The information previously contained on these webpages were authorized under the 2009 H1N1 Influenza Emergency Use Authorizations (EUAs). As of June 23, 2010, the EUAs have been terminated and this information is no longer current.

Emergency use authorization only.

Roche

RealTime ready

Influenza A/H1N1 Detection Set

Package Insert

Version November 2009

Pre-mixed primers and hydrolysis probes for detection of 2009 H1N1 influenza A viral RNA in human samples using the LightCycler® 2.0 Instrument

REF 05 977 207 001

Kit for 2 × 100 reactions

Store at +2 to +8°C

Store protected from light

www.roche-applied-science.com

PROTOCOL

1. INTENDED USE

The **RealTime ready Influenza A/H1N1 Detection Set** is intended for use in CLIA High Complexity laboratories with the ability to perform nucleic acid extraction using the Roche MagNA Pure LC Instrument and PCR on the LightCycler® 2.0 Instrument for the *in vitro* qualitative detection of 2009 H1N1 influenza viral RNA in nasal swabs (NS), nasopharyngeal swabs (NPS), nasal washes (NW), or nasal aspirates (NA) from human patients with signs and symptoms of respiratory infection in conjunction with clinical and epidemiological risk factors. The **RealTime ready Influenza A/H1N1 Detection Set** is intended to be used in combination with the **RealTime ready RNA Virus Master**.

Testing with the Roche **RealTime ready Influenza A/H1N1 Detection Set** in combination with the **RealTime ready RNA Virus Master** should not be performed unless the patient meets clinical and epidemiologic criteria for testing suspect specimens. The identification of 2009 H1N1 influenza A should be performed along with clinical and epidemiological assessment.

Negative results do not preclude Influenza virus infection and should not be used as the sole basis for treatment or other patient management decisions.

The **RealTime ready Influenza A/H1N1 Detection Set** is for use under the Food and Drug Administration's emergency use authorization only.

2. EXPLANATION OF THE TEST

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 detection of the conserved matrix protein 2 gene (M2). The respective subtype identification is based on detection of the hemagglutinin HA1 gene. A network of virologists has selected and verified two primer/hydrolysis probe mixes to detect the viral RNA genes of 2009 H1N1 influenza using actual 2009 H1N1 influenza clinical samples.

3. TEST PRINCIPLE/ SUMMARY

3.1 Specimen preparation

The specimen preparation is performed with the MagNA Pure LC Instrument and the **MagNA Pure LC Total Nucleic Acid Isolation Kit - High Performance** using the **"Total NA HS 200 External_Lysis"** Protocol.

3.2 Amplification and detection

The recommended testing procedure for the new 2009 H1N1 influenza virus includes the detection of the conserved M2 gene of Influenza A. The parallel assay targeting the specific HA1 gene of the new 2009 H1N1 influenza virus serves for differentiation of 2009 H1N1 influenza from seasonal Influenza A.

The primer/probe set for the detection of the Influenza A Matrix Protein 2 gene M2 has been recommended by the WHO for bird flu virus detection in 2007 (Ref. 1).

The primer/probe set for the detection of the specific Hemagglutinin HA1 gene of 2009 H1N1 influenza virus has been recommended by the Robert Koch Institute in Berlin, Germany in May 2009 (Ref. 2).

For the confirmation of correct specimen collection, successful extraction and absence of RT-PCR inhibitors, the kit includes an additional primer/probe mix to be used in combination with the Inf A/M2 assay. This allows for the simultaneous detection of a common human nucleic acid as an extraction control.

4. REAGENTS – WORKING SOLUTIONS

4.1 Kit contents

Vial/ Cap	Label	Contents/Function
1a blue cap	Primer/Probe Mix for Inf A/M2	• 1 vial, lyophilizate • Contains 1 nmol of primers and 0.5 nmol FAM-labeled probe (channel 530) • Dissolve in 300 µl Water, PCR Grade (vial 5) to a final conc. of 3.33 µM for primers and 1.67 µM for probe.
1b yellow cap	Primer/Probe Mix for Human Nucleic Acid (Extraction Control)	• 1 vial, lyophilizate • Contains 1 nmol of primers and 0.5 nmol of LightCycler® Yellow 555 labeled probe (channel 560) • Dissolve in 300 µl Water, PCR Grade (vial 5) to a final conc. of 3.33 µM for primers and 1.67 µM for probe.
2 blue cap	Positive control for Inf A/M2	• 1 vial, lyophilizate • Positive control containing 8×10^5 copies of Inf A/M2 gene • Dissolve in 400 µl Water, PCR Grade (vial 5) to a final conc. of 8×10^5 copies/ 400 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 conc. of 3.33 µM for primers and 1.67 µM for probe.
4 red cap	Positive control for Inf A/H1	• 1 vial, lyophilizate • Positive control containing 8×10^5 copies of Inf A/H1 gene • Dissolve in 400 µl Water, PCR Grade (vial 5) to a final conc. of 8×10^5 copies/ 400 µl.
5 color-less cap	Water, PCR Grade	6 vials, 1 ml each

5. PRECAUTIONS AND WARNINGS

5.1 Handling requirements

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

- Wear disposable gloves and change them frequently.
- Avoid touching surfaces that could cause RNase carryover.
- Use only the reagents listed in this package insert.
- Clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents against RNases and nucleic acids.
- Use only new RNase-free aerosol-blocking pipette tips and microcentrifuge tubes.
- Use a work area specifically designed for RNA work, and use reaction vessels and pipettors dedicated only for work with template RNA.

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

- If available use a PCR hood.
- Wipe and UV-illuminate PCR workstations and Biosafety Cabinets each time before use.
- Have separate areas for sample preparation, PCR reaction set-up and PCR amplification.
- Discard pipette tips in sealed containers to prevent airborne contamination.
- The control plasmids provided with this kit have to be handled with care: Open and dissolve the lyophilizates (vial 2 and 4) in a separate place.
- Avoid opening LightCycler® Capillaries containing amplification products.

5.2 Laboratory procedures

All human sourced material and all 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.
- Clean and decontaminate work areas and instruments, including pipettes, with commercially available decontamination reagents.
- Avoid microbial and nuclease contamination of reagents when removing aliquots from reagent bottles. Use sterile disposable pipette tips.
- Wash hands thoroughly after handling specimens and kit reagents.

5.3 Waste handling

- Dispose unused reagents and waste in accordance with country, federal, state, and local regulations.
- Material Safety Data Sheets (MSDS) are available on the Roche Applied Science homepage, or upon request from the local Roche office.

5.4 Specimen preparation

- For preparation of viral RNA from nasal swabs, nasopharyngeal swabs, and nasal washes, use the **MagNA Pure LC Total Nucleic Acid Isolation Kit – High Performance**, on the MagNA Pure LC Instrument.
- Refer to the safety instructions in the package insert of the **MagNA Pure LC Total Nucleic Acid Isolation Kit – High Performance** for handling and disposal information.

5.5 Amplification and detection

- Consult the LightCycler® 2.0 Instrument Operator's Manual before use.
- Create a written record for each run providing the kit lot number, the LightCycler® Sample Carousel position of each specimen and the target which is tested (*e.g.,* Inf A/M2 or H1).
- Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries.

5.6 Reagent handling

- Store all reagents of the **RealTime ready Influenza A/H1N1 Detection Set** protected from light.

6. STORAGE AND STABILITY (REAGENTS)

6.1 Kit and reagents

- The **RealTime ready Influenza A/H1N1 Detection Set** is shipped at +15°C to +25°C.
- Store the kit after arrival at +2°C to +8°C in the dark.
- After resuspending the primer/probe mix lyophilizates (vial 1a, 1b and 3), aliquot and store at -15°C to -25°C in the dark. Avoid repeated freezing and thawing.
- After resuspending the control lyophilizates (vial 2 and 4), aliquot and store at -15°C to -25°C. Avoid repeated freezing and thawing.

6.2 Specimen collection

- Human nasal washes
- Human nasal swabs
- Human nasopharyngeal swabs
- Human nasal aspirates

⚠ For transport and storage please follow the recommendations of the WHO.

7. MATERIALS

7.1 Materials provided

See “REAGENTS - WORKING SOLUTIONS”

7.2 Materials and devices required but not provided

- Standard laboratory equipment
- Microcentrifuge Tubes, 1.5 ml sterile, nuclease-free
- LightCycler® 2.0 Instrument (Cat. No. 03 531 414 001)
- LightCycler® Software Version 4 (Cat. No. 04 898 915 001 or Cat. No. 04 717 392 001)
- LightCycler® Capillaries (20 µl) (Cat. No. 04 929 292 001)
- LightCycler® Centrifuge Adapters (Cat. No. 11 909 312 001)
- LightCycler® Capping Tool (Cat. No. 03 357 317 001)
- MagNA Pure LC 1.0 Instrument (Cat. No. 12 236 931 001) or
- MagNA Pure LC 2.0 Instrument (Cat. No. 05 197 686 001)
- MagNA Pure LC Total Nucleic Acid Isolation Kit - High Performance (Cat. No. 05 323 738 001)
- RealTime ready RNA Virus Master (Cat. No. 05 619 416 001)

7.3 Optional materials

- LC Carousel Centrifuge 2.0 including rotor buckets (Cat. Nos. 03 709 507 001 and 03 709 582 001) or
- LC Carousel Centrifuge (Cat. Nos. 03 030 512 001 and 12 189 682 001) and LC Carousel Centrifuge 2.0 Rotor Set (Cat. No. 03 724 697 001)

8. ASSAY PROCEDURE

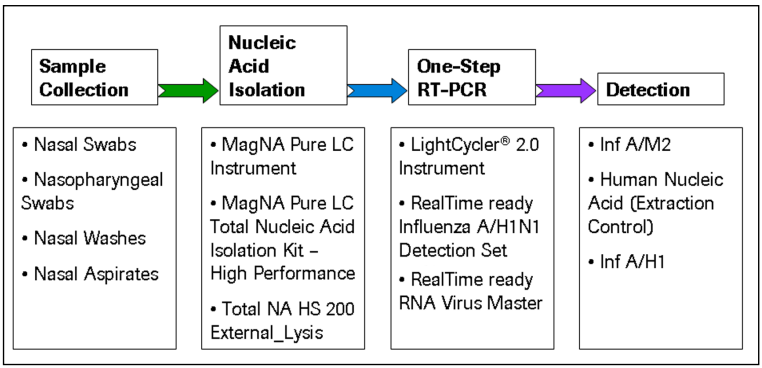

Fig. 1: Overview of the workflow

8.1 Specimen preparation on the MagNA Pure LC Instrument

Automated specimen preparation from nasal swabs and/or nasopharyngeal swabs and/or nasal washes and/or nasal aspirates is performed on the MagNA Pure LC Instrument, using the **MagNA Pure LC Total Nucleic Acid Kit – High Performance** (Cat. No. 05 323 738 001).

For maximum recovery and sensitivity, the preparation of viral RNA is performed using the "**Total NA HS 200 External_Lysis**" Protocol. Samples are lysed manually outside the MagNA Pure LC Instrument. For external lysis use 200 µl specimen volume and add 300 µl Lysis/Binding Buffer. Proceed as described in the package insert of the kit under "External Lysis Protocol". The external lysis volume is 500 µl and the elution is performed in 100 µl Elution Buffer.

For a detailed description on how to use the MagNA Pure LC Instrument, please refer to the corresponding Operator's Manual.

For a detailed description on how to use the **MagNA Pure LC Total Nucleic Acid Kit – High Performance**, particularly as concerns the storage of the eluates, please refer to the corresponding package insert.

The protocol name, "**Total NA HS 200 External_Lysis**", should appear in the protocol selection of the 'Sample Ordering' screen of the MagNA Pure LC 1.0 Instrument, or on the 'Ordering' sub-tab of the MagNA Pure LC 2.0 Instrument. If not previously installed, order the protocol free of charge. For additional details, please contact your local Roche representative.

⚠ **First time use of the RealTime ready Influenza A/H1N1 Detection Set on a specific LightCycler® 2.0 Instrument:**
Prepare in addition human nucleic acid from an uninfected individual (*e.g.,* from a nasal swab). This is required to perform the instrument-specific Color Compensation Protocol, which is described in the next section.

8.2 Amplification and detection on the LightCycler® 2.0 Instrument

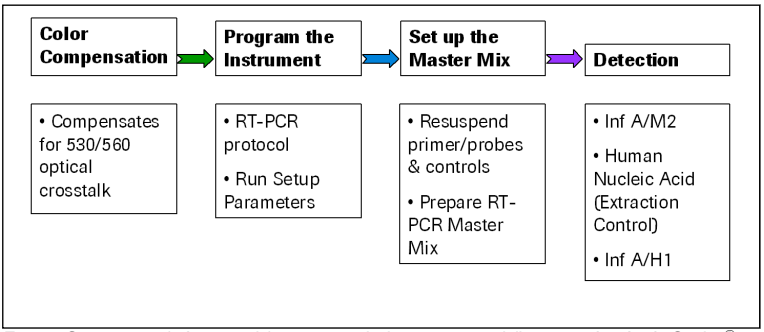

Fig.2: Overview of the amplification and detection workflow on the LightCycler®2.0 Instrument

If using this **RealTime ready Influenza A/H1N1 Detection Set** for the first time on a specific LightCycler® 2.0 Instrument, it is essential to perform the instrument-specific Color Compensation Protocol described below. If the Color Compensation Protocol has been performed on the specific LightCycler® 2.0 Instrument, please continue with **8.2.2. Program RT-PCR Parameters in the LightCycler® Software.**

Please note that a Color Compensation file is specific for the individual instrument. Prepare one Color Compensation file per instrument.

8.2.1 Color Compensation Protocol

The completion of a 530/560 nm color compensation is a prerequisite for running the Inf A/M2 test, in combination with the extraction control test for human nucleic acid. The generated color compensation file is used to compensate for crosstalk between the individual detection channels when performing dual-color experiments. A color compensation calibration run is performed by running a blank capillary (containing Water, PCR Grade), and two capillaries with one dye each, in a RT-PCR program, followed by a melting curve analysis.

Preparation of Primer/Probe Working Solutions for Color Compensation

Resuspend primer/probe lyophilizates for Inf A/M2 (vial 1a) and for Human Nucleic Acid Extraction Control (vial 1b) in 300 µl Water, PCR Grade (vial 5, each). Prepare appropriate aliquots and store at -15°C to -25°C in the dark.

Before use, thaw the primer/probe mixtures and heat to +85°C for 10 min, then chill on ice. Spin briefly before use.

Protocol for the LightCycler® 2.0 Instrument

The following tables show the parameters that have to be programmed for a LightCycler® Color Compensation calibration run with the **RealTime ready Influenza A/H1N1 Detection Set**.

⚠ Program the LightCycler® 2.0 Instrument before preparing the calibration mixes.

For information on how to login to the software and for details on how to program experimental protocols, refer to the LightCycler® Software (Version 4) Operator's Manuals.

Tab. 1: RT-PCR Protocol for Color Compensation

Analysis Mode	Cycles	Segment	Target Temp. (°C)	Hold Time (hh:mm:ss)	Ramp Rate (°C/s)	Acquisition Mode
Reverse Transcription						
None	1	1	58	00:08:00	20	None
Initial Denaturation						
None	1	1	95	00:00:30	20	None
Amplification						
Quantification	45	Denaturation	95	00:00:01	20	None
		Annealing	60	00:00:20	20	Single
		Extension	72	00:00:01	2	None
Temperature Gradient Step						
Color Compensation	1	1	95	00:00:01	20	None
		2	40	00:00:05	20	None
		3	95	00:00:00	0.2	Continuous
Cooling Step						
None	1	1	40	00:00:30	20	None

Tab. 2: Run Setup Parameters

Parameter	Setting
LightCycler® Software Version 4	
Seek Temperature	58°C
Default Channel	Fluorescence Channels 530 or 560
Fluorescence Gains	Not required
"Max Seek Pos."	Enter the total number of capillaries used.
"Instrument Type"	"6 Ch." for LightCycler® 2.0 Instrument (selected by default)
"Capillary Size"	"20 µl"

1. Open the LightCycler® Sample List to enter sample names in the Capillary View tab as follows:

Pos.	Sample Name
1	**Water**
2	**530, FAM**
3	**560, Yellow 555**

2. Select the Analysis Type button and choose "Color Compensation". On the Color Comp tab, enter the Dominant Channel, as follows:

Pos.	Sample Name	Dominant Channel
1	**Water**	**Water**
2	**530, FAM**	**530**
3	**560, Yellow 555**	**560**

3. Then select 530 and 560 in the "Selected Channels" area.

Preparation of the Calibration Mixes

⚠ Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries.

1. Place three LightCycler® Capillaries into precooled LightCycler® Centrifuge Adapters.
2. Prepare the three capillaries, as follows:

Component	Water	530, FAM	560, Yellow 555
Enzyme blend 50 × conc. *(RealTime ready RNA Virus Master/vial 1*)	-	0.4 µl	0.4 µl
Reaction buffer 5 x conc. (*RealTime ready RNA Virus Master/vial 2*)	-	4.0 µl	4.0 µl
Primer/Probe Mix for Inf A/M2 (vial 1a)	-	3.0 µl	-
Primer/Probe Mix for Human Nucleic Acid Extraction Control (vial 1b)	-	-	3.0 µl
Water, PCR Grade	20.0 µl	7.6 µl	7.6 µl
Template: Control for Inf A/M2	-	5.0 µl	-
Template: Human Nucleic Acid*	-	-	5.0 µl
Total Volume	**20.0 µl**	**20.0 µl**	**20.0 µl**

* Please note that human nucleic acid is not part of the kit. Human nucleic acid can be prepared from the nasal swab of an uninfected individual according to the procedure described in this package insert.

3. Seal each capillary with a stopper using the LightCycler® Capping Tool.
4. 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 s (3,000 rpm in a standard benchtop microcentrifuge). Alternatively, use the LC Carousel Centrifuge for spinning the capillaries.

5. Place the capillaries in the following order in the sample carousel of the LightCycler® 2.0 Instrument:

   rotor position 1: Water
   rotor position 2: 530, FAM
   rotor position 3: 560, Yellow 555

6. Start run to cycle the samples as described above in Table 1 and Table 2.

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 and click the **Save CC Object** tab. Place the object in the "Special Data\CCC" folder under your user name.

You can now apply the specific 'CC Object' you created to any dual color hydrolysis probe experiment that is performed with the same FAM-LightCycler®Yellow 555 dye combination.

8.2.2 Program RT-PCR parameters in the LightCycler® Software

Before you begin with the master mix preparation, program the RT-PCR parameters into the LightCycler® Software according to the Tables 3 and 4 below.

For information on how to log in to the software, and for details on how to program experimental protocols, refer to the LightCycler® Software (Version 4) Operator's Manuals.

Tab. 3: RT-PCR Protocol for the LightCycler® 2.0 Instrument

Analysis Mode	Cycles	Segment	Target Temp. (°C)	Hold Time (hh:mm:ss)	Ramp Rate (°C/s)	Acquisition Mode
Reverse Transcription						
None	1	1	58	00:08:00	20	none
Initial Denaturation						
None	1	1	95	00:00:30	20	none
Amplification						
Quantification	45	Denaturation	95	00:00:01	20	none
		Annealing	60	00:00:20	20	single
		Extension	72	00:00:01	2	none
Cooling Step						
None	1	1	40	00:00:30	20	none

Tab. 4: : Run Setup Parameters

Parameter	Setting
LightCycler® Software Version 4	
Seek Temperature	58°C
Default Channel	Fluorescence Channels 530 or 560
Fluorescence Gains	Not required
"Max Seek Pos."	Enter the total number of capillaries used.
"Instrument Type"	"6 Ch." for LightCycler® 2.0 Instrument (selected by default)
"Capillary Size"	"20 µl"

Sample Data

Enter specimen names in the sample list in "Samples". In addition, enter test/target name and the dilution, if applicable.

8.2.3 Quality Control

⚠ Always run appropriate negative and positive controls for each of the two targets (Inf A/M2 and Inf A/H1).

Negative control/ no template control (ntc)

To prepare a negative control, use Water, PCR Grade (vial 5) instead of specimen material in the sample preparation and the RT-PCR. The negative control is mandatory to observe potential contamination issues.

Positive controls

Run a control reaction using the positive controls for Inf A/M2 and for Inf A/H1 (vials 2 and 4 of the detection set) in the respective assays along with the samples. In addition, we recommend testing a known positive specimen as positive control in each run, to check the complete procedure including sample preparation and RT-PCR.

⚠ Please handle the control reagents (vials 2 and 4) with care to avoid carry-over contamination. Resuspend and handle the lyophilizates of the positive control plasmids in a separate place.
Resuspend positive control lyophilizates in 400 µl Water, PCR Grade (vial 5), each. Both positive controls, for Inf A/M2 and Inf A/H1, contain gene copy numbers of 10^4/5 µl.

8.3 Preparation of the master mix

Preparation of primer/probe working solutions:

Resuspend each primer/probe mixture (vial 1a, 1b and 3) in 300 µl Water, PCR Grade (vial 5). Prepare appropriate aliquots and store at -15°C to -25°C in the dark.

Before use, thaw the primer/probe mixtures and heat to +85°C for 10 min, then chill on ice. Spin briefly before use.

⚠ Each time before using the primer/probe mixtures, a heating step at +85°C for 10 min is required. Please heat the primer/probe mixtures only once. Subsequently, chill primer/probe mixtures on ice, and spin briefly before use.

Preparation of the RT-PCR master mix

⚠ Only prepare the required amount of master mix, immediately prior to use.

1. Thaw completely the following reagents of the **RealTime ready RNA Virus Master**: Enzyme Blend, Reaction Buffer, and Water, PCR Grade. To ensure recovery of all contents, briefly spin vials 1 and 2 in a microcentrifuge before opening, and mix carefully by pipetting up and down. Store all vials on ice.
2. Place one LightCycler® Capillary per sample, and one for each of the controls, into precooled LightCycler® Centrifuge Adapters.
3. Prepare the RT-PCR Master Mix by multiplying the amount in the columns below, by the number of reactions to be run (*i.e.,* prepared specimen NA,

negative and positive controls), calculate extra volume for the RT-PCR Master Mix by adding one reaction.

4. In a 1.5 ml reaction tube on ice, add the following components in the order listed below:

Component	Inf A/M2 plus extraction control for human NA	Inf A/H1
Enzyme blend 50 × conc. (*RealTime ready RNA Virus Master/vial 1*)	0.4 µl	0.4 µl
Reaction buffer 5 × conc. *(RealTime ready RNA Virus Master/vial 2*)	4.0 µl	4.0 µl
Primer/Probe Mix for Inf A/M2 (vial 1a)	3.0 µl	-
Primer/Probe Mix for Human NA Extraction Control (vial 1b)	3.0 µl	-
Primer/ Probe Mix for Inf A/H1 (vial 3)	-	3.0 µl
Water, PCR Grade	4.6 µl	7.6 µl
Total Volume	**15.0 µl**	**15.0 µl**

To test for Influenza Matrix Protein 2, please use primer/probe mixtures in vial 1a and 1b, and the positive control for Influenza A/M2 (vial 2). To test for Influenza Hemagglutinin HA1, please use primer/probe mixture in vial 3, and the positive control for Influenza A/H1 (vial 4).

5. Mix gently by pipetting up and down. Do not vortex.
6. For each 20 µl reaction, dispense 15 µl RT-PCR Master Mix into a precooled LightCycler® Capillary. Add 5 µl sample. Always start with the negative control, followed by the prepared specimen. Positive controls are pipetted last.

⚠ Seal each LightCycler® Capillary with a stopper, using the LightCycler® Capping Tool immediately after addition of each sample before pipetting the next

7. If using the LC Carousel Centrifuge, proceed to step 9. If using a standard benchtop centrifuge (*e.g.*, the Biofuge 13 from Heraeus Instruments or Kendro Laboratory Products), place each capillary into a precooled centrifuge adapter in a standard benchtop microcentrifuge.

⚠ Place the centrifuge adapters in a balanced arrangement within the centrifuge. Centrifuge at 700 × g (3,000 rpm) for 5 s, then proceed with step 9.

8. Place the capillary containing the negative control in position 1, then the capillary containing the positive control in position 2 of the LightCycler® Sample Carousel. Starting with position 3, place the capillaries containing the purified specimen nucleic acid in the LightCycler® Sample Carousel.

9. Place the LightCycler® Sample Carousel into the LightCycler® 2.0 Instrument.
10. Start run to cycle the samples as described above in Table 3 and Table 4.

8.3.1 Analysis of data

When the experiment is finished click on the **Analysis** button and select **Absolute Quantification (Amplification Analysis)** from the Analysis Menu. Automatically the **Channel (530)** is displayed.

To designate the "CC Object" click on **Color Compensation** in either the "Run" or the "Analysis" module, then choose the appropriate object under "Select Color Compensation".

When a dialog box opens and displays the compensated channels, click **OK**.

The experimental data and the analysis graphs will now be redrawn using the compensated data. Notice that the Color Compensation menu label now reads "on".
The result table displays the calculated crossing points (Cp) for the amplification curves.

To view the extraction control results select **Channel (560)**.

Check all amplification curves by visual inspection.

9. INTERPRETATION OF RESULTS

Use the Absolute Quantification Mode for analysis. Analyse Inf A/H1 and Inf A/M2 in channel 530. Analyse the extraction control in channel 560.

9.1 Analysis of amplification results

Tab. 5: Interpretation of RT- PCR Cp values for the Inf A/M2 and the Inf A/H1 assay

Cp value	Result
No value or Cp > 40	Negative
Cp ≤ 40	Positive

If a Cp value is indicated in brackets, i.e. [ ], check the corresponding amplification curve visually for an exponential rise and plausibility of the Cp value. The RT-PCR should be repeated for a sample for which the initial Cp result was reported in brackets [ ]. If the result is not resolved by repeated testing, the result should be reported as invalid.

9.2 Negative control/ no template control (ntc)

The assay result for a negative control shall always be "negative". If the result is "positive", all specimen results controlled by the corresponding negative control are invalid because of a potential contamination of the reagents. In the case of a "positive" result for the negative control, sample preparation and RT-PCR with the appropriate detector must be repeated.

9.3 Positive controls

The assay result for the positive controls shall always be "positive". If the result is "negative", all specimen results controlled by the corresponding positive control are invalid, and the respective RT-PCR with the appropriate detector must be repeated.

9.4 Specimen results

Check if the results of the negative and positive controls in the run are valid, and interpret the specimen results for each of Inf A/H1, Inf A/M2, and Extraction Control according to Table 5.

9.5 Extraction control/ internal control

If specimen results are "negative" for Inf A/M2, the simultaneous extraction control measured in channel 560 shall be "positive" to prove that there actually was specimen material in the reaction and that the RT-PCR was not inhibited. For Inf A/M2 positive samples, with a high amount of Inf A/M2 RNA, the extraction control may be "negative" because of the competition of the two reactions.

9.6 Interpretation

For a valid test batch (including negative controls with a "negative" result and positive controls with a "positive" result), verify each individual specimen for the Inf A/M2 result, the extraction control result, and the Inf A/H1 result.

Specimen results are interpreted as follows:

Tab. 6: Interpretation of specimen results

Inf A M2 (Channel 530)	Extraction control (Channel 560)	Inf A/H1 (Channel 530)	Interpretation	
Positive	Positive	Positive	Influenza A RNA: 2009 H1 RNA:	Detected Detected
		Negative	Influenza A RNA: 2009 H1 RNA:	Detected Not detected
Positve	Negative	Positive	Influenza A RNA: 2009 H1 RNA:	Detected Detected
		Negative	Influenza A RNA: 2009 H1 RNA:	Detected Not detected
Negative	Positive	Positive	Invalid*	
		Negative	Influenza A RNA: 2009 H1 RNA:	Not detected Not detected
Negative	Negative	Positive	Invalid*	
		Negative	Invalid*	

* For invalid specimen results, the specimen preparation and the RT-PCR analysis must be repeated.

10. LIMITATIONS

1. This test has been evaluated for use with human specimen material, only.
2. Reliable results are dependent on adequate specimen collection, transport, storage and processing procedures.
3. Use of this product should be limited to personnel trained in techniques of PCR and of RNA preparation.
4. Negative results do not exclude Influenza infection with other Influenza A or B viruses. Additional testing for Influenza A or B, or other respiratory infections may be required. Results that are positive for a 2009 H1N1 influenza virus do not definitively identify a specific Influenza A virus subtype.
5. Optimum specimen types and timing for peak viral levels during infections caused by 2009 H1N1 influenza virus have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus.
6. False negative results may occur if a specimen is improperly collected, transported or handled. False negative results may occur if inadequate numbers of organisms are present in the specimen.
7. Positive and negative predictive values are highly dependent on prevalence. False positive test results are likely when prevalence of disease due to 2009 H1N1 influenza virus is low or non-existent in a community.
8. If the virus mutates in the target region, 2009 H1N1 influenza virus may not be detected or may be detected less predictably. Performance of this kit with other strains of swine influenza virus has not been established. Inhibitors or other types of interference may produce a false negative result. This test is a qualitative test and does not provide the quantitative value of detected organism present.
9. The performance of this test has not been established for monitoring treatment of Influenza A or 2009 H1N1 influenza virus infections. The performance of this test has not been established for screening of blood or blood products. This test cannot rule out diseases caused by other bacterial or viral pathogens.

11. SPECIFIC PERFORMANCE DATA

11.1 Analytical sensitivity (LoD)

The Limit of Detection (LoD) was determined for 2009 H1N1 influenza virus and for Seasonal Influenza A virus by limiting dilution studies using viral stocks of 2009 H1N1 influenza virus (A/Hamburg/05/09) and of Seasonal Influenza A (A/Brisbane/59/07 like).

Both viruses were spiked in two different matrices leading to 4 sample types:

- 2009 H1N1 influenza virus in nasal swab
- 2009 H1N1 influenza virus 2009 in nasal wash
- Seasonal Influenza A in nasal swab
- Seasonal Influenza A in nasal wash

The data was statistically evaluated by Probit analysis and the LoD was calculated for the 95% confidence interval (LoD_95).

The result summary is presented in the following tables.

Tab. 7: Summary of LoD results for **2009 H1N1 influenza virus by Influenza A/H1 assay** in nasal swabs and nasal washes

	Inf A/H1 assay					
	2009 H1N1 influenza virus in nasal swabs			**2009 H1N1 influenza virus in nasal washes**		
	Virus concentration			Virus concentration		
Dilution	$TCID_{50}$*/ml	copies/ PCR	Results	$TCID_{50}$/ml	copies/ PCR	Results
1	161.0	790	21/21	161.0	790	21/21
1:5	32.2	158	21/21	32.2	158	21/21
1:10	16.1	79	21/21	16.1	79	21/21
1:50	3.2	16	7/21	3.2	16	10/21
1:100	1.6	8	3/21	1.6	8	2/21
LoD_95	**11.0**	**54**	**N/A**	**8.0**	**39**	**N/A**

* TCID: Tissue Culture Infective Dose

Tab. 8: Summary of LoD results for **2009 H1N1 influenza virus by Influenza A/M2 assay** in nasal swabs and nasal washes

	Inf A/M2 assay					
	2009 H1N1 influenza virus in nasal swabs			**2009 H1N1 influenza virus in nasal washes**		
	Virus concentration			Virus concentration		
Dilution	$TCID_{50}$/ml	copies/ PCR	Results	$TCID_{50}$/ml	copies/ PCR	Results
1	161.0	790	21/21	161.0	790	16/16
1:5	32.2	158	20/21	32.2	158	16/16
1:10	16.1	79	16/21	16.1	79	13/16
1:50	3.2	16	6/21	3.2	16	5/16
1:100	1.6	8	1/21	1.6	8	0/16
LoD_95	**36.0**	**175**	**N/A**	**23.8**	**117**	**N/A**

Tab. 9: : Summary of LoD results for **Seasonal Influenza A by Influenza A/M2** in nasal swabs and washes

	Inf A/M2 assay					
	Seasonal Influenza A in nasal swabs			**Seasonal Influenza A in nasal washes**		
	Virus concentration			Virus concentration		
Dilution	$TCID_{50}$/ml	copies/ PCR	Results	$TCID_{50}$/ml	copies/ PCR	Results
1	24600	300	16/16	24600	300	21/21
1:5	4920	60	16/16	4920	60	17/21
1:10	2460	30	16/16	2460	30	14/21
1:50	492	6	6/16	492	6	4/21
1:100	246	3	0/16	246	3	1/21
LoD_95	**2460***	**30***	**N/A**	**10400**	**128**	**N/A**

*Not determined by Probit Analysis

Over all sample materials, the results of the Probit analysis of the 95% confidence intervals show that the LoD values for two assays are as follows or lower:

LoD of Inf A/H1 assay:
54 copies per PCR or 11 $TCID_{50}$/ml for 2009 H1N1 influenza virus
LoD of Inf A/M2 assay:
175 copies per PCR or 36 $TCID_{50}$/ml for 2009 H1N1 influenza virus
LoD of Inf A/M2 assay: 128 copies/PCR or 10,400 $TCID_{50}$/ml for Seasonal Influenza A

11.2 Analytical reactivity (Inclusivity)

Tab. 10: Different subtypes of Influenza A were tested using the **RealTime ready Influenza A/H1N1 Detection Set**. The Influenza A subtypes listed were collected in different geographical regions and over a period of three decades. All isolates (high 10^3 and low 10^2 [pfu/PCR], each) were tested in a RT-PCR reaction for the Influenza A/M2 gene and the Influenza A/H1 2009 gene. All tested Influenza A subtypes show the expected positive results for Inf A/M2. All tested Influenza A subtypes show the expected negative results for Inf A/H1. This indicates that the Inf A/H1 assay is specific and detects 2009 H1N1 influenza virus, only.

No	ID	Subtype	Influenza A	H1N1 2009
1	A/FM/1/47	H1N1	positive	negative
2	A/PR8/34	H1N1	positive	negative
3	A2/Aichi2/68	H3N2	positive	negative
4	A/WSN/33 (6/99)	H1N1	positive	negative
5	A/turkey/England	H7N7	positive	negative
6	A/Brisbane/59/07 like	H1N1	positive*	negative
7	A/New Jersey/8/76	H1N1	positive	negative
8	A/Port Chalmers/1/73	H3N2	positive	negative
9	A/New Caledonia/20/99	H1N1	positive	negative
10	A/Victoria/3/75	H3N2	positive	negative
11	A2/Wisconsin/67/2005	H3N2	positive	negative
12	A/Moscow/10/99	H3N2	positive	negative
13	A/Brisbane/10/07	H3N2	positive	negative
14	A/California/7/04	H3N2	positive	negative

* weak signal

11.3 Analytical specificity (Cross-reactivity)

Tab. 11: Different viruses and bacteria were tested with Inf A/H1 and Inf A/M2. All of the tested viral and bacterial pathogens show negative results. This indicates that the **RealTime ready Influenza A/H1N1 Detection Set** is specific for 2009 H1N1 influenza virus and the assay does not cross-react with the tested pathogens. The data show 100% concordance with the expected results.

Viral pathogens		Concentration [copies/PCR]	Influenza A	H1N1 2009
No	**Organism**			
1	Adenovirus	9.5×10^5	negative	negative
2	Adenovirus	2.5×10^5	negative	negative
3	Human Corona Virus	2.0×10^2	negative	negative
4	Cytomegalovirus	2.0×10^2	negative	negative
5	Enterovirus	8.5×10^4	negative	negative
6	Epstein Barr Virus (EBV)	8.4×10^2	negative	negative
7	Human parainfluenza Type 1	1.7×10^2	negative	negative
8	Human parainfluenza Type 3	1.0×10^7	negative	negative
9	Respiratory syncytical virus (RSV)	6.0×10^5	negative	negative

Bacterial pathogens		Concentration [copies/PCR]	Influenza A	H1N1 2009
No	**Organism**			
1	*Bordetella pertussis*	$> 10^6$	negative	negative
2	*Chlamydia pneumoniae*	$> 10^6$	negative	negative
3	*Corynebacterium diphteriae*	$> 10^6$	negative	negative
4	*Escherichia coli*	$> 10^6$	negative	negative
5	*Haemophilus influenza*	$> 10^6$	negative	negative
6	*Lactobacillus rhamnosus*	$> 10^6$	negative	negative
7	*Legionella pneumophila*	$> 10^6$	negative	negative
8	*Moraxella catharralis*	$> 10^6$	negative	negative
9	*Mycobacterium tuberculosis*	$> 10^6$	negative	negative
10	*Mycoplasma pneumoniae*	$> 10^6$	negative	negative
11	*Neisseria meningitidis*	$> 10^6$	negative	negative
12	*Neisseria mucosa*	$> 10^6$	negative	negative
13	*Pseudomonas aeruginosa*	$> 10^6$	negative	negative
14	*Staphylococcus aureus*	$> 10^6$	negative	negative
15	*Staphlyococcus epidermidis*	$> 10^6$	negative	negative
16	*Streptococcus pneumoniae*	$> 10^6$	negative	negative
17	*Streptococcus pyogenes*	$> 10^6$	negative	negative
18	*Streptococcus salivarius*	$> 10^6$	negative	negative

11.4 Clinical performance

Clinical performance characteristics of the **RealTime ready Influenza A/H1N1 Detection Set** using the LightCycler® 2.0 Instrument was assessed by testing one hundred and twenty-nine upper respiratory specimens that had been previously tested with either the CDC rRT-PCR assay for 2009 H1N1 influenza or the Focus Diagnostics Diagnostics Influenza A H1N1 (2009) Real-Time RT-PCR assay. Clinical samples consisted of 53 nasopharyngeal swabs, 55 nasal washes, 16 nasal swabs, 3 throat swabs, and 2 swabs of undetermined origin. There was a total of 50 samples positive for Seasonal Influenza A and 26 samples positive for 2009 H1N1 influenza A virus included in the study. The results of the clinical study are summarized in Table 12.

Summary of the clinical performance evaluation

Tab. 12: Results of clinical performance studies

		Comparator*				
		Negative	**Seasonal Influenza A Positive**	**H1N1 2009 Positive**	**Total**	
RealTime ready InfluenzA A/H1N1 Detection Set	Negative	52	1	0	53	98% Agreement **Negatives** 95% CI (90%-100%)
	Seasonal Influenza A Positive	1	49	0	50	98% Agreement Seasonal **Influenza A** 95% CI (89%-100%)
	H1N1 2009 Positive	0	0	26	26	100% Agreement **H1N1 2009** 95% CI (87%-100%)
	Total	53	50	26	129	

* The comparator method was the CDC rRT-PCR Swine Flu Panel for all 50 seasonal influenza A positives and 26 H1N1 2009 influenza A positive samples and for 20 of the negative samples. The Focus Diagnostics Influenza A H1N1 (2009) Real-Time RT-PCR assay was the comparator for the other 33 negative samples.

Overall, 129 samples were included in this analysis. Positive agreement values were determined using the Statistical Analysis Software (SAS) from the SAS Institute. The calculation of the confidence intervals was done according to the Clopper-Pearson method.

The positive agreement found for the 2009 H1N1 influenza virus is 100% overall.

The positive agreement found for the Seasonal Influenza A is 98%.

One (1) of 50 samples, positive for Seasonal Influenza A using the comparator assay, was determined to be negative using the **RealTime ready Influenza A/H1N1 Detection Set.**

The negative percent agreement (Influenza A negative / H1N1 2009 negative) is 98%.

One (1) of 53 samples typed negative using the comparator assay was determined to be positive for Seasonal Influenza A using the **RealTime ready Influenza A/H1N1 Detection Set.**

12. SUPPLEMENTARY INFORMATION

12.1 Symbols

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

Symbol	Description
⚠	Information Note
Ⓘ	Important Note
REF	Catalogue Number
LOT	Batch code
⌛	Use by
	Temperature limitation (Store at)
D	Distributor
Cont.	Contents of kit
	Manufacturer

12.2 Changes to previous version

First version

12.3 References

1 Ward, C.L., Dempsey, H.M., Ring, C.J.A., Kempson, R.E., Zhang, L., Gor, D., Snowden, B.W., Tisdale, M. (2004) Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. *J. of Clin Virology*, **29**, p 179-88.

2 Panning M, Eickmann M, Landt O, Monazahian M, Ölschläger S, Baumgarte S, Reischl U, Wenzel JJ, Niller HH, Günther S, Hollmann B, Huzly D, Drexler JF, Hellmer A, Becker S, Matz B, Eis-Hübinger AM, Drosten C. (2009) Detection of Influenza A(H1N1)v virus by real-time RT-PCR. *Euro Surveill.*,**14(36)**, pii=19329 (Available online:http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19329.)

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