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Roche

RealTime ready

Influenza A/H1N1 Detection Set

Pre-mixed primers and hydrolysis probes for detection of Influenza A Matrix Protein 2 and Hemagglutinin H1 (Mexico) genes

Cat. No. 05 640 393 001 Kit for 2 × 100 reactions

Version June 2009

⚠ Store at +2 to +8°C
⚠ Store protected from light!

1. What this Product Does

Introduction

Influenza A is a negative strand single-stranded RNA virus from the Orthomyxovirus family which infects birds and mammals. It is characterized using the Hemagglutinin (HA) and Neuraminidase (NA) genes. In April 2009, a new H1N1 virus was reported in Mexico. This H1N1 influenza virus has a NA gene similar to H5N1, but a very specific HA gene which can be used for its identification. RT-PCR detection of Influenza A is based on the conserved matrix protein 2 gene (M2). The respective subtype identification is based on detection of the hemagglutinin HA1 gene. A network of virologist has selected and verified two primer/hydrolysis probe mixes to detect the viral RNA genes of influenza using actual H1N1 clinical samples.

The recommended procedure for testing for the new Influenza A/H1N1 virus is the detection of the M2 gene for Influenza A. Positive samples are then tested targeting the specific H1 gene or parallel testing of both targets is performed.

The primer/probe set for detection of the Influenza A Matrix Protein 2 gene M2 (Ward *et al.*, 2004) has been recommended by the WHO for bird flu virus detection (2007).

The primer/probe set for detection of Influenza A Hemagglutinin HA1 (Panning *et al.*, 2009, submitted) has been recommended by the Robert Koch Institute in Berlin, Germany (May 2009).

Number of Tests

The kit is designed for the detection of Influenza A Matrix Protein 2 gene M2 and the specific H1 gene for Influenza A/H1N1, 100 reactions each, with a final reaction volume of 20 µl each using the LightCycler® 480 System or using the LightCycler® Carousel-Based System.

Kit Contents

Vial/Cap	Label	Contents/Function
1 blue cap	Primer/Probe Mix for Inf A/M2	• 1 vial, lyophilizate, contains 1 nmol of primers and 0.5 nmol FAM-labeled probe; • dissolve in 300 µl Water, PCR-grade (vial 5) to a final concentration of 3.33 µM for primers and 1.67 µM for probes.
2 blue cap	Control for Inf A/M2	• 1 vial, lyophilizate; • dissolve in 40 µl Water, PCR-grade (vial 5), positive control containing Inf A/M2 gene copy numbers of 8×10^5/40 µl.
3 red cap	Primer/Probe Mix for Inf A/ H1	• 1 vial, lyophilizate, contains 1 nmol of primers and 0.5 nmol FAM-labeled probe; • dissolve in 300 µl Water, PCR-grade (vial 5) to a final concentration of 3.33 µM for primers and 1.67 µM for probes.
4 red cap	Control for Inf A/ H1	• 1 vial, lyophilizate; • dissolve in 40 µl Water, PCR-grade (vial 5), positive control containing Inf A/H1 gene copy numbers of 8×10^5/40 µl.
5 colorless cap	Water, PCR-grade	• 2 vials

Storage and Stability

The kit is shipped at +15°C to +25°C. Store the kit after arrival at +2°C to +8°C in the dark.

- After dissolving the primer/probe mix lyophilizates (vials 1 and 3), store at +2°C to +8°C in the dark. This solution will be stable for four weeks. Alternatively, this solution can be stored at −15°C to −25°C for one year. Please note that the frozen solution can only be thawed once. Repeated freezing and thawing should be avoided.
- After dissolving the control lyophilizates (vials 2 and 4), store at +2°C to +8°C.

Additional Equipment Required

Required

- Standard laboratory equipment
- Nuclease-free pipet tips
- 1.5 ml RNase-free microcentrifuge tubes for preparing master mixes and dilutions
- To minimize risk of RNase contamination, autoclave all vessels.

Gloves should be worn at all times.

For qPCR:

- Real-Time PCR systems, such as LightCycler® 480 Instrument (96 well)* LightCycler® 2.0 Instrument* and LightCycler® 1.x Instrument*.
- LightCycler® 480 Multiwell Plate 96* and LightCycler® Capillaries (20 µl)*.
- Standard swing-bucket centrifuge with rotor for multiwell plates.

Optional

For Virus RNA purification:

- MagNA Pure LC Total Nucleic Acid Isolation Kit - High Performance*
- MagNA Pure LC Total Nucleic Acid Isolation Kit*
- MagNA Pure LC Total Nucleic Acid Isolation Kit – Large Volume*
- MagNA Pure LC RNA Isolation Kit – High Performance*
- MagNA Pure Compact Nucleic Acid Isolation Kit I*
- MagNA Pure Compact Nucleic Acid Isolation Kit I – Large Volume*
- MagNA Pure Compact RNA Isolation Kit*
- High Pure Viral RNA Kit*
- High Pure Viral Nucleic Acid Kit*
- High Pure Viral Nucleic Acid Large Volume Kit*

For RT-PCR:

- RealTime ready RNA Virus Master*

Assay Time

The RealTime ready Influenza A/H1N1 Detection Set can be used for fast RT-PCR protocols with run times of 25 – 50 min using the RealTime ready RNA Virus Master and a LightCycler® 480 System or a LightCycler® Carousel-Based System.

2. How to Use this Product

2.1 Before You Begin

Precautions

Using 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 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.

Sample Material

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

- either the MagNA Pure LC Instrument* or the MagNA Pure Compact Instrument* and a dedicated MagNA Pure Nucleic Acid Isolation Kit (for automated isolation), or
- a High Pure Nucleic Acid Isolation Kit (for manual isolation).

For details see the Roche Applied Science catalogue or the website: www.roche-applied-science.com

⚠ Use up to 10^6 viral RNA copies. Higher concentrations may result in inhibition of the reaction.

Negative Control

Always run a negative control with the samples. To prepare a negative control, replace the viral template RNA with PCR-grade water. A contamination problem can be observed using the negative control.

Positive Control

- Always run a control reaction for Inf A/M2 and/or for Inf A/ H1 with the samples.
- Please handle this solution with care to avoid contamination.

⚠ We recommend preparing a 10 to 10,000 fold dilution series per 5 µl sample volume. Dilutions of the positive control solution should be prepared fresh each time.

2.2 Procedure

2.2.1 One-Step RT-PCR Procedure

Follow the procedure below to prepare one 20 µl standard reaction with the RealTime ready RNA Virus Master:

⚠ Do not touch the surface of the LightCycler® 480 Multiwell Plate or the LightCycler® Capillaries.

1. Thaw vials 1 to 4 of this Detection Set for testing samples for Influenza A Matrix Protein 2 and Hemagglutinin H1 (Mexico) genes. To check only for Influenza A Matrix Protein 2, please thaw vial 1 only and the control for Influenza A /M2 (vial 2). In addition, thaw the 3 vials of the RealTime ready RNA Virus Master: the Enzyme Blend, Reaction Buffer and Water, PCR-grade. To ensure recovery of all the contents, briefly spin vials 1 and 2 in a microcentrifuge before opening and mix carefully by pipetting up and down.
2. Prepare the RT-PCR master mix, see pipetting protocol below.
3. Dispense equal amounts of reaction mixture and sample dilutions to the respective wells of the LightCycler® 480 Multiwell Plate 96 or LightCycler® Capillaries (5 µl sample volume each for a reaction volume of 20 µl).
4. Seal the LightCycler® 480 Multiwell Plate 96 plate with adequate sealing film or when using the LightCycler® 1.x or 2.0 Instrument, seal each LightCycler® Capillary with a stopper.
5. Place 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 min at 1,500 × *g*.
   When using the LightCycler® 1.x or 2.0 Instrument: If a LC Carousel Centrifuge is available spin the capillaries in a LightCycler® Sample Carousel in the LightCycler® Carousel Centrifuge.
   - Alternatively, place the capillaries in cooled adapters in a standard benchtop microcentrifuge, centrifuge at 700 × *g* (3,000 rpm) for 5 s, and transfer the capillaries to the LightCycler® Sample Carousel.

   Place the centrifuge adapters in a balanced arrangement within the centrifuge.
6. Load the multiwell plate into the LightCycler® 480 Instrument. Or alternatively place the LightCycler® Sample Carousel in the LightCycler® Carousel-Based System.
7. Start the PCR program.

In a 1.5 ml reaction tube on ice, prepare the RT-PCR Mix for a 20 µl reaction by adding the following components in the order listed below, then mix gently up and down:

Component Volume for a 20 µl Reaction

Pipetting Protocol:

	Volume per 20 µl reaction	Final conc.
Water, PCR-grade	7.6 µl	
Enzyme blend, 50-fold conc. of the RealTime ready RNA Virus Master	0.4 µl	1-fold
Reaction buffer, 5-fold conc. of the RealTime ready RNA Virus Master	4 µl	1-fold
Primer/Probe Mix vial 1 or 3 (3.33 µM primers, 1.67 µM probes)	3 µl	0.5 µM primers 0.25 µM probe
Viral RNA Sample *or* Positive Control *or* Negative Control	5 µl	10 to 10^6 copies for viral RNA samples 10 to 10^5 copies for positive control

⚠ To prepare the RT-PCR mix for more than one reaction, multiply the amount in the "Volume" column above by the number of reactions. Please note that you will use up more liquid during the pipetting steps. Please calculate extra volume for the RT-PCR by adding one reaction to your mix.

For a 20 µl reaction: Pipet 15 µl RT-PCR mix into each precooled LightCycler® Capillary or for the LightCycler® 480 Multiwell Plate 96, and add 5 µl of the Sample, Positive Control and Negative Control.

A) LightCycler® 480 System Protocol

- The following procedure is optimized for use with the LightCycler® 480 System.
- Program the LightCycler® 480 Instrument before preparing the reaction mixes.

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

- Reverse Transcription of viral template RNA
- Denaturation: of cDNA/RNA hybrid
- Amplification of the cDNA
- Cooling the rotor and thermal chamber

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

Protocol for Use with LightCycler® 480 Multiwell Plate 96

The following table shows the RT-PCR parameters that must be programmed for a LightCycler® 480 System RT-PCR run with the RealTime ready RNA Virus Master using a LightCycler® 480 Multiwell Plate 96.

Setup				
Detection Format		**Block Type**		
Mono Color Hydrolysis Probe / UPL Probe		96		
Programs				
Program Name		**Cycles**		
Reverse Transcription		1		
Initial Denaturation		1		
Cycling		45		
Cooling		1		
Temperature Targets				
Target [°C]	Acquisition Mode	Hold [hh:mm:ss]	Ramp Rate [°C/s]	Acquisitions [n/°C)]
Reverse Transcription				
55	None	00:08:00	4.4	–
Initial Denaturation				
95	None	00:00:30	4.4	–
Amplification				
95	None	00:00:01	4.4	–
60	Single	00:00:20	2.2	–
72	None	00:00:01	4.4	–
Cooling				
40	None	00:00:30	1.0	–

B) LightCycler® Carousel-Based System Protocol

- The following procedure is optimized for use with the LightCycler® Carousel-Based System.
- Program the LightCycler® 2.0 or 1.x Instrument before preparing the reaction mixes.

A LightCycler® 2.0 or 1.x Instrument protocol that uses RealTime ready RNA Virus Master contains the following programs:

- Reverse Transcription of viral template RNA
- Denaturation: of cDNA/RNA hybrid
- Amplification of the cDNA
- Cooling the rotor and thermal chamber

For details on how to program the experimental protocol, see the LightCycler® Software 4.1 Operator's Manual.

Protocol for Use with the LightCycler® Carousel-Based System

The following table shows the PCR parameters that must be programmed for a LightCycler® Carousel-Based PCR run with the RealTime ready RNA Virus Master using LightCycler® Capillaries (20 µl).

Setup						
Analysis Mode	**Cycles**	**Segment**	**Target [°C]**	**Hold [hh:mm:ss]**	**Ramp Rate [°C/s]**	**Acquisition Mode**
Reverse Transcription						
None	1	-	55	00:08:00	20	None
Initial Denaturation						
None	1	-	95	00:00:30	20	None
Amplification						
Quantification	45	Denaturation	95	00:00:01		None
		Annealing	60	00:00:20		Single
		Extension	72	00:00:01		None
Cooling						
None	1	-	40	00:00:30	20	None

2.2.2 Two-Step RT-PCR Procedure

In addition to the one-step RT-PCR procedure using the RealTime ready RNA Virus Master, it is also possible to perform a two-step RT-PCR. To do this, use the Transcriptor First Strand cDNA Synthesis Kit* with the LightCycler® 480 Probes Master* on LightCycler® 480 Instruments, or with the LightCycler® TaqMan® Master* on LightCycler® Carousel-Based Instruments.

Detailed protocols are listed below:

Reverse Transcription (RT) with Transcriptor First Strand cDNA Synthesis Kit:

- Thaw all frozen reagents before use.
- Briefly centrifuge them before starting the procedure.
- Keep all reagents on ice during the set-up of the reactions.
- In a 1.5 ml reaction tube on ice, prepare the RT Mix for a 20 µl reaction by adding the following components in the order listed below

	Volume per 20 µl reaction	**Final concentration**
Random Hexamer Primer 600 µM (vial 6)	2 µl	
Transcriptor Reverse Transcriptase Reaction Buffer, 5× conc. (vial 2)	4 µl	1-fold (8 mM MgCl2)
Protector RNase Inhibitor, 40 U/µl (vial 3)	0.5 µl	1-fold
Deoxynucleotide Mix, 10 mM each (vial 4)	2 µl	1 mM each
Transcriptor Reverse Transcriptase 20 U/µl	0.5 µl	10 U
Water, PCR-grade	1 µl	
RNA template (usually 1:4 diluted in water, PCR-grade) *or* Positive Control *or* Negative Control	10 µl	10 to 10^6 copies for viral RNA samples 10 to 10^5 copies for viral RNA samples

⚠ To prepare the RT mix for more than one reaction, multiply the amount in the "Volume" column above by the number of the reactions. Please note that you will use up more liquid during the pipetting steps. Please calculate extra volume for the RT by adding one reaction to your mix.

- Centrifuge the tube briefly to collect the liquid on the bottom of the tube.
- Place the tube in a thermal block cycler with a heated lid (to minimize evaporation).

Reverse Transcription Protocol for use with a Thermal Block Cycler:

- Incubate the RT reaction 10 min at +25°C, followed by 30 min at +55°C.
- Inactivate the Transcriptor Reverse Transcriptase by heating at +85°C for 5 min.
- Stop the reaction by placing the tube on ice immediately.
- At this point the reaction tube can be stored at −15°C to −25°C for several weeks.

A) Component Volume for a 20 µl Reaction with LightCycler® 480 Probes Master:

Pipetting Protocol:

	Volume per 20 µl reaction	Final concentration
Water, PCR-grade	-	
Primer/Probe Mix (vial 1 of the Influenza A/H1N1 Detection Set, 3.33 µM primers, 1.67 µM probes)	3 µl	0.5 µM primers 0.25 µM probe
LightCycler® 480 Probes Master 2-fold	10 µl	1-fold
cDNA of RNA template *or* cDNA of Positive Control *or* Negative Control	7 µl	

⚠ To prepare the PCR mix for more than one reaction, multiply the amount in the "Volume" column above by the number of the reactions. Please note that you will use up more liquid during the pipetting steps. Please calculate extra volume for the PCR by adding one reaction to your mix.

B) Component Volume for a 20 µl Reaction with LightCycler® TaqMan® Master:

Pipetting Protocol:

	Volume per 20 µl reaction	Final concentration
Water, PCR-grade	6 µl	
Primer/Probe Mix (vial 1 of the Influenza A/H1N1 Detection Set, 3.33 µM primers, 1.67 µM probes)	3 µl	0.5 µM primers 0.25 µM probe
LightCycler® TaqMan® Master 5-fold	4 µl	1-fold
cDNA of RNA template *or* cDNA of Positive Control *or* Negative Control	7 µl	

⚠ To prepare the PCR mix for more than one reaction, multiply the amount in the "Volume" column above by the number of the reactions. Please note that you will use up more liquid during the pipetting steps. Please calculate extra volume for the PCR by adding one reaction to your mix.

PCR Protocol for use with LightCycler® 480 Probes Master:

Setup				
Detection Format		**Block Type**		
Mono color Hydrolysis Probes or Multicolor Hydrolysis Probes		96		
Programs				
Program Name		**Cycles**		
Initial Denaturation		1		
Cycling		45		
Cooling		1		
Temperature Targets				
Target [°C]	**Acquisition Mode**	**Hold [hh:mm:ss]**	**Ramp Rate [°C/s]**	**Acquisitions [n/°C)]**
Initial Denaturation				
95	None	00:10:00	4.4	–
Amplification				
95	None	00:00:10	4.4	–
60	None	00:00:30	2.2	–
72	Single	00:00:01	4.4	–
Cooling				
40	None	00:00:30	2.2	–

PCR Protocol for use with LightCycler® TaqMan® Master:

Setup						
Analysis Mode	**Cycles**	**Segment**	**Target [°C]**	**Hold [hh:mm:ss]**	**Ramp Rate [°C/s]**	**Acquisition Mode**
Initial Denaturation						
None	1	-	95	00:10:00	20	None
Amplification						
Quantification	45	Denaturation	95	00:00:10		None
		Annealing	60	00:00:30		None
		Extension	72	00:00:01		Single
Cooling						
None	1	-	40	00:00:30	20	None

2.3 Quality Control

Each lot of RealTime ready Influenza A/H1N1 Detection Set is tested to meet specifications of qRT-PCR using the LightCycler® 480 Instrument.

3. Results

The following results were obtained using the RealTime ready Influenza A/H1N1 Detection Set and the RealTime ready RNA Virus Master on the LightCycler® 480 Instrument with 1,000 copies of plasmid control DNA for H1 (✱ - - -✱- -); and M2 (-✱——✱-) and one positive viral sample.

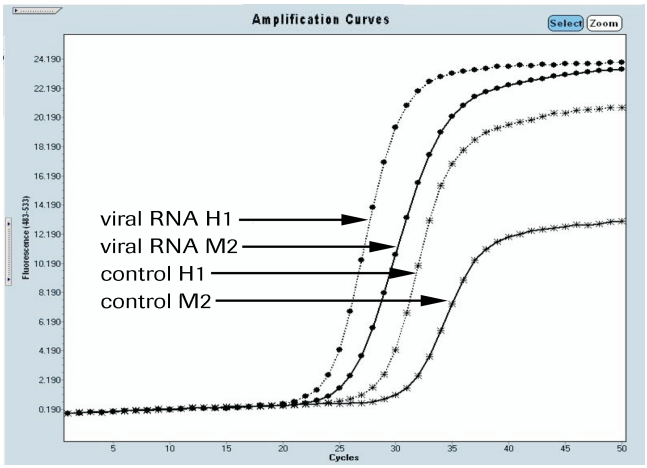

Fig. 1: Real-time PCR amplification curves obtained using 1,000 copies per well of the **control H1** (-✱- - -✱- -, Cp = 28.71); and **control M2** (-✱——✱- , Cp = 31.08) plasmid DNA, and one positive viral sample. RT-PCR was performed in a reaction volume of 20 µl per well in a LightCycler® 480 Multiwell plate, 96. Influenza Hemagglutinin H1 virus RNA was readily detected in the viral sample using the **H1 primer/probe set** (- ●- - -●- , Cp = 23.99) and the **M2 primer/probe set** (-●——●-, Cp = 26.21).

Interpretation of Data

Possible analysis results for detection of Influenza A Matrix Protein 2.

Influenza A Matrix Protein 2 (Sample)	Positive control containing Inf A/M2 gene	Negative Control	Result
No signal after PCR	Detectable	Negative	Negative
Signal after PCR	Detectable	Negative	Positive
No signal after PCR	Not detectable	Not relevant	PCR failure, repeat experiment
Signal after PCR	Not relevant	Positive	Contamination, repeat experiment

Possible analysis results for detection of Hemagglutinin H1 (Mexico) genes

Hemagglutinin H1 (Mexico) genes (Sample)	Positive Control containing Inf A/H1 gene	Negative Control	Result
No signal after PCR	Detectable	Negative	Negative
Signal after PCR	Detectable	Negative	Positive
No signal after PCR	Not detectable	Not relevant	PCR failure, repeat experiment
Signal after PCR	Not relevant	Positive	Contamination, repeat experiment

4. Supplementary Information

Changes to Previous Version
- Detailed protocols for the two-step RT-PCR procedure are described.
- Editorial changes.

Text Conventions

To make information consistent and understandable, the following text conventions are used in this Instruction Manual:

Text Convention	Use
Numbered instructions labeled ❶, ❷, etc.	Steps in a procedure that must be performed in the order listed.
Asterisk *	Denotes a product available from Roche Applied Science.

Symbols

Symbols are used in this Instruction Manual 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.

Ordering Information

Roche Applied Science 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.roche-applied-science.com and these Special Interest Sites:
- LightCycler® Carousel-Based System: http://www.lightcycler.com
- LightCycler® 480 System: http://www.lightcycler480.com
- Automated Sample Preparation (MagNAPure LC System and MagNAPure Compact System): http://www.magnapure.com
- DNA & RNA preparation - Versatile Tools for Nucleic Acid Purification: http://www.roche-applied-science.com/napure

Instrument and Accessories

Product	Pack Size	Cat. No.
LightCycler® 480 Instrument II, 96 well	1 instrument (96 well)	05 015 278 001
LightCycler® 480 Block Kit 96 Silver	1 block kit for 96-well PCR Multiwell Plates	05 015 197 001
LightCycler® 480 Multiwell Plate 96, white	50 plates and foils	04 729 692 001
LightCycler® 480 Sealing Foil	50 foils	04 729 757 001
LightCycler® 480 Software, Version 1.5	1 software package	04 994 884 001
LightCycler® 2.0 Instrument	1 instrument plus accessories	03 531 414 001
LightCycler® 1.5 Instrument	1 instrument plus related products and data station	04 484 495 001
LC Carousel Centrifuge 2.0	1 centrifuge plus rotor and bucket	03 709 507 001 (115 V) 03 709 582 001 (230 V)
LightCycler® Software 4.1	1 software package	04 898 915 001
LightCycler® Capillaries (20 µl)	1 pack (containing 5 boxes, each with 96 capillaries and stoppers)	04 929 292 001
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

	Product	Pack Size	Cat. No.
RNA Virus Isolation Kits	MagNA Pure LC Total Nucleic Acid Isolation Kit - High Performance	1 kit for up to 288 isolations	05 323 738 001
	MagNA Pure LC Total Nucleic Acid Isolation Kit	1 kit (192 isolations)	03 038 505 001
	MagNA Pure LC Total Nucleic Acid Isolation Kit - Large Volume	1 kit (192 isolations)	03 264 793 001
	MagNA Pure LC RNA Isolation Kit - High Performance	1 kit (192 isolations)	03 542 394 001
	MagNA Pure Compact Nucleic Acid Isolation Kit I	1 kit for 32 isolations	03 730 964 001
	MagNA Pure Compact Nucleic Acid Isolation Kit I - Large Volume	1 kit for 32 isolations	03 730 972 001
	MagNA Pure Compact RNA Isolation Kit	1 kit for 32 isolations	04 802 993 001
	High Pure Viral RNA Kit	1 kit for up to 100 purifications	11 858 882 001
	High Pure Viral Nucleic Acid Kit	1 kit for up to 100 purifications	11 858 874 001
	High Pure Viral Nucleic Acid Large Volume Kit	1 kit for up to 40 purifications	05 114 403 001
Associated Kits and Reagents	RealTime ready RNA Virus Master	1 kit	05 619 416 001
	Transcriptor First Strand cDNA Synthesis Kit	1 kit for 100 reactions	04 896 866 001
	LightCycler® 480 Probes Master	5 × 1 ml	04 707 494 001
	LightCycler® TaqMan® Master	1 kit for 480 reactions	04 735 536 001

Contact and Support

To ask questions, solve problems, suggest enhancements or report new applications, please visit our **Online Technical Support Site** at:

www.roche-applied-science.com/support

To call, write, fax, or email us, visit the Roche Applied Science home page, www.roche-applied-science.com, and select your home country. Country-specific contact information will be displayed. Use the Product Search function to find Pack Inserts and Material Safety Data Sheets.

Roche Diagnostics GmbH
Roche Applied Science
68298 Mannheim
Germany