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Manual

MutaPLEX®
RespiraSys 1

real time RT-PCR kit

Test for the in vitro detection of RNA of the influenza A virus, the influenza B virus and the respiratory syncytial virus in clinical samples

Valid from 2015-10-29

REF **KG198432**
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1 INTENDED USE

The MutaPLEX® RespiraSys 1 real time RT-PCR TM is an assay for the detection of RNA of influenza A and B virus as well as respiratory syncytial virus (RSV) in clinical specimens (e. g. throat swabs, nasal swabs, bronchoalveolar lavage, liquor) using open real time PCR microplate systems.

2 PATHOGEN INFORMATION

MutaPLEX® RespiraSys 1 is a multiplex real time RT-PCR for the detection of causative agents of respiratory diseases. The MutaPLEX® RespiraSys 1 real time RT-PCR is designated for pathogens with a RNA genome: influenza A virus, influenza B virus, respiratory syncytial virus (RSV). MutaPLEX® RespiraSys 1 real time RT-PCR allows a fast, efficient and cost effective diagnostic.

Influenza viruses belong to the family of *Orthomyxoviridae* and are the causative agent of the flu. Influenza A and B viruses have a single stranded RNA genome, consisting of 8 RNA segments. The genome of influenza A viruses is characterized by a high mutation frequency, the so-called antigenic drift. Numerous subtypes of influenza A viruses are known. They can be categorized by their surface antigens H (haemagglutinin) and N (neuraminidase): Influenza A (H1N1) virus, influenza A (H5N1) virus etc. Therefore, yearly *in silico* analysis of the sequences of newly emerged subtypes is done, to prevent false negative results caused by primer and/or probe mismatches.

Respiratory syncytial viruses are enveloped negative-sense, single stranded RNA viruses of the *Paramyxoviridae* familiy. RSV is a member of the subfamily *Pneumovirinae*, genus *Pneumovirus*. RSV are divided into subgroups A and B. RSV is a virus that causes infections of the lungs and respiratory tract. It's so common that most children have been infected with the virus by age 2. RSV can also infect adults.

In adults and older, healthy children, the symptoms of RSV infections are mild and typically mimic the common cold. Self-care measures are usually all that is needed to relieve any discomfort. Infection with RSV can be severe in some cases, especially in premature babies and infants with underlying health conditions. RSV can also become serious in older adults, adults with heart and lung diseases, or anyone with a very weak immune system (immunocompromised).

3 PRINCIPLE OF THE TEST

The MutaPLEX® RespiraSys 1 real time RT-PCR kit contains specific primers and dual-labelled probes for the amplification and detection of RNA of influenza A and B virus as well as respiratory syncytial virus (RSV) in clinical specimens (e. g. throat swabs,

nasal swabs, bronchoalveolar lavage, liquor) after the extraction of RNA from the sample material.

The reverse transcription (RT) of viral RNA to cDNA and the subsequent amplification of RespiraSys 1-specific fragments are performed in an one-step RT-PCR. The amplification can be detected when specific probes are hydrolysed by the polymerase. The presence of nucleic acid is detected by an increase in fluorescence due to hydrolysis of the probes during amplification.

The fluorescence of the pathogen-specific probes is measured in the FAM (influenza A), Cy5 (influenza B) or ROX (RSV) channel.

Furthermore, MutaPLEX® RespiraSys 1 real time RT-PCR kit contains a control RNA, which is added during RNA extraction and detected in the same reaction by a differently labelled probe.

The control RNA allows the detection of RT-PCR inhibition and acts as control for the isolation of the nucleic acid from the clinical specimen.

The fluorescence of the control RNA is measured in the VIC®/HEX/JOE/TET channel.

4 PACKAGE CONTENTS

The reagents supplied are sufficient for 32 (KG198432) or 96 (KG198496) reactions, respectively.

Table 1: Components of the MutaPLEX® RespiraSys 1 real time RT-PCR kit.

Label	Lid Colour	Content	
		32	96
Reaction Mix	yellow	1 x 506 µl	2 x 759 µl
Enzyme	blue	1 x 6.4 µl	1 x 19.2 µl
Positive control	red	1 x 50 µl	1 x 100 µl
Negative control	green	1 x 50 µl	1 x 100 µl
Control RNA	colourless	1 x 160 µl	2 x 240 µl

5 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- RNA isolation kit (e. g. MutaCLEAN® Universal RNA/DNA, KG1038)
- PCR grade water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter

- Table centrifuge
- Vortex mixer
- Real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation
- Optional: VLP-RNA (virus-like particles, please see chapter 11 for details).

  * Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

6 TRANSPORT, STORAGE AND STABILITY

The MutaPLEX® RespiraSys 1 real time RT-PCR-Kit is shipped on dry ice or cool packs. All components must be stored at maximum -18 °C in the dark immediately after receipt. Up to 20 freeze and thaw cycles are possible.

Do not use reagents after the date of expiry printed on the package. Opened reagents can be stored at 2–8 °C for up to 6 months.

Protect kit components from direct sunlight during the complete test run.

7 IMPORTANT NOTES

- The MutaPLEX® RespiraSys 1 real time RT-PCR must be performed by qualified personnel only.
- Good Laboratory Practice (GLP) has to be applied.
- Clinical samples must always be regarded as potentially infectious material and all equipment used has to be treated as potentially contaminated.

8 GENERAL PRECAUTIONS

- Stick to the protocol described in the instructions for use.
- Set up different laboratory areas for the preparation of samples and for the set up of the RT-PCR in order to avoid contaminations.
- Pipettes, tubes and other materials must not circulate between those different laboratory areas.
- Always use filter tips.
- Regulary decontaminate equipment and benches with ethanol-free decontaminant.

- Do not combine MutaPLEX® RespiraSys 1 real time RT-PCR-Kit components of different lot numbers.

9 SAMPLE MATERIAL

Starting material for the MutaPLEX® RespiraSys 1 real time RT-PCR is viral RNA isolated from clinical specimens (e. g. throat swabs, nasal swabs, bronchoalveolar lavage, liquor).

10 SAMPLE PREPARATION

The MutaPLEX® RespiraSys 1 real time RT-PCR is suitable for the detection of RNA isolated from clinical specimens with appropriate isolation methods.

Commercial kits for RNA isolation such as MutaCLEAN® Universal RNA/DNA (KG1038) are recommended.

Important: In addition to the samples, always run a water control in your extraction. Treat this water control analogous to a sample.

Comparing the amplification of the control RNA in the samples to the amplification of the internal control in the water control will give insights on possible inhibitions of the real time RT-PCR. Furthermore, possible contaminations during RNA extraction will be detectable.

Please note chapter 11 "Control RNA".

If the real time RT-PCR is not performed immediately, store extracted RNA according to the instructions given by the RNA extraction kit's manufacturer.

11 CONTROL RNA

A control RNA is supplied and can be used as extraction control or only as inhibition control. This allows the user to control the RNA isolation procedure and to check for possible real time RT-PCR inhibition.

The virus-like particles (VLP-RNA) are not supplied.

a) Control RNA or VLP-RNA used as extraction control

MutaPLEX® RespiraSys 1 control RNA or VLP-RNA is added to the RNA extraction.

Add 5 µl control RNA or VLP-RNA per extraction (5 µl x (N+1)). Mix well. Perform the RNA isolation according to the manufacturer's instructions. Please follow protocol A.

The control RNA must be added to the lysis buffer of the extraction kit.

b) Control RNA used as internal control of the real time RT-PCR

If only inhibition will be checked, please follow protocol B.

12 REAL TIME RT-PCR

12.1 Important points before starting

- Please pay attention to chapter 7 "Important Notes".
- Before setting up the real time RT-PCR familiarise yourself with the real time PCR instrument and read the user manual supplied with the instrument.
- The programming of the thermal profile should take place before the RT-PCR set up.
- In every RT-PCR run, one positive control and one negative control should be included.
- Before each use, all reagents – except the enzyme – should be thawed completely at room temperature, thouroughly mixed (do NOT vortex the reaction mix but mix by pipetting up and down repeatedly), and centrifuged very briefly.
- We recommend to keep reagents and samples at 2–8 °C (e. g. on ice or a cooling block) at all times.

12.2 Procedure

If the control RNA or VLP-RNA is used to control both, the real time RT-PCR and the RNA isolation procedure, please follow protocol A. If the control RNA is solely used to detect possible inhibition of the real time RT-PCR, please follow protocol B.

Protocol A

The control RNA or VLP-RNA was added during RNA extraction (see chapter 11 "Control RNA"). In this case, prepare the master mix according to table 2.

The master mix contains all of the components needed for RT-PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Table 2: Preparation of the master mix (control RNA was added during RNA extraction)

Volume per reaction	Volume master mix
15.8 µl Reaction Mix	15.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)

Protocol B

The control RNA is used for the control of the real time RT-PCR only (see chapter 11 "Control RNA"). In this case, prepare the master mix according to table 3.

The master mix contains all of the components needed for real RT-PCR except the sample. Prepare a volume of master mix for at least one sample more than required, in order to compensate for pipetting inaccuracy.

Important: Dilute the **control RNA 1:10** in ultra pure water (e.g. 1 µl control RNA + 9 µl ultra pure water) before adding it to the master mix.

Table 3: Preparation of the master mix (control RNA is added directly to the master mix)

Volume per reaction	Volume master mix
15.8 µl Reaction Mix	15.8 µl x (N+1)
0.2 µl Enzyme	0.2 µl x (N+1)
0.2 µl Control RNA* **diluted 1:10**	0.2 µl x (N+1)*

*The increase in volume caused by adding the control RNA is not taken into account when preparing the PCR assay. The sensitivity of the detection system is not impaired.

Protocol A and B: real time RT-PCR set up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument.
- Pipet 16 µl of the master mix into each optical PCR reaction tube.
- Add 4 µl of the eluates from the RNA isolation (including the eluate of the water control), the positive control and the negative control to the corresponding optical PCR reaction tube (table 4).
- Close the optical PCR reaction tubes immediately after filling in order to reduce the risk of contamination.

Table 4: Preparation of the real time RT-PCR

Component	Volume
Master mix	16.0 µl
Sample	4.0 µl
Total volume	20.0 µl

12.3 Instrument settings

For the real time RT-PCR, use the thermal profile shown in table 5.

Table 5: real time RT-PCR thermal profile

Description	Time	Temperature	No of cycles
Reverse Transcription	10 min	45 °C	1
Initial Denaturation	5 min	95 °C	1
Amplification of DNA			45 Aquisition at the end of this step
Denaturation	10 s	95 °C	
Annealing	40 s	60 °C	

Dependent on the real time instrument used, further instrument settings have to be adjusted according to table 6.

Table 6: Overview of the instrument settings required for the MutaPLEX® RespiraSys 1 real time RT-PCR.

Real time RT-PCR Instrument	Parameter	Detection Channel	Notes		
LightCycler 480I	Influenza A RSV Control RNA Influenza B	483–533 558–610 523–568 615–670	Color compensation kit needed*		
LightCycler 480II			Color compensation kit needed*		
			Melt factor	Quant factor	Max integration time [s]
	Influenza A	465–510	1	10	1
	RSV	533–610	1	10	2
	Control RNA	533–580	1	10	2
	Influenza B	618–660	1	10	3
Stratagene Mx3000P/ Mx3005P	Influenza A RSV Control RNA Influenza B	FAM ROX HEX Cy5	Gain 8 Gain 1 Gain 1 Gain 4	Reference Dye: None	
ABI 7500	Influenza A RSV Control RNA Influenza B	FAM ROX HEX Cy5	Option Reference Dye ROX: NO		

Real time RT-PCR Instrument	Parameter	Detection Channel	Notes	
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Influenza A RSV Control RNA Influenza B	Green Orange Yellow Red	Gain 5 Gain 5 Gain 5 Gain 5	

* can be ordered at Immundiagnostik on request

13 DATA ANALYSIS

The influenza A-specific amplification is measured in the FAM channel, RSV in the ROX channel and influenza B in the Cy5 channel. The amplification of the control RNA is measured in the VIC®/HEX/JOE/TET channel.

The following results can occur:

- **A signal in the FAM channel is detected:**

  The result is positive, the sample contains influenza A RNA.

  In this case, detection of a signal of the control RNA in the VIC®/HEX/JOE/TET channel is inessential, as high concentrations of influenza A cDNA may reduce or completely inhibit amplification of the control RNA.

- **A signal in the ROX channel is detected:**

  The result is positive, the sample contains RSV RNA.

  In this case, detection of a signal of the control RNA in the VIC®/HEX/JOE/TET channel is inessential, as high concentrations of RSV cDNA may reduce or completely inhibit amplification of the control RNA.

- **A signal in the Cy5 channel is detected:**

  The result is positive, the sample contains influenza B RNA.

  In this case, detection of a signal of the control RNA in the VIC®/HEX/JOE/TET channel is inessential, as high concentrations of influenza B cDNA may reduce or completely inhibit amplification of the control RNA.

- **No signal in the FAM/ROX/Cy5 channels, but a signal in the VIC®/HEX/JOE/TET channel is detected:**

  The result is negative, the sample does not contain influenza A, B or RSV RNA.

  The signal of the control RNA excludes the possibilities of RNA isolation failure (in case the control RNA is being used as an extraction control) and/or real

time RT-PCR inhibition. If the CT value of a sample differs significantly from the CT value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see „Troubleshooting“).

- **Neither in the FAM/ROX/Cy5 channels nor in the VIC®/HEX/JOE/TET channel a signal is detected:**

  A diagnostic statement cannot be made.

  The RNA isolation was not successful or an inhibition of the RT-PCR has occurred. In case the control RNA was added during RNA isolation and not directly to the PCR master mix, the negative control is negative in both channels.

Figure 1 and figure 2 show examples for positive and negative real time RT-PCR results.

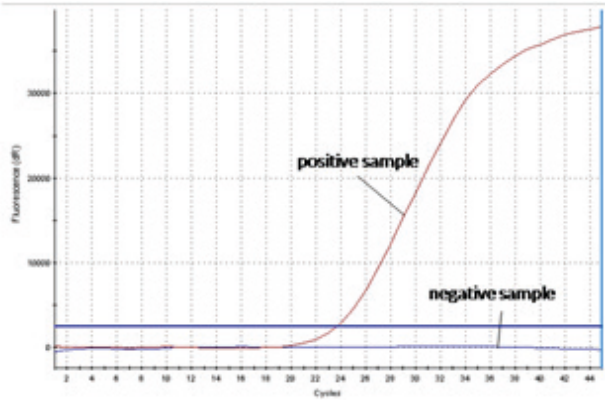

Figure 1: The positive sample shows virus-specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

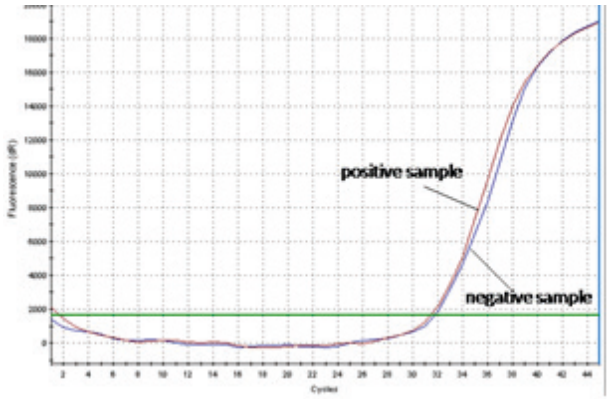

Figure 2: The positive sample as well as the negative sample show a signal in the control RNA-specific VIC®/HEX/JOE/TET channel. The amplification signal of the control RNA in the negative sample shows that the missing signal in the virus-specific FAM channel is not due to RT-PCR inhibition or failure of RNA isolation, but that the sample is a true negative.

14 ASSAY VALIDATION

Set a threshold as follows:

Negative controls

All negative controls should be below the threshold. If there is a potential contamination (appearance of a curve in the negative control or a cluster of curves in specimens at high CT – for example above 36), results obtained are not interpretable and the whole run (including extraction) has to be repeated.

Positive controls

All the positive controls must show a positive (i. e. exponential) amplification curve. The positive controls must fall below a CT of 30.

Internal controls

All internal controls must show a positive (i. e. exponential) amplification curve. The internal control must fall below a CT of 33. If the internal control is above CT 34, this points to a purification problem or a strong positive sample that can inhibit the IC. In the latter case, the assay is valid. If a water control run is performed, the IC must fall below a CT of 33.

15 LIMITATIONS OF THE METHOD

The results must always be considered in relation to the clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.

A negative test result does not exclude an influenza A, B or RSV infection.

16 TROUBLESHOOTING

The following troubleshooting guide is included to help you with possible problems that may arise when performing a real time RT-PCR.

No fluorescence signal in the FAM, ROX and Cy5 channel of the positive control

The selected channel for analysis does not comply with the protocol

Select the FAM/ROX/Cy5 channel for analysis of the influenza A-, B- or RSV-specific amplification and the VIC®/HEX/JOE/TET channel for the amplification of the control RNA.

Incorrect configuration of the real time RT-PCR

Check your work steps and compare with chapter "Procedure".

The programming of the thermal profile is incorrect

Compare the thermal profile with the protocol (table 5).

Incorrect storage conditions for one or more kit components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter "Transport, Storage and Stability".

Weak or no signal of the control RNA and simultaneous absence of a signal in the virus-specific FAM/ROX/Cy5 channel

real time RT-PCR conditions do not comply with the protocol

Check the real time RT-PCR conditions (chapter 12).

real time RT-PCR inhibited

Make sure that you use an appropriate isolation method (see "Sample preparation") and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffer of the isolation kit has been completely removed. An additional centrifugation step at high speed is recommended before elution of the RNA.

RNA loss during isolation process

In case the control RNA was added before extraction, the lack of an amplification signal can indicate that the RNA isolation was not successful. Make sure that you use an appropriate isolation method (commercial kits are recommended) and stick to the manufacturer's protocol.

Incorrect storage conditions for one or more components or kit expired

Check the storage conditions and the date of expiry printed on the kit label. If necessary, use a new kit and make sure kit components are stored as described in chapter "Transport, Storage and Stability".

Detection of a fluorescence signal in the FAM/ROX/Cy5 channel of the negative control

Contamination during preparation of the RT-PCR

Repeat the real time RT-PCR in replicates. If the result is negative in the repetition, the contamination occured when the samples were pipetted into the optical PCR reaction tubes. Make sure to pipet the positive control last and close the optical PCR reaction tube immediately after adding the sample. If the same result occurs, one or more of the kit components might be contaminated. Make sure that work space and instruments are decontaminated regularly. Use a new kit and repeat the real time RT-PCR.

17 KIT PERFORMANCE

17.1 Diagnostic Sensitivity and Specificity

During the validation study of the MutaPLEX® RespiraSys 1 real time RT-PCR kit, 100 positive and 39 negative samples were tested. The diagnostic sensitivity was found to be 100 % and the diagnostic specificity 100 % (table 7).

The positive predictive value was found to be 100 %, the negative predictive value showed to be 100 %.

Table 7: Overview of the amount of samples tested and the resulting positive and negative predictive values

	positive samples	negative samples
MutaPLEX® RespiraSys 1 positive	100	0
MutaPLEX® RespiraSys 1 negative	0	39
Sensitivity	100 %	
Specificity	100 %	

17.2 Analytical Sensitivity

The limit of detection (LoD) of the MutaPLEX® RespiraSys 1 real time RT-PCR kit was determined using serial dilutions of influenza virus A, influenza virus B and RSV in virus transport medium in a Stratagene Mx3000 real time PCR instrument. Before total nucleic acids extraction, each sample was supplemented with 5 µl control-RNA. Total nucleic acids were eluted with 50 µl and 4 µl of the eluates were applied to the subsequent real time RT-PCR.

The LoD of the MutaPLEX® RespiraSys 1 real time RT-PCR kit for influenza virus A, influenza virus B and RSV is ≥ 10 genome copies per reaction each.

The sensitivity of the MutaPLEX® RespiraSys 1 real time RT-PCR kit was also analysed by testing round robin samples of known status. All samples of the QCMD influenza A and B panel were detected correctly. Likewise the samples of the RSV ring trial (INSTAND e.V.). Results are shown in table 8.

Table 8: Samples tested for the validation of the sensitivity of the MutaPLEX® RespiraSys 1 real time RT-PCR Kit.

Sample	Sample content	Expected result	Result MutaPLEX® RespiraSys 1	Sample type
13-01	Influenza A H5N1	positive	positive	educational
13-02	Influenza B Yamagata	positive	positive	educational
13-03	Influenza A H3N2	positive	positive	core
13-04	Influenza B Victoria	positive	positive	educational
13-05	negative	negative	negative	core
13-06	Influenza A H1N1 pdm09	positive	positive	educational
13-07	Influenza A H3N2	positive	positive	educational
13-08	Influenza B Yamagata	positive	positive	educational

Sample	Sample content	Expected result	Result MutaPLEX® RespiraSys 1	Sample type
13-09	Influenza A H1N1 pdm09	positive	positive	core
13-10	Influenza B Victoria	positive	positive	educational
RSV14-01	RSV negative	negative	negative	core
RSV14-02	RSV A	positive	positive	core
RSV14-03	RSV B	positive	positive	core
RSV14-04	RSV A	positive	positive	educational
RSV14-05	RSV B	positive	positive	educational
RSV14-06	RSV B	positive	positive	core
RSV14-07	RSV B	positive	positive	core
RSV14-08	RSV A	positive	positive	core
359021	RSV A	positive	positive	-
359022	RSV B	positive	positive	-
359023	RSV A	positive	positive	-
359024	RSV A	positive	positive	-

17.3 Analytical Specificity

The specificity of the MutaPLEX® RespiraSys 1 real time RT-PCR was evaluated with different influenza virus A, RSV, and influenza virus B strains as well as with other relevant viruses and bacteria found in clinical samples. The MutaPLEX® RespiraSys 1 real time RT-PCR showed positive results for all samples containing influenza A, influenza B, or RSV, whereas samples containing other pathogens were reliably tested negative. The results are shown in table 9.

Table 9: Bacterial and viral pathogens tested for the determination of the analytical sensitivity of the MutaPLEX® RespiraSys 1 real time RT-PCR kit.

Strain	Expected Result	Result
Influenza virus A A/ Brisbane H1N1 59/2007 E40/08	positive	positive
Influenza virus A Indonesia H5N1 05/2005	positive	positive
Influenza virus A New Caledonia 20/99 H1N1	positive	positive
Influenza virus A Panama H3N2 2007/99	positive	positive
Influenza virus B B/ Brisbane 60/2008 E09/09	positive	positive

Strain	Expected Result	Result
Influenza virus B Jiangsu 10/2003	positive	positive
RSV strain A2 ATCC-VR-1540	positive	positive
RSV strain B WV/14617/85 ATCC-VR-1400	positive	positive
Parainfluenza virus type 3 str. C243 VR93	negative	negative
Mycoplasma pneumoniae ATCC 15531	negative	negative
Chlamydophila pneumoniae str. CM-1, ATCC-VR-1360	negative	negative
Adenovirus	negative	negative
Legionella pneumophila serogroup 2	negative	negative
Rhinovirus type 3 FEBVR483	negative	negative
Streptococcus agalactiae	negative	negative
Coxsackie virus B5	negative	negative
Borrelia burgdorferi Strain 4681	negative	negative

18 ABBREVIATIONS AND SYMBOLS

cDNA	complementary Deoxyribonucleid Acid	CONTROL –	Negative control
CT	Cycle Threshold	CONTROL RNA IC	Control RNA
nn	not known	→REF	To be used with
PCR	Polymerase Chain Reaction	REF	Catalog number
RNA	Ribonucleid Acid		Contains sufficient for <n> test
RSV	Respiratory Syncytial Virus		Upper limit of temperature
RT	Reverse Transcription		Manufacturer
VLP	Virus-Like Particles		Use by

VTM	Virus Transport Medium	LOT	Lot number
REACTION MIX	Reaction Mix	CONT	Content
ENZYME	Enzyme		Consult instructions for use
CONTROL +	Positive control	IVD	In vitro diagnostic medical device

19 REFERENCES

Lothar Thomas, Labor und Diagnose: Indikation und Bewertung von Laborbefunden für die medizinische Diagnostik, 8. Auflage, 2012, TH-Books, ISBN-10: 3980521583