For general laboratory use.

Print Protocol | Print Changes to Previous Version | Print

LightCycler® Multiplex RNA Virus Master

Version: 09

Content version: February 2018

Easy-to-use reaction mix for one-step RT-qPCR using the LightCycler® 480, LightCycler® 96, LightCycler® Nano, or the LightCycler® 2.0 Real-Time PCR Systems

Cat. No. 06 754 155 001	1 kit 200 reactions of 20 µl final volume each
Cat. No. 07 083 173 001	1 kit 1,000 reactions of 20 µl final volume each

Store the kit at −15 to −25°C.

1. General Information

1.1. Contents

Vial / Bottle	Cap	Label	Function / Description	Catalog Number	Content
1	blue	LightCycler® Multiplex RNA Virus Master, RT Enzyme Solution, 200x conc.	Contains Reverse Transcriptase.	06 754 155 001	1 vial, 28 µl
				07 083 173 001	5 vials, 28 µl each
2	red	LightCycler® Multiplex RNA Virus Master, RT-qPCR Reaction Mix, 5x conc.	Contains RT-qPCR Reaction Buffer, AptaTaq Polymerase, dATP, dCTP, dGTP, dUTP, $MgCl_2$, and proprietary additives.	06 754 155 001	1 vial, 880 µl
				07 083 173 001	5 vials, 880 µl each
3	colorless	LightCycler® Multiplex RNA Virus Master, Water, PCR Grade	To adjust the final reaction volume.	06 754 155 001	3 vials, 1 ml each
				07 083 173 001	15 vials, 1 ml each

1.2. Storage and Stability

Storage Conditions (Product)

The kit is shipped on dry ice.
When stored at −15 to −25°C, the kit is stable through the expiration date printed on the label. The kit is stable at +2 to +8°C for 4 weeks.

Vial / Bottle	Cap	Label	Storage
1	blue	RT Enzyme Solution, 200x conc.	Store at −15 to −25°C or store at +2 to +8°C for a maximum of 4 weeks. ⚠ ***Close lid immediately after use.***
2	red	RT-qPCR Reaction Mix, 5x conc.	Store at −15 to −25°C. ⚠ ***Avoid repeated freezing and thawing (more than 5 times).*** Aliquot Vial 2 and freeze or store Vial 2 at +2 to +8°C for a maximum of 4 weeks.
3	colorless	Water, PCR Grade	Store at −15 to −25°C or store at +2 to +8°C for a maximum of 4 weeks.

Storage Conditions (Working Solution)

Although we recommend working on ice and preparing the reagents right before use, the working solution, see **Setup of the RT-qPCR Reaction**, 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 Reagents 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.
  Wear gloves at all times.

For RT-qPCR

- Real-Time PCR systems such as the LightCycler® 480 Instrument*, LightCycler® 96 Instrument*, LightCycler® Nano Instrument, or the LightCycler® 2.0 Instrument*
- LightCycler®480 Multiwell Plate 96* or 384*
- LightCycler® 8-Tube Strips*
- LightCycler® 8-Tube Strip Adapter Plate*
- LightCycler® Capillaries (20 µl)*
- Standard swinging-bucket centrifuge with rotor for multiwell plates
- Real-Time PCR systems such as Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR System, Applied Biosystems 7500 Real-Time PCR System, Applied Biosystems 7900HT Fast Real-Time PCR System, Applied Biosystems StepOnePlus™Real-Time PCR System, and Bio-Rad CFX96™ Real-Time PCR Detection System

For RT-qPCR Primer and Probe Design

- Universal ProbeLibrary Assay Design Center at www.universalprobelibrary.com
- To design and order RT-qPCR assays and panels: www.realtimeready.roche.com

Optional For Virus 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*

1.4. Application

The LightCycler® Multiplex RNA Virus Master is designed for fast, highly sensitive and specific real-time one-step RT-qPCR analysis of viral RNA.
The 2-vial composition (separate RT enzyme and qPCR Mix) is ideally suited for use with RT-minus controls. The proprietary reaction buffer allows a fast and convenient hot start RT-qPCR without pre-activation of the Taq DNA Polymerase. The kit is optimized for hydrolysis probes, as well as Universal ProbeLibrary (UPL) probes and does not require optimization with $MgCl_2$.

1.5. Preparation Time

Typical run time

The LightCycler® Multiplex RNA Virus Master can be used for multiplex RT-qPCR protocols. For example, a triplex protocol using 45 cycles including a 10-minute reverse transcription step requires 65 minutes using the LightCycler® 480 System or 67 minutes using the LightCycler® 96 System.

2. How to Use this Product

2.1. Before you Begin

Sample Materials

Use any viral template RNA suitable for RT-qPCR in terms of purity, concentration, and absence of RT-PCR inhibitors. For reproducible isolation of nucleic acids, we recommend:
- 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).

Control Reactions

Negative Control and RT Minus Control Reactions

Always run a negative control with the samples. To prepare a negative control, replace the template RNA with Water, PCR Grade water (Vial 3). A contamination problem can be observed using the negative control.
For the RT-minus control, omit the RT enzyme in the mix. With the RT-minus control, you can verify whether your signal comes from the RNA target or from DNA contamination.

Primers

Suitable concentrations of PCR primers 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 PCR). 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.

Mg^{2+} Concentration

The master mix of this kit is optimized with a fixed concentration of $MgCl_2$, which works with nearly all primer combinations. There is no need for adjustment.

General Considerations

Precautions

Always 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 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 siliconized reaction 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.

2.2. Protocols

LightCycler® 480, LightCycler® 96, LightCycler® 2.0, or LightCycler® Nano System Protocol

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 current LightCycler® 480 Instrument Operator's Manual, the LightCycler® 96 System Operator's Guide, LightCycler®2.0 Instrument Operator's Manual B, or the LightCycler® Nano System Operator's Guide.

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

- **Reverse Transcription** of the viral template RNA
- **Pre-Incubation** for activation of DNA polymerase and denaturation of the cDNA/RNA hybrid
- **Amplification** of the cDNA
- **Cooling** of the thermal bloc

Protocol for Use with the LightCycler® 480 Instrument II (Multiwell Plate 96 or 384)[1]

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

Setup					
Block Type			Reaction Volume [µl]		
96 (384)			20 (10)		
Detection Format		Excitation Filter		Emission Filter	
For example: Mono Color Hydrolysis Probe / UPL Probe					
FAM		465		510	
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 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® 480 Instrument II

For a multicolor measurement, the application of a color compensation file is necessary to compensate for optical crosstalk between the different channels. For the LightCycler® 480 Instrument II, an instrument-specific color compensation file is mandatory for multicolor experiments, and a color compensation object can be generated as shown below.

The LightCycler® 480 Instrument II protocol contains the following programs:

- **Reverse Transcription** of the viral template RNA
- **Pre-Incubation** for activation of DNA polymerase and denaturation of the 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.

Programming a Customized Detection Format for the LightCycler® 480 System Filter Combination Selection

The Detection Format in the LightCycler® 480 Software, Version 1.5 setup must be customized for the applied multicolor hydrolysis probe format used in the RT-qPCR detection. In the **Tool** module, the **Detection Formats** option allows creating a new detection format specified by the user, including a **Detection Format** list, a **Filter Combination** selection area, and a **Selected Filter Combination List**. Different filter settings for the LightCycler® 480 II Instrument are defined.

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

Setup					
Block Type			Reaction Volume [µl]		
96			20		
Detection Format	Excitation Filter		Emission Filter		
For example: 3 Color Hydrolysis Probe					
FAM Red 610 Cy 5	465 533 618		510 610 660		
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		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 volume column by the number of reactions you need (minimum of 3 to 5 replicates) plus additional reactions since there will be a slight loss of liquid during the pipetting steps. In order to ensure accuracy, do not pipette volumes less than 1 µl. For each dye, set up the following reactions:

Reagent	1x Buffer [µl]	1x for each Dye [µl]
RT Enzyme Solution, 200x conc. (Vial 1)	0.1	0.1
RT-qPCR Reaction Mix, 5x conc. (Vial 2)	4.0	4.0
Detection mix for each dye	–	X (depending on the assay)
Water, PCR Grade (Vial 3)	15.9	Y (depending on the assay)
Template, such as viral RNA or positive samples eluates	–	5.0
Total Volume	**20.0**	**20.0**

1. Pipette the three replicates of each different calibrator mix into a precooled LightCycler® 480 Multiwell Plate 96.
2. Seal the LightCycler® 480 Multiwell Plate with a LightCycler® 480 Sealing Foil.
3. Place the Multiwell Plate in a standard swinging-bucket centrifuge containing a rotor for multiwell plates with suitable adaptors. Balance it with a suitable counterweight (*e.g.,* another plate), and centrifuge for 2 minutes at 1,500 × *g*.
4. Load the Multiwell Plate into the LightCycler® 480 Instrument 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 respectively (*e.g.,* FAM, Red 610, Cy5 for the example mentioned above).
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 multicolor experiment performed with FAM, Red 610, and Cy5 on the same instrument.

Protocol for use with the LightCycler® 96 Instrument[1]

Run Editor				
Detection Format			Reaction Volume [µl]	
For example: Dyes 1: FAM			20	
Programs				
	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 aptamer 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.

Protocol for Use with the LightCycler® Nano Instrument[1]

Run Settings				
Optics Settings			Reaction Volume [µl]	
For example: Hydrolysis Probes Normal Quality			20	
Profile/Programs				
	Temp. [°C]	Ramp [°C/s]	Hold [s]	Acquire
Reverse Transcription	50[2]	5	600[3]	–
Pre-Incubation	95	5	30	–
2-Step Amplification	No. of Cycles: 45[4]			
	95	5	10[5]	–
	60[6]	4	30	Yes

[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] 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] Note that for the LightCycler® Nano Instrument, it is not possible to program hold times shorter than 10 seconds. Program 10 seconds instead of 5 seconds for LightCycler® Nano Instruments.

[6] 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® Nano Instrument

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

Setup of the RT-qPCR Reaction for the LightCycler® 480, LightCycler® 96, and LightCycler® Nano Instruments

Follow the procedure below to prepare at least ten 20 µl standard reactions:
Do not touch the surface of the LightCycler® 480 Multiwell Plate or the LightCycler® 8-Tube Strips.

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.
   – Place samples on ice.

2. Prepare a 20x conc. solution of your primers and a 20x conc. solution of your probes.

3. In a 1.5 ml reaction tube, prepare the RT-qPCR Mix and put on ice.
   – For best results, prepare at least 10 reactions in order to reduce pipetting errors. To prepare more reactions, multiply the amount in the "Volume 1 Reaction" column below by the number of reactions to be run, plus at least one additional reaction.

Reagent[1]	Volume 1 Reaction [µl]		Volume 10 Reactions [µl]		Final conc.
	96-well plate	384-well plate	96-well plate	384-well plate	
Water, PCR Grade (Vial 3)	8.9	4.45	89.0	44.5	–
RT-qPCR Reaction Mix, 5x conc. (Vial 2)	4.0	2.00	40.0	20.0	1x
Primer Mix, 20x conc.[2]	1.0	0.50	10.0	5.0	1x
Probe Mix, 20x conc.	1.0	0.50	10.0	5.0	1x
RT Enzyme Solution, 200x conc. (Vial 1)	0.1	0.05	1.0	0.5	1x
Total Volume	**15.0**	**7.50**	**150.0**	**75.0**	

[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 Grade Water volume.
[2] Due to possible primer/primer interactions that occur during storage, it may be necessary to preheat the PCR primer mix for 1 minute at +95°C before pipetting the full mixture. This extra step will ensure optimum sensitivity.

4. Mix carefully by pipetting up and down or vortex briefly.
   – Place on ice.
   – Although we recommend working on ice and preparing the reagents right before use, the 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.

5. Prepare sample concentration of the viral RNA and/or DNA.

6. Pipette 15 µl (7.5 µl) RT-qPCR Mix into a precooled multiwell plate or precooled LightCycler® 8-Tube Strip.
   – Add 5 µl (2.5 µl) of the RNA and/or DNA template.
   – Seal multiwell plate with a LightCycler® 480 Sealing Foil or seal the LightCycler® 8-Tube Strips using the corresponding lid.

7. Place the Multiwell Plate 96 into a standard swinging-bucket centrifuge with a suitable adapter and balance it with a suitable counterweight (*e.g.,* another multiwell plate), or place the 8-Tube Strips into a standard Multiwell Plate 96 and balance them in the centrifuge.
   – Centrifuge at 1,500 × *g* for 0.5 to 2 minutes.

8 Load the reaction vessels into the LightCycler® 480, LightCycler® 96, or LightCycler® Nano Instrument.

9 Start the PCR program described above.
– If you use reaction volumes other than 20 µl, be sure to adapt the right volume in the running protocol. To start, we recommend using the same hold times as for the 20 µl volume.

Protocol for Use with the LightCycler® 2.0 Instrument

A LightCycler® 2.0 Instrument protocol that uses the LightCycler® Multiplex RNA Virus Master contains the following programs:

- **Reverse Transcription** of the viral template RNA
- **Pre-Incubation** for activation of DNA polymerase and denaturation of the cDNA/RNA hybrid
- **Amplification** of the cDNA
- **Cooling** the rotor and thermal chamber

The following table shows the RT-qPCR parameters that must be programmed for a LightCycler® 2.0 Instrument RT-qPCR run with the LightCycler® Multiplex RNA Virus Master using the LightCycler® Capillaries (20 µl).

LightCycler® Software Version 4.1				
Programs				
Setup[1]	Setting			
Default Channel	Fluorescence Channel			
Seek Temperature	30°C			
Max Seek Pos.	Enter the total number of sample positions for which the instrument should look.			
Instrument Type	“6 Ch.” for LightCycler® 2.0 Instrument			
Capillary Size	Select “20 µl” as the capillary size for the experiment.			
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]	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisition Mode [per °C]
Reverse Transcription	50[3]	00:10:00[4]	20	None
Pre-Incubation	95	00:00:30	20	None
Amplification	95	00:00:05	20	None
	60[5]	00:00:30	20	Single
Cooling	40	00:00:30	20	None

[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® 2.0 Instrument

The following procedure is optimized for use with the LightCycler® 2.0 System. Program the LightCycler® 2.0 Instrument before preparing the reaction mixes. A LightCycler® 2.0 Instrument color compensation protocol that uses LightCycler® Multiplex RNA Virus Master contains the following programs:

- **Reverse Transcription** of the viral template RNA
- **Pre-Incubation** for activation of DNA polymerase and denaturation of the cDNA/RNA hybrid
- **Amplification** of the cDNA
- **Temperature Gradient Step** for Color Compensation
- **Cooling** of the cDNA

For details on how to program the experimental protocol, see the LightCycler® 2.0 Instrument Operator's Manual B.

Color Compensation Protocol

The performance of a color compensation is a prerequisite for running a dual-color experiment. The generated color compensation file is used to compensate for crosstalk between the individual detection channels when performing multi-color experiments. A color compensation calibration run is performed by running a blank capillary (containing Water, PCR Grade), and individual capillaries with one dye each (monocolor PCR reactions), in a RT-qPCR program, followed by a color compensation analysis.

The following tables show the parameters that must be programmed for a LightCycler® Instrument Color Compensation calibration run with the LightCycler® Multiplex RNA Virus Master.

LightCycler® Software Version 4.1				
Programs				
Setup	Setting			
Default Channel	Fluorescence Channel			
Seek Temperature	30°C			
Max Seek Pos.	Enter the total number of sample positions for which the instrument should look.			
Instrument Type	"6 Ch." for LightCycler® 2.0 Instrument			
Capillary Size	Select "20 µl" as the capillary size for the experiment.			
Programs				
Program Name	Cycles	Analysis Mode		
Reverse Transcription	1	None		
Pre-Incubation	1	None		
Amplification	45	Quantification		
Temperature Gradient	1	Color Compensation		
Cooling	1	None		
Temperature Targets				
	Target [°C]	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisition Mode [per °C]
Reverse Transcription	50	00:10:00	20	None
Pre-Incubation	95	00:00:05	20	None
Amplification	95	00:00:01	20	None
	60	00:00:15	20	Single
Temperature Gradient	95	00:00:01	20	None
	40	00:01:00	20	None
	95	00:00:00	0.2	Continuous
Cooling	40	00:00:30	20	None

Preparation of the Color Compensation Mixes

⚠ ***Do not touch the surface of the LightCycler® Capillaries.***

1. Place three LightCycler® Capillaries (20 µl) into precooled LightCycler® Centrifuge Adapters.

2. Prepare the capillaries (20 µl, each), as shown in Step 3.

3. For each dye, set up the following reactions:

Reagent	Volume for each Dye [µl]	Capillary with Water [µl]
RT Enzyme Solution, 200x conc. (Vial 1)	0.1	–
RT-qPCR Reaction Mix, 5x conc. (Vial 2)	4.0	–
Detection mix for each dye	X (depending on the assay)	–
Water, PCR Grade (Vial 3)	Y (depending on the assay)	20.0
Bovine Serum Albumin (20 µg/µl)[(1)]	0.2	–
Template, such as viral RNA or positive samples eluates	5.0	–
Total Volume	**20.0**	**20.0**

[(1)] Molecular biology-grade Bovine Serum Albumin is recommended.

4. Seal each capillary with a stopper using the LightCycler® Capping Tool.

5. Place the centrifuge adapters (containing the capillaries) into a standard benchtop microcentrifuge.
   – Place the centrifuge adapters in a balanced arrangement within the centrifuge.
   – Centrifuge at 700 × *g* for 5 seconds (3,000 rpm in a standard benchtop microcentrifuge). Alternatively, use the LC Carousel Centrifuge for spinning the capillaries.

6. Place the capillaries in the following order in the sample carousel of the LightCycler® 2.0 Instrument:
   Carousel rotor position 1: Water
   Carousel rotor position 2: Monocolor PCR for Dye 1
   Carousel rotor position 3: Monocolor PCR for Dye 2

7. Cycle the samples as described above and edit the dominant channel in the “Analysis Type” - “Color Comp” accordingly.

Create Color Compensation Objects

When the experiment is finished, click on the **Analysis** button and select **Color Compensation** (Other Methods) from the **Analysis** Menu. Save the experiment by clicking the **Save CC Object** button. Place the object in the “Special Data\C” folder under your user name.
After doing this, you can apply the specific “CC Object” you created to any dual-color hydrolysis probe experiment that is performed with the same dye combination.

Setup of the RT-qPCR Reaction for the LightCycler® 2.0 Instrument

This setup can also be used in a RT-qPCR protocol for the LightCycler® 1.x Instrument.
Follow the procedure below to prepare at least ten 20 µl standard reactions:

⚠ ***Do not touch the surface of the LightCycler® Capillaries.***

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.
   – Place samples on ice.

2. Prepare a 20x conc. solution of your primers and a 20x conc. solution of your probes.

3. In a 1.5 ml reaction tube, prepare the RT-qPCR Mix and put on ice.
   – For best results, prepare at least 10 reactions in order to reduce pipetting errors. To prepare more reactions, multiply the amount in the "Volume 1 Reaction" column below by the number of reactions to be run, plus at least one additional reaction.

Reagent	Volume 1 Reaction [µl]	Volume 10 Reactions [µl]	Final conc.
Water, PCR Grade (Vial 3)	8.7	87.0	–
RT-qPCR Reaction Mix, 5x conc. (Vial 2)	4.0	40.0	1x
Primer Mix, 20x conc.[1]	1.0	10.0	1x
Probe Mix 20x conc.	1.0	10.0	1x
RT Enzyme Solution, 200x conc. (Vial 1)	0.1	1.0	1x
Bovine Serum Albumin (20 mg/ml)[2]	0.2	2.0	0.2 µg/µl
Total Volume	**15.0**	**150.0**	

[1] Due to possible primer/primer interactions that occur during storage, it may be necessary to preheat the PCR primer mix for 1 minute at +95°C before pipetting the full mixture. This extra step will ensure optimum sensitivity.
[2] Molecular biology-grade Bovine Serum Albumin is recommended.

4. Mix carefully by pipetting up and down or vortex briefly.
   – Place on ice.
   – Although we recommend working on ice and preparing the reagents right before use, the 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.

5. Prepare sample concentration of the viral RNA and/or DNA.

6. Pipette 15 µl RT-qPCR Mix into a LightCycler® Capillary.
   – Add 5 µl of the RNA and/or DNA template.

7. Seal the LightCycler® Capillaries with a stopper using the LightCycler® Capping Tool.

8. If you are using the LC Carousel Centrifuge 2.0, proceed to Step 10.

9 Alternatively, place the capillaries in cooled adapters in a standard benchtop microcentrifuge in a balanced arrangement.
– Centrifuge at 700 × *g* (3,000 rpm) for 5 seconds, and transfer the capillaries to the LightCycler® Sample Carousel.

10 Place the LightCycler® Sample Carousel in the LightCycler® Carousel-Based Instrument.

11 Start the RT-qPCR program.

3. Results

The following results were obtained using the LightCycler® Multiplex RNA Virus Master on the LightCycler® 480 Instrument. A duplex reaction using primers and UPL probes specific for G6PD (FAM) and β2M (Yellow 555) was run. Human total RNA served as the template. Total RNA from different tissues in a dilution series [50 ng, 5 ng, 500 pg, 50 pg, and 10 pg and a no template control (NTC)] was analyzed using real-time PCR.

FAM Channel (465 – 510)

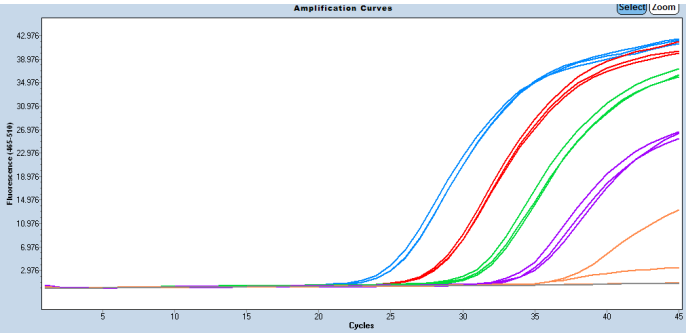

Fig. 1: The FAM channel shows the results for G6PD. Amplification curves shown were obtained from dilutions of 50 ng (far left,blue), 5 ng (red), 500 pg (green), 50 pg (purple), and 10 pg (far right, orange) human total RNA per well, including a no template control (flat line, grey). Duplex RT-qPCR was performed in a reaction volume of 20 µl per well in a LightCycler® 480 Multiwell Plate 96.

Yellow 555 Channel (533 – 580)

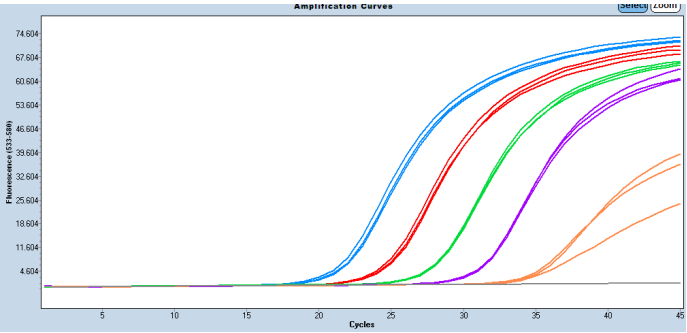

Fig. 2: The Yellow 555 channel shows the results for β2M. Amplification curves shown were obtained from dilutions of 50 ng (far left, blue), 5 ng (red), 500 pg (green), 50 pg (purple), and 10 pg (far right, orange) human total RNA per well, including a no template control (flat line, grey). Duplex RT-qPCR was performed in a reaction volume of 20 µl per well in a LightCycler® 480 Multiwell Plate 96.

4. Troubleshooting

Observation	Possible cause	Recommendation
Increase specificity.		Some assays show higher specificity when using a higher reverse transcription temperature and/or higher annealing temperature.
Fluorescence intensity varies.	Some of the reagent is still in the upper part of the microwell/8-tube strip, or an air bubble is trapped in the microwell/8-tube strip.	Repeat centrifugation, but allow sufficient centrifugation time so all reagent is at the bottom of the microwell/8-tube strip and air bubbles are expelled.
	Skin oils or dirt on the surface of the microwell/8-tube strip.	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 away from light.
		Store the reagents at −15 to −25°C and avoid repeated freezing and thawing.
	Poor PCR efficiency (reaction conditions not optimized).	Primer concentration should be in the range of 0.2 to 0.5 µM; probe concentration should be in the range of 0.2 to 0.5 µM and half of the primer concentration.
		Check annealing temperature of primers and probes.
		Check experimental protocol.
		Optimize annealing temperature in the reverse transcription step or in the PCR reaction.
		Always run a positive control along with your samples.
	Chosen imaging time is too low.	Choose the appropriate detection format in combination with “dynamic” detection mode, or
		Increase imaging time when using “manual” detection mode. *For details, see the LightCycler® 480 Software Instrument Operator’s Manual.*
	RT-qPCR primers and probes are not optimized.	Check sequence and location of the hydrolysis probe on the PCR product.
		Check RT-qPCR product on an agarose gel.
	PCR has not been optimized.	Check primer design (quality).
		Check RT-qPCR product on an agarose gel.
	RNA is degraded during isolation or improper storage.	If possible, check RNA quality on a gel.
		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. Please use special RT-qPCR setup working areas.

5. Additional Information on this Product

5.1. Test Principle

How this Product Works

The LightCycler® Multiplex RNA Virus Master consists of 3 different vials:
- Vial 1: RT Enzyme Solution (Reverse Transcriptase)
- Vial 2: RT-qPCR Reaction Mix
- Vial 3: Water, PCR Grade

The separate vial of Reverse Transcriptase makes it possible to prepare a RT-qPCR reaction mix for running RT-minus controls (lacking reverse transcriptase). This is important for verifying whether the obtained results are derived from RNA transcripts or from residual (contaminating) genomic DNA. The kit also provides sufficient vials of Water, PCR Grade to ensure that fresh (unopened) vials can be used. This minimizes the risk of contamination of RT-qPCR reaction mixes by RNases and other substances.
The Reverse Transcriptase provided in this kit is a recombinant reverse transcriptase with higher stability than native reverse transcriptase. This feature allows for higher reverse transcription temperatures of up to +55°C. This Roche recombinant Reverse Transcriptase also has lower affinity for DNA than other commonly used reverse transcriptases. The resulting RT-qPCR reaction mix also has AptaTaq DNA Polymerase, nucleotides, and additives, ensuring a hot start amplification system with high specificity. The mix contains an optimized concentration of $MgCl_2$, eliminating the need for additional adjustments. For greater convenience, the RT-qPCR mixture, including Reverse Transcriptase, can be used for both RNA and/or DNA templates in parallel.

5.2. Quality Control

Each lot of the LightCycler® Multiplex RNA Virus Master is tested to meet specifications of the RT-qPCR using a duplex RT-qPCR assay on the LightCycler® 480 Instrument II.

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.
❶ ❷ ❸ 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

The typing error [Target °C] on page 14 in the color compensation protocol for the reverse transcription has been corrected.
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 homepage lifescience.roche.com.

Product	Pack Size	Cat. No.
Accessories general (hardware)		
LightCycler® Centrifuge Adapters	1 set	11 909 312 001
LightCycler® Capping Tool	1 capping tool	03 357 317 001
LightCycler® 480 Block Kit 96 Silver	1 block kit	05 015 219 001
LightCycler® 480 Block Kit 384 Silver	1 block kit	05 015 197 001
LC Carousel Centrifuge 2.0	1 centrifuge plus rotor and rotor bucket (115 V)	03 709 507 001
	1 centrifuge plus rotor and rotor bucket (230 V)	03 709 582 001
LightCycler® 8-Tube Strip Adapter Plate	1 piece, adapter plate, The adapter plate can be used multiple times	06 612 598 001
Accessories software		
LightCycler® 480 Software, Version 1.5	1 software package	04 994 884 001
Consumables		
LightCycler® Capillaries (20 µl)	5 x 96 capillaries, containing 5 boxes, each with 96 capillaries and stoppers, 5 boxes, each with 96 capillaries and stoppers	04 929 292 001
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
LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
MagNA Pure 96 Internal Control Tube		06 374 905 001
LightCycler® 8-Tube Strips (white)	10x 12 white strips and clear caps.	06 612 601 001
Instruments		
LightCycler® 2.0 Instrument	1 instrument	03 531 414 001
LightCycler® 480 Instrument II	1 instrument	05 015 278 001
	1 instrument	05 015 243 001
MagNA Pure 96 Instrument		06 541 089 001
LightCycler® 96 Instrument	1 instrument	05 815 916 001
Reagents , kits		
Bovine Serum Albumin	20 mg, 1 ml	10 711 454 001
High Pure Viral Nucleic Acid Kit	1 kit, up to 100 isolations	11 858 874 001
MagNA Pure LC Total Nucleic Acid Isolation Kit - High Performance	1 kit, up to 288 isolations	05 323 738 001
MagNA Pure Compact Nucleic Acid Isolation Kit I	1 kit, 4 x 8 sealed cartridges, 32 isolations	03 730 964 001
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

6.4. Trademarks

APTATAQ, HIGH PURE, LIGHTCYCLER and MAGNA PURE are trademarks of Roche.
All third party product names and trademarks are the property of their respective owners.

6.5. License Disclaimer

For patent license limitations for individual products please refer to: http://technical-support.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 **lifescience.roche.com**, to download or request copies of the following **Materials**:
- Instructions for Use
- Safety Data Sheets
- 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.

0218.07168055001®

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim
Germany