Instruction for Use

virellaASFV seqc

real time PCR Kit

In vitro veterinary diagnostic tool for simultaneous detection of pathogen DNA of African swine fever (African Swine Fever Virus, ASFV) and an Internal Process Control as well as an Internal System Control in eluates of single or pooled samples from serum, plasma, EDTA blood, saliva, swab and tissue samples from pigs and boar.

The German version of the Instruction for Use is approved according to German law TierGesG § 11 section 2.

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- 18°C		

Contents

1 Intended Use

The virellaASFV seqc real time PCR Kit is an in vitro veterinary diagnostic tool for simultaneous detection of pathogen DNA of African swine fever (African Swine Fever Virus, ASFV) and an Internal Process Control as well as an Internal System Control in eluates of single or pooled samples from serum, plasma, EDTA blood, saliva, swab and tissue samples from pigs and boars.
The pooling of samples is possible if local regulations allow it. For additional information (German language only) visit www.fli.de/de/publikationen/amtliche-methodensammlung.

2 Pathogen Information

African swine fever is a notifiable animal disease that affects domestic pigs and boar. In the African countries of origin, Argasidae (soft ticks) transmit ASFV. These are irrelevant for transmission in Central Europe where transmission takes place through direct contact with infected animals (secretions, blood, sperm), the consumption of food waste or pork products as well as other indirect transmission paths (vehicles, contaminated equipment including hunting equipment, agricultural equipment and machines, clothing). Contact with blood is the most efficient way of transmission. After infection, the animals develop very severe but unspecific general symptoms. African swine fever is not a zoonosis, an infectious disease that can be transmitted between animals and humans and is therefore not dangerous to humans.

3 Principle of the Test

The virellaASFV seqc real time PCR Kit contains specific primers and dual-labelled probes as well as additional material for the simultaneous detection of pathogen DNA of African swine fever (African Swine Fever Virus, ASFV) and an Internal Process Control (IPC) as well as an Internal System Control (ISC) in eluates of single samples or pool samples from serum, plasma, EDTA blood, saliva, swab and tissue samples from pigs and boar using real time PCR in open real time PCR systems (e.g. Stratagene Mx3000P / 3005P, Agilent AriaMx, Roche LightCycler 480II, Life Technologies ABI 7500 and QuantStudio 5, Qiagen Q-Cycler, BioRad CFX96, BMS mic qPCR Cycler).
The amplification is detected in real time by the hybridization and subsequent hydrolysis of the ASFV-specific Cy5-labeled fluorescence probes. In addition, the virellaASFV seqc real time PCR kit contains a Control DNA which is added during extraction and is detected in a heterologous amplification system. The amplification of the Control DNA (Internal Process Control, IPC) is detected in the VIC®/HEX channel. Furthermore, a cellular gene region (succinate dehydrogenase, Internal System Control, ISC) is amplified in a further heterologous amplification system. The amplification of the ISC is detected in the ROX channel. The control of the sample and extraction quality (seqc - sample and extraction quality control) with the help of the ISC and IPC enables the detection of errors during extraction, possible inhibitory

effects in the PCR and provides information about the quality of the extracted cell-containing sample material.

4 Package Contents

The reagents supplied are sufficient for 96 or 384 reactions, respectively.

Table 1: Components of the virellaASFV seqc real time PCR Kit.

Label	Lid Colour	Content G01123-96	Content G01123-384
Reaction Mix	yellow	1 x 1344 µl	4 x 1344 µl
Positive Control	red	1 x 150 µl	1 x 150 µl
Negative Control	green	1 x 150 µl	1 x 150 µl
Control DNA	colourless	1 x 480 µl	4 x 480 µl

5 Equipment and Reagents to be Supplied by User

- Extraction kit (e. g. NukEx Mag RNA/DNA, gerbion Cat. No. G05012)
- sterile reaction tubes
- Pipets (adjustable volume)
- DNase/RNase-free disposable pipet tips with aerosol barriers
- Table centrifuge
- Vortexer
- real time PCR instrument
- Color Compensation Kit Multiplex 1 for Roche LightCycler 480II (gerbion Cat. No. G070MP1-CC)
- Optical PCR reaction tubes or optical PCR reaction plates
- optional: liquid handling system for automation

6 Transport, Storage and Stability

The virellaASFV seqc real time 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 kit label. For convenience, opened reagents can be stored at +2-8°C for up to 6 months. If the reagents are stored at -18 ° C or lower temperatures, up to 20 thawing and freezing cycles are possible. Protect kit components from direct sunlight during the complete test run.

7 Important Notes

- The virellaASFV seqc real time PCR must be performed in laboratories suitable for this purpose and by qualified personnel only.
- The guidelines of Good Laboratory Practice (GLP) have to be followed.
- All samples must be considered as potentially infectious and all objects in contact with the samples must be considered as potentially contaminated.

8 General Precautions

- The instructions in the Instruction for Use must be followed.
- Areas for sample preparation and the preparation of the PCR master mix should be strictly separated.
- Always use DNase/RNase-free disposable pipet tips with aerosol barriers.
- Regularly clean pipets and workspaces with a suitable decontamination solution (no ethanol-containing agents).
- Components from different lots of the virellaASFV seqc real time PCR Kit must not be used together.

9 Sample Material

The starting material for the detection reaction is DNA, which has been extracted from single samples or pool samples of serum, plasma, EDTA blood, saliva, swab and tissue samples from pigs and boar using suitable methods.

10 Sample Preparation

It is recommended to use commercially available extraction kits, e.g.:

- NukEx Mag RNA/DNA, gerbion Cat. No. G05012
- NukEx Pure RNA/DNA, gerbion Cat. No. G05004
- QIAamp® cador® Pathogen Mini Kit, Qiagen Cat. No. 54104/54106
- MagMax Core Nucleic Acid Purification Kit, ThermoFisher Cat. No. A32700/A32702

Important: Independent of the sample material used, a water control (ultrapure water) should be extracted in addition to the samples, which will give insights on possible inhibitions of the real time PCR or possible contaminations during DNA extraction. This water control must be treated analogously to a sample to be examined. A delay in reaching the C_T value of the IPC (HEX channel) in the eluate of a sample of > 4 C_T compared to the C_T value of the IPC in the eluate of the extracted water indicates the presence of inhibitors in the detection reaction.

Please note the chapter ‚Control of sample and extraction quality'.

If the real time PCR is not performed immediately, store extracted nucleic acids according to the instructions given by the manufacturer.

For more information on isolating nucleic acids, please refer to the Instruction for Use of the extraction kit or the extraction kit manufacturer's technical service.

Pooling of samples
Please inform yourself about the current status at www.fli.de/de/publikationen/amtliche-methodensammlung.

EDTA blood samples, serum and plasma samples, saliva samples
Pool aliquots of the same size (e.g. 100 µl each) from single samples and mix well (vortex), then extract according to the manufacturer's instructions.

Tissue samples and swab samples
Pool aliquots of the same size (e.g. 10 µl each) of the digestion supernatants from single samples and then mix well (vortex). Extract the pool according to the manufacturer's instructions.

11 Control of sample and extraction quality

The virellaASFV seqc real time PCR kit contains a Control DNA (IPC), which is added during extraction and detected in the VIC®/HEX channel. The amplification of a cellular gene region (succinate dehydrogenase, Internal System Control, ISC) is detected in the ROX channel. The detection of these two controls enables the detection of errors in the extraction as well as possible inhibitions of the PCR and provides information about the quality of the extracted sample material. This reduces the risk of false negative results.

11.1 Control DNA used as Extraction Control

Add 5 µl Control DNA per extraction (5 µl x (N+1)). Mix well. Perform the DNA isolation according to the manufacturer's instructions.

The Control DNA must be added to the Lysis Buffer of the extraction kit.

12 Real time PCR

12.1 Important Points Before Starting:

- Please note the 'Important Notes' on page 5.
- In every PCR run one Positive Control and one Negative Control should be included.
- Before each use, all reagents should be thawed completely at room temperature, thoroughly mixed, and centrifuged very briefly.

12.2 Procedure

The Master Mix is prepared as described in Table 2. The Master Mix contains all the components needed for real time PCR except the sample. Prepare a volume of Master Mix for at least one sample more than required to compensate for pipetting inaccuracy.

Table 2: Preparation of the Master Mix

Volume per Reaction	Volume Master Mix
14.0 µl Reaction Mix	14.0 µl x (N+1)

real time PCR set-up

- Place the number of optical PCR reaction tubes needed into the respective tray of the real time PCR instrument / take an optical PCR reaction plate.
- Pipet **14 µl** of the Master Mix into each optical PCR reaction tube / the optical PCR reaction plate.

- Add **6 µl** of the eluates from the DNA isolation (including the eluate of the water control), the Positive Control and the Negative Control to the corresponding optical PCR reaction tube / the optical PCR reaction plate.
- Close the optical PCR reaction tubes / the optical PCR reaction plate immediately after the samples have been added to reduce the risk of contamination.
- When using a Roche LC480II real time PCR instrument the closed the closed optical PCR reaction plate must be centrifuged briefly.

Table 3: Preparation of the real time PCR.

Component	Volume
Master Mix	14.0 µl
Sample	6.0 µl
Total Volume	20.0 µl

12.3 Instrument Settings

For the real time PCR use the thermal profile shown in Table 4.

Table 4: real time PCR thermal profile.

Description	**Time**	**Temperature**	**Number of Cycles**
Reverse Transcription*	20 min	50°C	1
Initial Denaturation	15 min	95°C	1
Amplification of DNA			
Denaturation	15 sec	95°C	45
Annealing	30 sec Acquisition at the end of this step	57°C	
Extension	30 sec	72°C	

*The thermal profile corresponds to the profile for all gerbion detection methods for detecting the pathogens of classical swine fever (CSFV) and African swine fever (ASFV).

Depending on the real time PCR instrument used, further instrument settings have to be adjusted.

Table 5 gives an example of an overview of the necessary settings for common real time PCR instruments. For information on settings on other real time PCR instruments, please contact our service at info@gerbion.com.

Table 5: Overview of the instrument settings required for the virellaASFV seqc real time PCR.

Real time PCR Instrument	**Parameter Reaction Mix**	**Detection Channel**	**Notes**		
LightCycler 480II			Color Compensation Kit Multiplex 1 (G070MP1-CC) required		
			Melt Factor	Quant Factor	Max Integration Time (sec)
	ASFV	CY5 (618-660)	1	10	3
	IPC	HEX (533-580)	1	10	2
	ISC	ROX (533-610)	1	10	2
Stratagene Mx3000 / Mx3005	ASFV	CY5	Gain 4		
	IPC	HEX	Gain 1	Reference Dye: None	
	ISC	ROX	Gain 1		
AriaMx CFX96	ASFV	CY5			
	IPC	HEX		Reference Dye: None	
	ISC	ROX			
ABI 7500 QuantStudio 5	ASFV	CY5			
	IPC	HEX	Option Reference Dye ROX: NO		
	ISC	ROX			
Rotor-Gene Q, Rotor-Gene 3000 Rotor-Gene 6000	ASFV	Red	Gain 5		
	IPC	Yellow	Gain 5		
	ISC	Orange	Gain 5		
mic qPCR Cycler	ASFV	Red	Gain 10		
	IPC	Yellow	Gain 10		
	ISC	Orange	Gain 10		

13 Data Analysis

The amplification of the DNA of the pathogen of African swine fever is shown in the Cy5 channel (red). The amplification of the Control DNA (IPC) is measured in the VIC®/HEX channel (yellow). The detection of the amplification of the Internal System Control (ISC) takes place in the ROX channel (orange).

Following results can occur:

Ct Values			**Interpretation**
Cy5 (red) **ASFV**	**HEX** (yellow) **IPC**	**ROX** (orange) **ISC**	
positive	positive or negative	positive or negative	**Positive result, the sample contains DNA of ASFV. Valid result.** The results of the IPC and ISC are irrelevant.
negative	positive	positive	**Negative result, the sample contains no DNA of ASFV. Valid result.**
negative	negative	positive	**Extraction problems or PCR inhibition. Invalid result.** Dilute the sample or extract the sample again and repeat the PCR.
negative	positive	negative	**Not enough or bad sample material. Invalid result.** Extract fresh sample material and repeat the PCR.
negative	negative	negative	**Invalid result.** No diagnostic statement can be made. The real time PCR is either inhibited or errors occurred during the extraction.

*Depending on the real time PCR instrument and the extraction method used, the C_T areas of the Control DNA may shift slightly. The C_T value of the extracted water control serves as a reference. With the extraction procedures mentioned in Chapter 10 ‚Sample Preparation', the C_T value of the extracted water control is $\leq$34. If the C_T value for the water control in the HEX channel differs greatly from the C_T value of the sample (> 4 C_T delayed signal compared to the water control), there is a partial inhibition that can lead to weakly positive samples cannot be detected (see 'Troubleshooting', page 13).

Figure 1, Figure 2 and Figure 3 show examples of positive and negative real time PCR results.

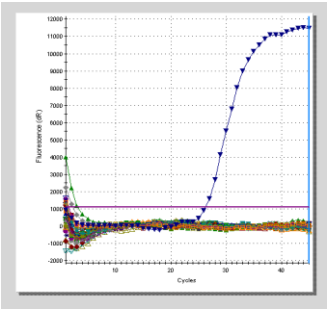


Figure 1: Detection of amplified ASFV DNA in the Cy5 channel. No fluorescence signal is detected for negative samples.

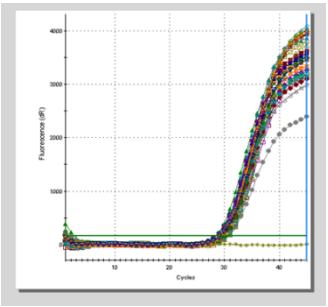


Figure 2: Detection of the IPC amplification in the VIC®/HEX channel.

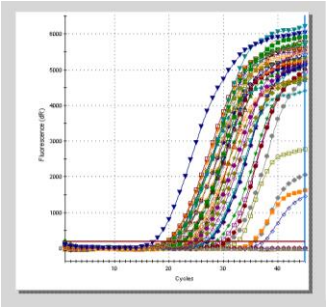


Figure 3: Detection of the ISC amplification in the ROX channel. Different C_T values result from the respective quality and quantity of the DNA in the sample material/eluate.

14 Assay Validation

Negative Control
The Negative Control must be below the threshold in the Cy5 channel (red) in the ROX channel (orange). In the event of potential contamination of this control (occurrence of a curve), the results of the test cannot be evaluated. The test must be repeated.

Positive Control
The Positive Control must show a sigmoid curve in the Cy5 channel (red). The C_T value of the Positive Control must be < 30. A Positive Control outside of this range indicates a problem with the amplification. In this case the test must be repeated.

Internal System Control, ISC
The ISC must show a sigmoid curve in the ROX channel (orange). The C_T values should be below 35. Higher C_T values indicate too little or too much decomposed sample material.
Strongly positive ASFV samples can lead to a loss of amplification of the ISC. Conversely, a competitive effect on weakly positive ASFV samples, triggered by a heavy loading of an eluate with cellular DNA, was experimentally excluded.

Internal Process Control, IPC
The IPC must show a sigmoid curve in the HEX channel (yellow). No amplification should be observed in the Negative Control and the Positive Control. The C_T values of the IPC in reaction batches with eluates from sample material should occur a maximum of 4 C_T values later than the C_T values in reaction batches with eluted water control. Strongly positive samples in the Cy5 channel can lead to a failure of the IPC in the HEX channel. In this case the result of the sample is valid.

15 Limitations of the Method

The virellaASFV seqc real time PCR cannot be carried out directly with biological materials. Appropriate nucleic acid extraction procedures must be performed before using the test.
As with any in vitro diagnostic test, the results obtained with the virellaASFV seqc real time PCR kit must always be interpreted in consideration of all clinical and laboratory findings.

16 Troubleshooting

The following troubleshooting guide is intended to help with any problems that may arise when performing a real time PCR. If you have any further questions, please contact our scientists at info@gerbion.com.

No fluorescent signal in the Cy5 channel of the Positive Control.	
The selected channel does not correspond to the channel specified in the protocol.	Select the Cy5 channel (red) for the analysis of ASFV-specific amplification, the ROX channel (orange) for the amplification of the ISC and the VIC®/HEX channel (yellow) for the amplification of the IPC.
Incorrect preparation of the real time PCR.	Check your work steps and compare them with the steps described in 'Procedure' on pages 7 ff.
The programming of the thermal profile is incorrect.	Compare the thermal profile with the protocol (Table 4, page 8).
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 4.
Weak or no signal of the Control DNA and simultaneous absence of a signal in the Cy5 channel.	
real time PCR conditions do not comply with the protocol.	Check the real time PCR conditions (page 7).
Real time PCR inhibited.	Make sure that you use an appropriate isolation method (see chapter ‚Sample Preparation', page 5) and follow the manufacturer's instructions. Make sure that the ethanol-containing washing buffers have been completely removed. If magnetic particle extraction is used, evaporation of ethanol residues should be ensured before the eluate is used in the real time PCR.
Loss of DNA during the purification process.	The absence of a signal for the IPC indicates an incorrect extraction. Make sure that you use a suitable extraction method. Follow 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 4.
Weak or no signal in the ROX channel and simultaneous absence of a signal in the Cy5 channel.	
Insufficient sample quantity.	Repeat the extraction with a larger amount of sample material.
Insufficient sample quality or insufficient sample digestion.	Check the date of expiry of the extraction kit used. If you extract a tissue sample, make sure that the tissue is mechanically and/or enzymatically digested. Depending on the extraction kit manufacturer, additional reagents and consumables may be required.
Detection of a signal in the Cy5 channel of the Negative Control.	
Contamination during preparation of the real time PCR.	Repeat the real time PCR in replicates. If the result of the repetitions is negative, the contamination occurred during the filling of the optical PCR reaction tubes. If the Negative Control in the repetition shows a signal in the Cy5 channel, this indicates that one or more kit components are contaminated. Ensure that workspace and equipment are regularly decontaminated. Repeat the real time PCR with a new kit.

17 Kit Performance

17.1 Analytical Sensitivity - ASFV

The limit of detection (LoD) of virellaASFV seqc real time PCR was determined using serial dilutions of DNA of ASFV of known concentration using a Stratagene Mx3005 real time PCR instrument. The LoD of virellaASFV seqc real time PCR is at least 10 target copies per reaction.

Table 6: Values of the virellaASFV seqc real time PCR detection limit test using a dilution series - Cy5 channel (ASFV).

Sample	Copies per Reaction	C_T Value	C_T Mean Value
		Cy5 channel	
ASFV	10000000	13.67 13.91 13.48	**13.69**
ASFV	1000000	16.72 17.10 16.72	**16.85**
ASFV	100000	19.81 20.64 20.21	**20.22**
ASFV	10000	23.60 23.35 23.30	**23.42**
ASFV	1000	26.82 27.23 27.13	**27.06**
ASFV	100	29.96 29.13 29.72	**29.60**
ASFV	10	31.83 32.75 32.74	**32.44**
ASFV	1.0	45.00 36.78 38.06	**39.95**

A C_T value of 45 was used to calculate the mean value for negative values.

17.2 Analytical Sensitivity – Internal System Control (ISC)

The sensitivity of the virellaASFV seqc real time PCR for the Internal System Control was determined using a dilution series of the ISC target sequence with known concentration and tested in a 3-fold approach.

The limit of detection of virellaASFV seqc real time PCR for the Internal System Control is 0.1 copies per reaction.

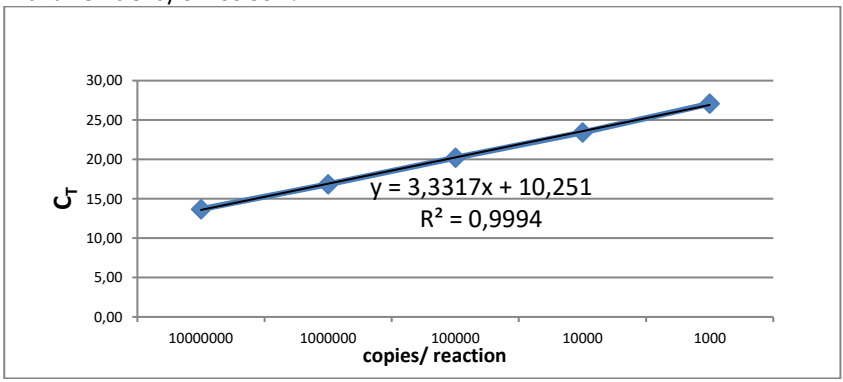
Table 7: Values of the virellaASFV seqc real time PCR detection limit test using a dilution series - ROX channel (ISC).

Sample	Copies per Reaction	CT Value	C_T Mean Value
		ROX Channel	
ISC	10000000	14.36 14.58 13.99	**14.31**
ISC	1000000	17.53 17.75 17.56	**17.61**
ISC	100000	20.32 20.08 20.41	**20.27**
ISC	10000	22.16 22.65 22.17	**22.33**
ISC	1000	25.31 25.43 25.12	**25.28**
ISC	100	28.01 28.53 27.90	**28.15**
ISC	10	31.48 31.34 31.23	**31.35**
ISC	1	34.51 35.68 34.73	**34.97**
ISC	0.1	37.89 36.87 38.04	**37.60**

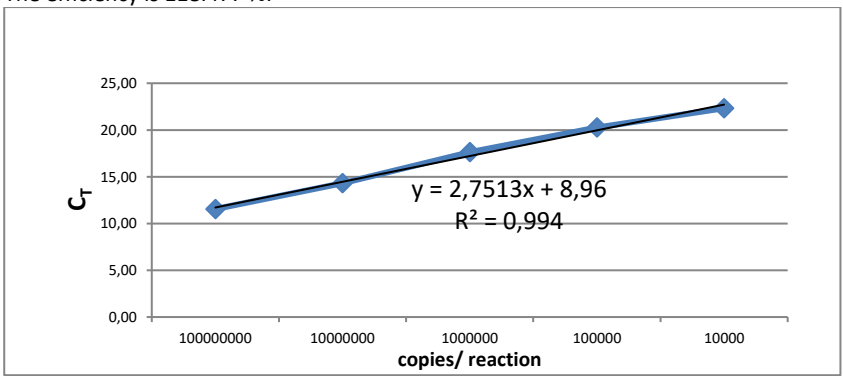
17.3 Linear Range

The linear range was determined using dilution series.

The R^2-value of the virellaASFV seqc real time PCR for the Cy5 channel (ASFV) is 0.9994 with an efficiency of 106.504 %.



The R^2-value of the virellaASFV seqc real time PCR for the ROX channel (ISC) is 0.994. The efficiency is 118.477 %.



17.4 Analytical Specificity and Sensitivity - Summary

Analytical specificity and sensitivity were determined using defined reference and field samples that were both positive and negative for ASFV, and defined ring trial and field samples that were positive for other pathogens. The analytical specificity and sensitivity of the virellaASFV seqc real time PCR are 100 % each.

Table 8: Sensitivity and specificity of virellaASFV seqc real time PCR.

	positive samples	**negative samples**
virellaASFV seqc **positive**	23	0
virellaASFV seqc **negative**	0	150
	Sensitivity (%)	**Specificity (%)**
	100	100

17.5 Precision

The precision of the virellaASFV seqc real time PCR was determined by intra-assay variability and inter-lot variability. Data on variability are presented using the standard deviation and the coefficient of variation.

Table 9: Precision of the virellaASFV seqc real time PCR.

ASFV (Cy5)	copies/reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	10	0.57	1.74
Inter-Lot Variability	10	0.01	0.02

IPC (HEX)	copies/reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	10.000	0.26	0.94
Inter-Lot Variability	10.000	0.18	0.65

ISC (ROX)	copies/reaction	Standard Deviation	Coefficient of Variation [%]
Intra-Assay Variability	10	0.26	0.81
Inter-Lot Variability	10	0.19	0.60

18 Abbreviations and Symbols

DNA	Deoxyribonucleic Acid	REF	Catalog number
PCR	Polymerase Chain Reaction	Σ	Content sufficient for x tests
ASFV	African Swine Fever Virus		Consult Instruction for Use
REACTION MIX	Reaction Mix	- 18°C	Upper limit of temperature
CONTROL +	Positive Control		Manufacturer
CONTROL −	Negative Control		Use by YYYY-MM-DD
CONTROL DNA IPC	Control DNA (IPC)	LOT	Batch code
		CONT	Content