR setup working areas.

## 5. Additional Information on this Product

**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<sup>1)</sup> that can be added to a large variety of sample materials without impairing the purification of other sample intrinsic nucleic acid<sup>2)</sup>, such as from RNA viruses. The RNA Process Control Kit contains reagents for  $\geq 6 \times 96$  MagNA Pure 96 sample purifications and 600 reactions using the LightCycler<sup>®</sup> Multiplex RNA Virus Master to detect the RNA and any possible RNA or DNA targets in the eluates.

**Test principle** 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 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.
  - a. The RNA Process Control detection assay specifically detects the internal control.
  - b. 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.

<sup>1)</sup> 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.

<sup>2)</sup> 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, Y555, HEX, or Red610 can be combined in multiplex setups.

## 6. Supplementary Information

### 6.1 Conventions



#### 6.1.1 Text Conventions

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

Text Convention	Usage
Numbered instructions labeled <b>1</b> , <b>2</b> etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Diagnostics.

#### 6.1.2 Symbols

In this document, the following symbols are used to highlight important information:

Symbol	Description
	Information Note: Additional information about the current topic or procedure.
	Important Note: Information critical to the success of the procedure or use of the product.

### 6.2 Changes to Previous Version

Editorial Changes.

### 6.3 Ordering Information

Roche offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page, [www.lifescience.roche.com](http://www.lifescience.roche.com), and our Special Interest Sites for:

- LightCycler<sup>®</sup> 480 System: [www.lightcycler480.com](http://www.lightcycler480.com)
- LightCycler<sup>®</sup> 96 System: [www.lightcycler96.com](http://www.lightcycler96.com)
- Automated Sample Preparation (MagNA Pure LC System, MagNA Pure 96 System, and MagNA Pure Compact System): [www.magnapure.com](http://www.magnapure.com)
- Manual Sample Preparation of Nucleic Acids: [www.lifescience.roche.com/shop/en/us/products/manual-sample-preparation-of-nucleic-acids](http://www.lifescience.roche.com/shop/en/us/products/manual-sample-preparation-of-nucleic-acids)

	<b>Product</b>	<b>Pack Size</b>	<b>Cat. No.</b>
<b>Instrument and Accessories</b>	LightCycler <sup>®</sup> 480 Instrument II, 96 well	1 instrument (96 well)	05 015 278 001
	LightCycler <sup>®</sup> 480 Instrument II, 384 well	1 instrument (384 well)	05 015 243 001
	LightCycler <sup>®</sup> 480 Block Kit 96 Silver	1 block kit for 96-well PCR Multiwell Plates	05 015 219 001
	LightCycler <sup>®</sup> 480 Block Kit 384 Silver	1 block kit for 384-well PCR Multiwell Plates	05 015 197 001
	LightCycler <sup>®</sup> 480 Multiwell Plate 96, white	5 × 10 plates with sealing foils	04 729 692 001
	LightCycler <sup>®</sup> 480 Multiwell Plate 384, white	5 × 10 plates with sealing foils	04 729 749 001
	LightCycler <sup>®</sup> 480 Sealing Foil	50 foils	04 729 757 001
	LightCycler <sup>®</sup> 8-Tube Strips (white)	10 x 12 white strips and clear caps	06 612 601 001
	LightCycler <sup>®</sup> 8-Tube Strip Adapter Plate		06 612 598 001
	LightCycler <sup>®</sup> 480 Software, Version 1.5	1 software package	04 994 884 001
	LightCycler <sup>®</sup> 96 Instrument	1 instrument plus accessories	05 815 916 001
	MagNA Pure 96 Instrument	1 instrument plus accessories	06 541 089 001
	MagNA Pure 96 Internal Control Tube		06 374 905 001

	<b>Product</b>	<b>Pack Size</b>	<b>Cat. No.</b>
	MagNA Pure LC 2.0 Instrument	1 instrument plus accessories	05 197 686 001
	MagNA Pure Compact Instrument	1 instrument plus accessories	03 731 146 001
<b>Associated Kits</b>	LightCycler® Multiplex RNA Virus Master Kit	200 reactions (20 µl)	06 754 155 001
<b>Viral RNA Isolation Kits</b>	MagNA Pure 96 DNA and Viral NA Small Volume Kit		06 543 588 001
	MagNA Pure 96 DNA and Viral NA Large Volume Kit		06 374 891 001
	MagNA Pure LC Total Nucleic Acid Isolation Kit - High Performance	1 kit for up to 288 isolations	05 323 738 001
	MagNA Pure Compact Nucleic Acid Isolation Kit I	1 kit for 32 isolations	03 730 964 001
	High Pure Viral Nucleic Acid Kit	1 kit for up to 100 purifications	11 858 874 001

#### 6.4 Disclaimer of License

For patent license limitations for individual products, please refer to [www.technical-support.roche.com](http://www.technical-support.roche.com).

#### 6.5 Trademarks

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#### 6.6 Regulatory Disclaimer

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