Instruction for Use

respiraRNA 2.0

real time RT-PCR Kit

For the *in-vitro* detection of the RNA of Influenzavirus A, Influenzavirus B and Respiratory Syncytial Virus in clinical specimens.

REF	G01084-32	G01084-96
Σ	32	96

- 18°C

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Index

1 Intended Use

The respiraRNA 2.0 real time RT-PCR Kit is an assay for the detection of RNA of Influenza A Virus, RSV, Influenza B Virus in clinical specimens (e.g. throat swabs, nasal swabs, bronchoalveolar lavage, liquor) using open real time PCR systems.

2 Pathogen Information

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 catergorized 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's 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 respiraRNA 2.0 real time RT-PCR Kit contains specific primers and hydrolysis probes for the detection of the RNA of Influenza A virus, RSV and Influenza B virus 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 virus specific fragments are performed in a one-step RT-PCR. The amplification can be detected when specific probes are hydrolysed by the Polymerase. The emitted fluorescence is measured in the FAM (*Influenza A virus*), ROX (*RSV*), and Cy5 (*Influenza B virus*) channel.

Furthermore, the respiraRNA 2.0 real time PCR Kit contains a Control RNA, which is detected in a second amplification system. Added during RNA extraction, the Control RNA allows not only for the detection of RT-PCR inhibition but also detects possible mistakes during RNA extraction. This greatly reduces the risk of false-negative results. The Control RNA can also be used solely as Internal Control by adding it directly to the Mastermix. 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 or 96 reactions respectively.

Table 1: Components of the respiraRNA 2.0 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 *Influenza A, Influena B, RSV*	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. NukEx Pure RNA/DNA, gerbion Cat. No. G05004 or NukEx Mag RNA/DNA, gerbion Cat. No. G05012)
- PCR grade Water
- Sterile microtubes
- Pipets (adjustable volume)
- Sterile pipet tips with filter
- Table centrifuge
- Vortexer
- Real time PCR instrument
- Optical PCR reaction tubes with lid
- Optional: Liquid handling system for automation
- Optional: VLP-RNA (please look at page 7 for details)

6 Transport, Storage and Stability

The respiraRNA 2.0 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. Do not use reagents after the date of expiry printed on the package.
Up to 20 freeze and thaw cycles are possible.
For convenience, 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 respiraRNA 2.0 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 instruction 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 respiraRNA 2.0 real time RT-PCR Kit components of different lot numbers.

9 Sample Material

Starting material for the respiraRNA 2.0 real time RT-PCR is viral RNA isolated from clinical specimens (e.g. throat swabs, nasal swabs, bronchoalveolar lavage, liquor).

10 Sample Preparation

The respiraRNA 2.0 real time RT-PCR is suitable for the detection of *Influenza A Virus, RSV, Influenza B Virus* in clinical specimens (e.g. throat swabs, nasal swabs, bronchoalveolar lavage, liquor) isolated with suitable isolation methods.

Commercial kits for RNA isolation such as the following are recommended:

- **NukEx Pure RNA/DNA**, gerbion Cat. No. G05004
- **NukEx Mag RNA/DNA**, gerbion Cat. No. G05012

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 sample 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 the chapter ‚Control RNA' on page 7.

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

Further information about RNA isolation is to be found in the extraction kit manual or from the extraction kit manufacturer's technical service.

11 Control RNA

A Control RNA is supplied to be used as Extraction 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, but must be added to the clinical or environmental samples directly. VLP-RNA can be used as patient-side extraction control and in automated extraction systems, when pipetting of the Control RNA to the first buffer of (e.g. binding buffer) of the respective extraction kit is not possible due to extraction instrument specifications.

RNA isolation from clinical specimens (e.g. throat swabs, nasal swabs, bronchoalveolar lavage, liquor).

a) Control RNA or VLP-RNA used as Extraction Control:
respiraRNA 2.0 real time RT-PCR 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 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 the ‚Important Notes' on page 5.
- 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 PCR set up.
- In every PCR run a 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.

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 ‚Control RNA', page 7). 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

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 ‚Control RNA', page 7). 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 PCR-grade dH_2O (e.g. 1 µl Control RNA + 9 µl PCR grade Water before adding it to the Master Mix.)

Table 3: Preparation of 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.

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 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	Number of Cycles
Reverse Transcription	10 min	45°C	1
Initial Denaturation	5 min	95°C	1
Amplification of cDNA			
Denaturation	10 sec	95°C	45
Annealing and Extension	40 sec Aquisition at the end of this step	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 respiraDNA real time PCR.

Real time PCR Instrument	Parameter	Detection Channel	Notes		
LightCycler 480I	Influenza A Virus	483-533	Color Compensation Kit Multiplex 1 (G070MP1-cc) required		
	RSV	558-610			
	Control RNA	523-568			
	Influenza B Virus	615-670			
LightCycler 480II			Color Compensation Kit Multiplex 1 (G070MP1-cc) required		
			Melt Factor	Quant Factor	Max Integration Time (sec)
	Influenza A Virus	465-510	1	10	1
	RSV	533-610	1	10	2
	Control RNA	533-580	1	10	2
	Influenza B Virus	618-660	1	10	3
Stratagene Mx3000P / Mx3005P	Influenza A Virus	FAM	Gain 8		
	RSV	ROX	Gain 1		Reference Dye: None
	Control RNA	HEX	Gain 1		
	Influenza B Virus	Cy5	Gain 4		
ABI 7500	Influenza A Virus	FAM	Option Reference Dye ROX: NO		
	RSV	ROX			
	Control RNA	JOE			
	Influenza B Virus	Cy5			
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	Influenza A Virus	Green	Gain 5		
	RSV	Orange	Gain 5		
	Control RNA	Yellow	Gain 5		
	Influenza B Virus	Red	Gain 5		

13 Data Analysis

The virus specific amplifications are measured in the FAM, ROX, Cy5 channels. The amplification of the Control RNA is measured in the VIC®/HEX/JOE/TET channel. The Positive Control contains in vitro transcripts of the respective nucleic acid sequences of Influenza A virus, Influenza B virus and Respiratory Syncytial Virus. For the Positive Control signals in the FAM, ROX, Cy5 channels must be detected.

Following results can occur:

- A signal in the FAM channel is detected:
  The result is positive, the sample contains Influenza A virus 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 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 Respiratory Syncytial Virus 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 cDNA may reduce or completely inhibit amplification of the Control RNA.

- A signal in the Cy 5 channel is detected:
  The result is positive, the sample contains Influenza B virus 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 cDNA may reduce or completely inhibit amplification of the Control RNA.

- No signal in the FAM, ROX and Cy5 channel, but a signal in the VIC®/HEX/JOE™/TET channel is detected:
  The result is negative, the sample does neither contain *Influenza A virus* RNA nor *Respiratory Syncytial Virus* RNA nor *Influenza B virus* 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 C_T value of a sample differs significantly from the C_T value of the water control, a partial inhibition occured, which can lead to negative results in weak positive samples (see „Troubleshooting“, page 15).

- **Neither in the FAM, ROX, Cy 5 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.

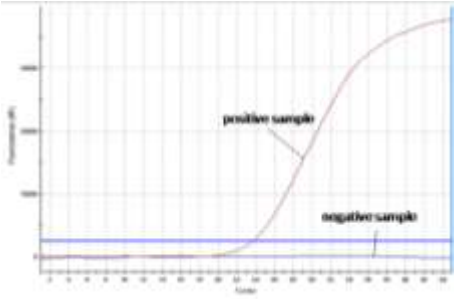

Figure 1: The positive sample shows virus-specific amplification in the FAM channel, whereas no fluorescence signal is detected in the negative sample.

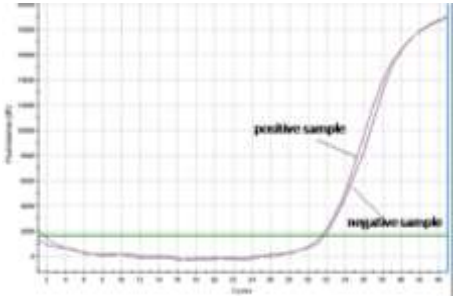

Figure 2: The positive sample as well as the negative sample show a signal in the Control RNA-specific VIC®/HEX/JOE™/TET channel.
The amplification signal of the Control RNA in the negative sample shows, that the missing signal in the virus-specific FAM channel is not due to RT-PCR inhibition or failure 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 C_T – 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 C_T of 30.

Internal Controls

All internal controls must show a positive (i.e. exponential) amplification curve. The internal control must fall below a C_T of 33. If the internal control is above C_T 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 C_T of 33.

15 Limitations of the Method

The results must always be considered in relation tot he clinical symptoms. Therapeutical consequences should be made in consideration of clinical data.
A negative test result does not exclude an *Influenza B Virus*, *RSV or Influenza A Virus* 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. If you have further questions, please do not hesitate to contact our scientists on info@gerbion.com.

No fluorescence signal in the FAM, ROX, Cy 5 channel of the Positive Controls	
The selected channel for analysis does not comply with the protocol	Select the FAM channel for analysis of the Influenza A specific amplification, the ROX channel for analysis of the RSV specific amplification and the Cy5 channel for analysis of the Influenza B Virus specific amplification (Reaction Mix). Select the VIC®/HEX/JOE™/TET channel for the amplification of the Control RNA. Due to amplification in the specific channels, amplification of the Internal Control can be inhibited in the Positive Control.
Incorrect configuration of the real time RT-PCR	Check your work steps and compare with ‚Procedure' on page 8.
The programming of the thermal profile is incorrect	Compare the thermal profile with the protocol (Table 5, page 10).
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 ‚Transport, Storage and Stability', page 5.
Weak or no signal of the Control RNA and simultaneous absence of a signal in the virus specific FAM channel, ROX channel or Cy 5 channel.	
real time RT-PCR conditions do not comply with the protocol	Check the real time RT-PCR conditions (page 8).
real time RT-PCR inhibited	Make sure that you use an appropriate isolation method (see ‚Sample Preparation', page 6) 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 ‚Transport, Storage and Stability', page 5.

Detection of a fluorescence signal in the FAM channel, ROX channel or Cy 5 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 respiraRNA 2.0 real time RT-PCR 100 positive and 39 negative samples were tested. The diagnostic sensitivity was found to be 100 % and the diagnostic specificity 100 %
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
respiraRNA 2.0 positive	100	0
respiraRNA 2.0 negative	0	39
Sensitivity	100 %	
Specificity	100 %	

17.2 Analytical Sensitivity

The limit of detection (LoD) of the respiraRNA 2.0 real time RT-PCR Kit was determined using serial dilutions of Influenzavirus A, Influenzavirus B und RSV in Virus transport medium in a Stratagene Mx3000 real time PCR instrument. Total nucleic acids were extracted using NukEx Pure RNA/DNA (gerbion) according to the manufacturer´s instructions. Each sample was supplemented with 5 µl Control-RNA prior to extraction. 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 respiraRNA 2.0 real time RT-PCR Kit for Influenzavirus A, Influenzavirus B and RSV is $\geq$10 genome copies per reaction each.

The sensitivity of the respiraRNA 2.0 real time RT-PCR kit was also analysed by testing round robin samples of known status.

Results:
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 respiraRNA 2.0 real time RT-PCR Kit.

Sample	Sample Content	Expected Result	Result respiraRNA 2.0	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
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 respiraRNA 2.0 real time RT-PCR was evaluated with different *Influenzavirus A, RSV, Influenazvirus B* strains as well as with other relevant viruses and bacteria found in clinical samples.

Results:

The respiraRNA 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 respiraRNA 2.0 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
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
Parainfluenzavirus Typ 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 Typ 3 FEBVR483	negative	negative
Streptococcus agalactiae	negative	negative
Coxsackievirus B5	negative	negative
Borrelia burgdorferi Strain 4681	negative	negative

18 Abbreviations and Symbols

cDNA	complementary Deoxyribonucleid Acid	REF	Catalog number
RNA	Ribonucleic Acid		Contains sufficient for <n> test
PCR	Polymerase Chain Reaction		Upper limit of temperature
RT	Reverse Transcription		Manufacturer
RSV	Respiratory Syncytial Virus		Use by YYYY-MM
REACTION MIX	Reaction Mix	LOT	Batch code
ENZYME	Enzyme	CONT	Content
CONTROL +	Positive Control		Consult instructions for use
CONTROL −	Negative Control	IVD	*In vitro* diagnostic medical device
CONTROL RNA IC	Control RNA	CE	European Conformity

19 Literature

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