LightMix® Modular Respiratory Virus I Multiplex

TIB MOLBIOL

1. Intended Use

The products listed in section 3 are intended to be used for multiplex or single pathogen genome detections from nucleic acid extracts obtained from nasopharyngeal swabs, throat swabs, tracheal aspirates, sputum, bronchial wash, or bronchoalveolar lavage from patients who present with signs and symptoms compatible with acute influenza, allowing to detect Influenza A virus (all types), identification of Influenza A H1N1 new variant (2009), and/or Influenza B virus (qualitative testing). Molecular test results must not be the only basis for a therapy decision.

2. Introduction

Respiratory disease can be caused by viruses, and less often by bacteria or fungi. These instructions describe the use the LightMix® Modular Kits for multiplex PCR genome detection of pathogens, using Roche LightCycler® 480 II systems. For pathogen information and study results refer to the documentation included with the respective product.

LightMix® Modular (ModularDx) Kit General Information. Up to six channels can be used to achieve maximum throughput, enabling the detection of five or more pathogens in a single reaction. The testing panel can be customized to the individual laboratory testing requirements; single assays can be run or panels can be built by combining, omitting or replacing individual assays. In addition to target selection the laboratory can choose to include a control to monitor the entire process from extraction to amplification. The 660 (Cy5) channel is reserved for the Control Reaction; choose either an internal control, a spiked extraction control, an extraction control, or a Roche Process Controls (RPC).

3. LightMix® Modular Kits Components and Ordering Information

				channel
50-0100-96	07792204001	InfA (H1N1)	CE-IVD	500
53-0101-96	07792182001	InfA	CE-IVD	530
58-0102-96	07792212001	InfB	CE-IVD	580
				610
				640
66-0909-96	07374330001	EAV	Extraction Control	660
or	07099592001	Roche RNA	Process Control	
90-0100-01		Respiratory	Pos Control	

4. Multiplex PCR Combinations (examples)

Respiratory Virus Multiplex PCR

Color Compensation 40-0320 is mandatory for Multiplex PCR

500	530	580	610	640	660
	InfA				EAV or Roche RPC
		InfB			
	InfA	InfB			
H1	InfA	InfB			

480 II	z 480			
X	X	2plex		
X	X	2plex		
X	X	3plex		
X		4plex		

5. LightMix® Modular Kit Storage

Kits are shipped at ambient temperature (not on ice). Products have been tested to be stable even after three days storage at 60℃ (Tropical Climate Transp ort Stability Study).

Upon arrival store complete Kits at room temperature or refrigerated (4℃ - 25℃) and protected from day light. Do not freeze lyophilized reagents.

Once dissolved positive controls must be stored frozen.

Once dissolved reagents can be stored for up to 30 days refrigerated (for daily use), for longer term storage, store frozen at -20℃ until expiry. Avoid repeat freeze-thaw cycles (< 10 cycles).

To minimize the potential for error associated with the use of self-labeled tubes, it is recommend to not aliquot reagents to secondary storage vials.

6. Additional Reagents Required and Instrument Information

LightCycler® Multiplex RNA Virus Master		Cat.-No. 06 754 155 001
RNA Process Control Kit	(optional)	Cat.-No. 07 099 592 001
Uracil-DNA Glycosylase (UNG)	(optional)	Cat.-No. 03 539 806 001
LightCycler® 480 II Instrument		Cat.-No. 05 015 278 001
cobas z 480 Analyzer, UDF software 1.5		Cat.-No. 05 200 881 001
LightCycler® 480 Multiwell Plate 96 white or		Cat.-No. 04 729 692 001
LightCycler® 480 Multiwell Plate 384 white		Cat.-No. 04 729 749 001
Color Compensation Kit Hexaplex 40-0320-00		Cat.-No. 06 296 971 001
Bovine Serum Albumin (BSA)	(optional)	Sigma B4287

7. Precautions and Warnings

Handling Requirements
The product is an *in-vitro* diagnostic device and therefore must be used by qualified personnel only. Before using this product, read the operator / safety instructions in the instrument Operator's Manual.

General precautions for the handling of generic laboratory materials are required.

The laboratory work-flow must conform to standard practices. Due to the risk of contamination, PCR preparation and PCR amplification must be performed in physically separated areas.

Do not mix reagents from different lots. Do not combine other assays than those described in this manual.

Do not use reagents after the expiration date. Use the version of manual delivered with the kit (kit label).

Laboratory Procedures
All materials of human origin and related waste must be considered potentially infectious. Thoroughly clean and treat all work surfaces with disinfectants approved by local authorities.

Do not eat, drink or smoke in the laboratory working area. Do not pipette by mouth.

Wear disposable protective gloves, laboratory coats and adequate eye protection during the handling of samples and set components. Thoroughly wash your hands after handling the samples and reagents.

Avoid microbial or nuclease contamination of the reagents. The use of disposable sterile tips is essential.

Handling of Waste Materials
Dispose of the unused reagents and waste materials according to the current local laws.

Reading the Results and Reporting
Reading the results involves a general-use software which is has not specifically been adapted for the analysis of these reagents. Software 'positive' and 'negative' result calls must be verified by the operator to avoid the reporting of false results.

Molecular testing reports genome-based results indicating the presence of a pathogen. Molecular test results should not be the only basis for a therapy decision. PCR methodology is extremely sensitive and may report more positive results than classical methods; the Cp values can help to classify the significance of the reported pathogen, in particular where dual infections are detected. Contamination events can cause false positive results.

8. Background Information

8.1. Medical Background

Respiratory infectious disease can be caused by viruses, bacteria, and less frequent by fungi.

Upper respiratory tract infections such as the common cold are mostly less severe than lower tract infections. Respiratory infections can be fatal, in particular in case of underlaying disease, weakness or immune suppression, as well as in case of secondary (bacterial) infections causing a pneumonia.

The prevalence of respiratory viruses depends on the seasons, but also the age of the patients. Most common are RSV and HRV, and most deadly are Influenza and some Corona viruses.

Influenza occurs in the winter season, with a peak in February (in the northern hemisphere).

Influenza A virus has different types defined by their HA and NA genes; the virus is rapidly rearranging these genes and mutating, allowing to escape from the immune response and causing pandemic outbreaks, whereas Influenza B is genetically more stable and the population is more thoroughly immunized.

Testing for influenza helps to improve the treatment of patients with respiratory symptoms.

8.2. Methodology and Assay Principle

During the extraction step all cells and viruses contained in the sample are lysed. The genomic Nucleic Acids (RNA and DNA) are extracted and purified for detection utilizing Real-time Polymerase Chain Reaction (PCR) based amplification. LightCycler® Reagent kits incorporate dUTP into the PCR products. Uracil-DNA Glycosylase may be incorporated to prevent carry over contamination.

Each kit contains a pair of primers designed to specifically amplify a short fragment of the respective pathogen genome; this PCR fragment is detected with a dual labeled hydrolysis probe, which, upon sequence specific-cleavage by the polymerase, develops a light signal which is detected by the PCR instrument. The cycle number (Cp) is proportional to the amount of starting material, enabling an estimation of the amount of pathogen contained in the sample to be calculated.

The hydrolysis probe contains one of six possible dye labels (see section 3), enabling the detection of up to six different target reactions simultaneously. Each multiplex reaction contains a Control Reaction in the 660 channel, for monitoring lysis (Roche Process Control only), extraction, and amplification.

9. Specification and Evaluation Data

The Analytical Sensitivity of ModularDx kits has been determined for both single and/or multiplex PCR. Each reagent lot has been verified to detect at least 10 copies of target nucleic acid per 20 µl reaction in a single PCR. Signal levels (normalized to a reference instrument), Cp values related to the amount of target and cut-off values are printed in the Certificate-of-Analysis (CoA), included with each kit.

Evaluation data and clinical study results are contained in the Technical Information document included with the product. Summary of evaluation results :

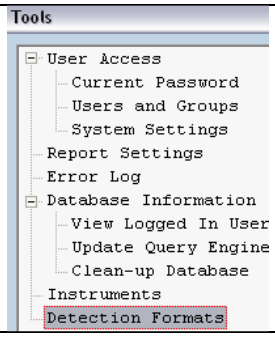
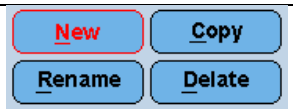
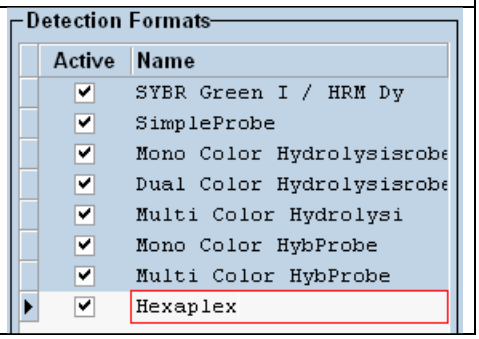
Channel	**500**	**530**	**580**		
Pathogen	**H1**	**InfA**	**InfB**		
LOD^{single} (copies/rx)	11.0	24.0	n.d.		
$LOD^{multiplex}$ (copies/rx)	10.5	9.7	8.7		
95% CI	8.1-17.3	7.7-13	6.4-17		
Specificity	passed [2]	passed [3]	passed [4]		
Diag. Sensitivity	100%	100%	100%		
Diag. Specificity	100%	100%	100%		
Prevalence					
PPV					
NPV					
Intra Assay Variation	0.19%	0.60%	0.17%		
Inter Assay Variation	0.45%	0.89%	0.85%		
Inter Lot Variation	0.43%	0.95%	0.98%		

n.d. = not determined
[1] PIV, RSV, EV, HRV, MPV, ADV, CoV and common respiratory bacteria tested negative
[2] InfA H3, H5, H7 tested negative [3] InfB negative [4] InfA negative

10. Instructions for Use

See the Instrument operator's manual for details. Start programming before preparing the solutions.

10.1. Dye Channels and Color Compensation

1) Open Tools	2) Select Detection Formats	3) New	4) Set name Hexaplex
			

Define Emission and Excitation filters for channels 500, 530, 580, 610, 640 and 660; (channel 500 is not available for cobas z 480). Set all parameters as describe below; see also the detailed instructions in the manual: ***LightMix 40-0320 Universal Color Compensation Hexaplex***

Name	500	530	580	610	640	660
LightCycler® 480	450-500	483-533	523-568	523-610	523-640	615-670
LightCycler® 480 II	440-488	465-510	533-580	533-610	533-640	618-660
cobas z 480	n.a.	465-510	540-580	540-610	540-645	610-670
Quant Factor *	10	10	10	10	10	10
Max Integration Time*	1 sec	1 sec	1 sec	2 sec	3 sec	3 sec

* Adaption of instrument settings is allowed (under the responsibility of the operator).

10.2. Programming Roche '480' Instruments

For use with Roche '480' instruments, software 1.5 and higher. See the Instrument operator's manual for details. Program the instrumentation prior to reagent preparation.

The protocol consists of four program steps:

- 1: Reverse Transcription of the viral RNA
- 2: Denaturation: sample denaturation and enzyme activation
- 3: Cycling: PCR-amplification
- 4: Cooling: cooling the instrument

Program Step:	***RT Step***	***Denaturation***	***Cycling***			***Cooling***
Parameter						
Analysis Mode	***None***	None	Quantification mode			None
Cycles	*1*	1	45			1
Target [°C]	55	95	95	60	72	40
Hold [hh:mm:ss]	00:05:00	00:05:00	00:00:05	00:00:15	00:00:15	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	4.4	2.2	4.4	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	4.6	2.4	4.6	2.0
Acquisition Mode	None	None	None	**Single**	None	None

10.3. Experimental Protocol

- **Sample material:** Use Nucleic Acid preparations (e.g. 'High Pure Viral Nucleic Acid Kit').
- **Positive Controls:** Every ModularDx kit is delivered with a single target Positive Control. These controls must be used in single reactions only. Mixtures of these controls not tested.
- **Premixed Controls:** Alternatively use the panel-specific premixed positive controls (2 vials) covering channels 500, 580 and 640 (Mix I) and 530, 610 (optional 640) and 660 (Mix II).
- **Negative Controls:** Use an extracted negative sample containing the Control Reaction target. If using water remember to add proteins (BSA) prior to extraction.

10.3.1. Preparation of Parameter-Specific Reagents (PSR):

Add 50 µl PCR-grade water to each reagent vial, mix the solution (vortex) and spin down.
For robotic pipetting the volume can be extended to 55 µl (signals will decrease by 10-20%).

►**Use 0.5 µl** reagent **per 20 µl** reaction.

10.3.2. Preparation of the Positive Control

Use Premixed Controls Mix I and II or single Positive Controls. One reagent vial contains for 32 reactions.

Add 160 µl PCR-grade water to the vial with the **black** cap. Mix by pipetting up and down 10 times: mixing by vortex may generate aerosol causing contamination.

►**Use 5 µl** positive control **per 20 µl** reaction.

10.3.3. Preparation of the Reaction Mix

The pipetting scheme for a single assay reaction is contained with every kit. The table below allows to set up pipetting from duplex to hexaplex assays with 20 µl. Choose between EAV (**blue**) and RPC (**purple**).

Include in every run one Positive Controls (for each target), and at least one 'No Template Control' (NTC). In a cooled tube, prepare the reaction mix by multiplying the single reaction volumes (left column) by the number of reactions including the controls plus one additional reaction; the smallest recommended pipetting volume is 1 µl; prepare a minimum of 10 reactions. Instructions for 96 well plates right column:

One reaction	Hexaplex Reaction Roche Multiplex Master	100 reactions
6.9 µl	**Water**, PCR-grade (colorless cap, provided with the Roche Master kit)	690 µl
0.5 µl	**EAV Control Reaction** mix or **Water**	50 µl
1.0 µl	**Water** or **RPC detection assay** (Roche vial 5)	100 µl
0.5 µl	**Reagent** mix (primers / probes) first target assay	50 µl
0.5 µl	**Reagent** mix second target assay or **Water**	50 µl
0.5 µl	**Reagent** mix third target assay or **Water**	50 µl
0.5 µl	**Reagent** mix fourth target assay or **Water**	50 µl
0.5 µl	**Reagent** mix fifth target assay or **Water**	50 µl
4.0 µl	**Roche Master** (see Roche manual)	400 µl
0.1 µl	**RT Enzyme**	10 µl
15.0 µl	**Volume of Reaction Mix**	**1500 µl**

One reaction	Duplex Reaction Multiplex DNA Master (example)	100 reactions
9.9 µl	**Water**, PCR-grade (colorless cap, provided with the Roche Master kit)	990 µl
0.5 µl	**EAV Control Reaction** mix	50 µl
0.5 µl	**Reagent** mix (primers / probes) first target assay	50 µl
4.0 µl	**Roche Master** (see Roche manual)	400 µl
0.1 µl	**RT Enzyme**	10 µl
15.0 µl	**Volume of Reaction Mix**	**1500 µl**

Mix gently, spin down and **transfer 15 µl** of master mix to each well. Stepping up the PCR reactions in a cooling plate is recommended.

Add 5 µl of sample or control DNA to each well. Seal plate and centrifuge. **Start run**

Do not touch the foil without gloves. Avoid prolonged waiting times before starting the instrument.

11. Reading the Results

Perform data analysis as described in the operator's manual. Use the Second Derivative Maximum method (Automated (F'' max). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. Repeat analysis for each dye channel used: (1) Open a new analysis, (2) Name the analysis after the channel and pathogen, (3) Apply the color compensation file, (4) Analyze and save.

Runs are valid if results generated for all controls are correct: Positive Controls are positive (within Cp range), Negative Controls are negative, and Control Reactions (where applicable) are positive.

11.1. Control Reaction - Validation of the run

Start the analysis from 660 Channel. Check the amplification curves are visible.
If the Control Reaction signals are missing the run is not valid and the pathogen results must be not used.

- If using the premixed Positive Controls and EAV an amplification curve should be visible for Mix II.
- If using with the single pathogen Positive Controls and EAV check the extracted Negative Control for the presence of an amplification curve with a Cp value in the range 27-33.
- If using the Roche Process Control check the extracted negative sample containing the Roche Control shows an amplification curve with a Cp range as outlined in the Roche manual.

Note: In case that the Negative Control used does not contain the Control Target (not recommended), the presence of Control Reaction signals in samples may be used to verify the functionality of the Control.

11.2. Pathogen Results

11.2.1. Verification of channel specific results

Perform analyses and review for each channel used, verify that each channel result is valid:

- Target specific ***Positive Control must be positive*** and within the Cp range of the CoA.
- Target specific ***Negative Control must be negative.***
- Target specific Positive Controls for other channels in use must be negative, indicating that the color compensation is switched on and is functional.

11.2.2. Target positive and target negative samples

Identify samples as target-positive, negative (below detection limit) or equivocal :

- Select **'Positive'** results.
- Identify all sample with a Cp value within the defined cut-off.
- Identify samples with erroneous false-positive calls by visual inspection and correct; false-positive calls can be generated as a result of incorrect interpretation of curves due to baseline or residual signals from a another channel, that has been not corrected by the color compensation (check other channels).
- Document all **'Positive'** samples and identify samples with manually corrected results.
- For high positive samples (Cp < 24) for one target and for positive samples with a negative Control Reaction result repeating the test with all other target single assays is recommended to avoid to miss low positive results in mixed infection samples.

- List all samples with a Cp value higher than the cut-off as '**Equivocal'**.
  The cut-off in the CoA is set based on the established limit-of-detection (LOD) of each respective assay. The LOD describes the amount of target that will be reliably detected in every run. Results with a later cycle are most likely true positive results, however they are outside the reporting range. It is highly recommend to repeat the test for '**Equivocal'** samples, preferably using a new extract from the same or a new collected specimen. If inhibition is not an issue, use more sample. If the test is not repeated report **'Not detectable'**.

- Select **'Negative'** results.
- Visually inspect the negative curves, identify any amplification curves that have been called falsely negative, correct the result and mark samples where the results have been manually corrected. False negative calls are rare.
- Determine **'Negative'** sample results in relation to the Control Reaction results (see 11.2.3)

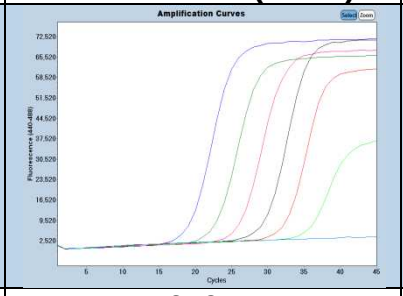
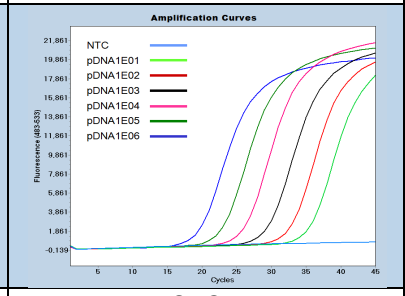
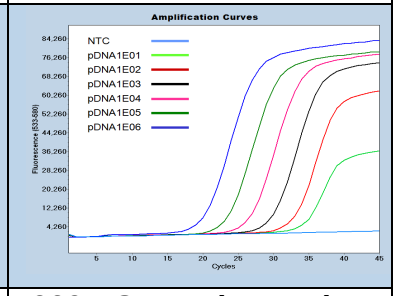
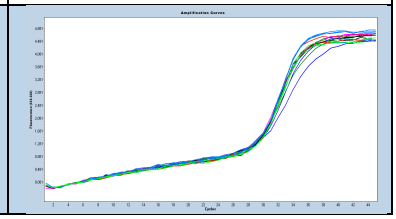
11.2.3. Determine true 'Negative' Results

- Identify samples which are negative for all pathogen channels
- Check that these samples have an amplification curve present in the Control Reaction (channel 660).
- Report the **'Negative'** samples with a positive result for the Control Reaction as **'Not detectable'** with the meaning **'below the defined detection limit'** for the target pathogen.
- Report the **'Negative'** samples with a positive result for any other pathogen as **'Not detectable'**.
- Report **'Negative'** samples with a negative result for the Control Reaction as "**Inhibited / failed to generate a result**" and request a new sample if applicable.

11.2.4. Report of Equivocal samples

Repeat analysis results for '**Equivocal**' samples not resolved as **'Positive'** or **'Not detectable'** must be reported as **'Potentially positive, below detection limit'**.

Document all manually corrected results and include the reason for adjustment.

	500 : InfA H1 (H1N1)	530 : Influenza A	580 : Influenza B
LC 480 II			
	610 :	**640 :**	**660 : Control Reaction**
LC 480 II			

11.3. Sample Results Interpretation :

The Control Reaction must be visible in all wells (Positive or Pass), if negative the sample is inhibited: repeat sample processing from extraction.
Any NTC positive in the target channels identifies a contamination: DO NOT report results, repeat entire amplification run.

500	530	580	610	640	660 Control	500-640 NTC	Result
negative	negative	negative	negative	negative	Detectable	negative	No virus detectable
Cp < cutoff	negative	negative	negative	negative	irrelevant*	negative	*InfA H1*
negative	Cp < cutoff	negative	negative	negative	irrelevant*	negative	*InfA - positive*
negative	negative	Cp < cutoff	negative	negative	irrelevant*	negative	*InfB - positive*
negative	negative	negative	Cp < cutoff	negative	irrelevant*	negative	*Pathogen 610 positive*
negative	negative	negative	negative	Cp < cutoff	irrelevant*	negative	*Pathogen 460 positive*
negative	negative	negative	negative	negative	negative	irrelevant	Inhibition/failure Repeat
irrelevant	irrelevant	irrelevant	irrelevant	irrelevant	irrelevant	positive	Contamination Repeat

* High positive samples might mask the amplification of other targets present in the sample. Positive target results combined with negative Control Reaction results can be an indication for inhibition. Repeat test with single target assays is recommended (see 11.2.2).

12. References

InfA H1N1
Detection of influenza A(H1N1)v virus by real-time RT-PCR. Panning et al., Euro Surveill 2009

InfA
Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Spackman Eet al., JCM 40 (2002)

InfA, InfB
Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. Ward CL, Dempsey MH, Ring CJ, Kempson RE, Zhang L, Gor D, Snowden BW, Tisdale M. J Clin Virol. 2004

13. MSDS

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the EU Directives 67/548/EC and 1999/45/EC any products which do not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

14. Version History

Notes in red mark: events requiring changes in procedures.

V151111	Draft version	2015-11-11
V160202	Editorial Changes, Corrections, Clinical Data added	2016-04-20

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