PROMISE
Preparing for RSV Immunisation and Surveillance in Europe

PROMISE RSV-LabNet Protocol Library

PCR - RSV A/B, Influenza B/Vic and B/Yam

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1. Reagents

RNA Extraction

MagNa Pure 96 System (Roche Material Number: 065 410 890 01) using MagNA Pure 96 DNA and Viral Nucleic Acid Small Volume Kit (Roche Catalog Number: 065 435 880 01)

Lightcycler PCR

PCR reactions should be performed on Lightcycler 480 with TaqMan™ Fast Virus 1-Step Master Mix (ThermoFisher Scientific Catalog Number: 444 443 2)

Primer Sets

RSV-A

2050F	TGA ACA ACC CAA AAG CAT CA
2117R	CCT AGG CCA GCA GCA TTG
2086probe2	*5'-6Fam* AAT TTC CTC ACT TCT CTA GTG TAG TAT TGG G *3'-BHQ1*

RSV-B

17fw2	GAT GGC TCT TAG CAA AGT CAA GTT GA
120	TGT CAA TAT TAT CTC CTG TAC TAC GTT GAA
PB45probe	*5'-Texas Red* TGA TAC ATT AAA TAA GGA TCA GCT GCT GTC ATC CA *3'-BHQ1*

Influenza B-HA (Specific B-Yam and B-Vic probes)

HA-444F2	ACC CTA CRA AAT TGG AAC CTC AGG
HA-444F3	ACC CTA CAG ACT TGG AAC CTC AGG
HA-524R	ACR GCC CAA GCC ATT GTT G
Yam501probe	*5'-Atto425* AAA TCC GAT TTT ACT GGT AG *3'-MGBeclipse*
Vic499probe	*5'-Atto532* ATC CTT TTC CAT TGG TAA *3'-MGBFQ*

Primers and Probes Information

RSV-infB mix	Label	Filter (Nm)	pmol in PCR-mix	Subset
RSV A 2050F			**15**	**RSVA**
RSV A 2117R			**15**	
RSV A 2086P2	FAM-BHQ1	483-533	**5**	
RSV B 17 fw2			**15**	**RSVB**
RSV B 120			**15**	
RSV B PB45	TXR-BHQ2	558-610	**5**	
INFB-HA-444F2			**9**	**VIC**
INFB-HA-444F3			**9**	**YAM**
INFB-HA-524R			**12**	
INFB-Yam501Pr	ATTO425-MGBeclipse	450-500	**3**	**YAM**
INFB-Vic499Pr	ATTO532-MGBeclipse	523-568	**2**	**VIC**

Target	Sequence	Filter (nm)	Subset name
RSVA	FAM-BHQ1	483-533	RSVA
RSVB	TXR-BHQ2	558-610	RSVB
InfB-Vic	ATTO532-MGBeclipse	523-568	VIC
InfB-Yam	ATTO425-MGBeclipse	450-500	YAM

2. Procedure

Preparation

1. Make appropriate dilutions of positive controls.
2. Isolate RNA by MagNA Pure 96 DNA and Viral Nucleic Acid Small Volume Kit (Roche Catalog Number: 065 435 880 01) Preheat two thermoblocks on 50ºC and 95ºC
3. Make PCR-mix for RSVA, RSVB, InfB-Vic, InfB-Yam (reagents lab)

PCR-Mix	conc.	[end-conc.]	µl
4x Taqman Fast Virus MM			5.0
RSV-InfB primer/probe mix			3.0
PCR Grade Water			7.0
Total volume			**15.0**

Sample Addition, Reverse Transcription, and PCR

1. Aliquot 15 ul portions of PCR-mix in 96-well plate according to plate layout template
2. Add 5 uL RNA to 15 ul PCR-mix
3. Seal the 96-well plate
4. Centrifuge briefly
5. Incubate directly 15 minutes at 50ºC (heating block)*****
6. Incubate directly 2 minutes at 95ºC (heating block)*****
7. Centrifuge briefly
8. Keep plate at 4ºC if you can't run directly
9. Run PCR Program on LightCycler 480
10. Analyse results

***Note –** RIVM uses thermoblock reverse transcription step instead of performing this step in the LightCycler to allow waiting times between preparation of the reaction 96-well plate and putting the plate in the LightCycler. If the plate is only kept cool during waiting time this reduces the performance of the RT-PCR. If immediately after preparing the reaction plate the reverse transcription is performed and after that the plate is kept cool, there is no impact on performance. If a lab has enough capacity to put the plate after reaction preparation immediately on the LightCycler, this separate thermoblock reverse transcription step can be avoided. However, in that situation, the reverse transcription step has to be programmed in the temperature programme of the LightCycler.

PCR-program LightCycler 480:

Detection Format:	**Multi Color Hydrolysis Probe**				
PCR Program	**Segment Number**	**Temp (ºC)**	**Hold Time (sec.)**	**Slope (ºC/sec.)**	**Acquisition Mode**
Reverse Transcription	1	50	900	EXTERNAL	
Inactivate RT, activate Pol.	1	95	120	EXTERNAL	
Denaturation	1	95	60	4.4	None
Amplification (Cycles: 50)	1 2	95 55	10 30	4.4 2.2	None Single
Cooling	1	40	30	4.4	None

Positive Control Information*

Control	**Name**
Positive Control	**Current strains from own repository**

***Note –** The positive control material used at RIVM are strains from the RIVM repository. RIVM can provide Influenza B viruses, but at this moment, RIVM cannot provide RSV.

Additional Note(s)

Assay validation report is available if requested. The PCR performs with 100% correct results at RIVM in QCMD influenza, RSV, and respiratory panels, WHO EQAP influenza panels, and in WHO RSV EQA pilot panel by NEQAS.