April 2025 | EN

IndiMix™ JOE

Technical Data Report

For real-time amplification of a single or multiple nucleic acid targets and an Internal Control

INDICAL BIOSCIENCE

Contents

1 Introduction

IndiMix JOE enables real-time amplification of single or multiple nucleic acid targets and contains both, reverse transcriptase and polymerase to amplify DNA and RNA targets. The primers and probe are premixed to identify the intype Internal Control.

For molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

For test procedure please see the respective Product Sheet (HB-2562).

2 Characteristics of the test

2.1 Sensitivity for several viral nucleic acids

2.1.1 Sensitivity testing *Porcine Reproductive and Respiratory Syndrome Virus* (PRRSV) North American (NA) genotype positive *in vitro* RNA

The sensitivity of the virotype PRRSV NA/EU Primers/Probes combined with IndiMix JOE was determined by testing an individual titration series of PRRSV NA genotype positive *in vitro* RNA. Tests were performed in triplicates of ten-fold dilutions on BioRad CFX96™ (BioRad Laboratories, Inc., Hercules, California, USA) and Applied Biosystems™ 7500 Fast (standard and fast mode; ABI 7500 Fast; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) thermocyclers.

Results/ Conclusion

Results are shown in Table 1 as well as Figure 1 - Figure 3. The IndiMix JOE in combination with virotype PRRSV NA/EU Primer/Probes is able to identify down to one PRRSV NA *in vitro* RNA copy per sample (Table 1, Figure 1 and Figure 2).

In this specific study, a high correlation between RNA copy number and amplification results could be shown. A correlation coefficient of 0.999 with an efficiency of 103.1 % for the *in vitro* RNA was calculated when testing the PRRSV NA *in vitro* RNA on the BioRad CFX96 instrument (Figure 3).

Table 1. Individual and mean C_T values of PRRSV NA *in vitro* RNA amplificates (Cy5 signal) tested in triplicates. The test was performed on the BioRad CFX96 as well as the ABI 7500 Fast (fast and standard modes) instruments.

		PRRSV NA					
Type	**Copy number**	**BioRad CFX96**		**ABI 7500 (fast)**		**ABI 7500 (standard)**	
		C_T	**C_T mean**	**C_T**	**C_T mean**	**C_T**	**C_T mean**
PRRSV NA	10^6	18.77		17.91		18.04	
	10^6	18.84	18.80	17.97	17.92	18.06	18.08
	10^6	18.80		17.88		18.14	
	10^5	21.89		21.46		21.37	
	10^5	21.90	21.89	21.46	21.47	21.46	21.40
	10^5	21.89		21.48		21.36	
	10^4	25.02		24.80		24.71	
	10^4	25.01	25.11	24.75	24.76	24.73	24.71
	10^4	25.30		24.73		24.68	
	10^3	28.37		28.25		28.11	
	10^3	28.65	28.44	28.10	28.15	28.16	28.15
	10^3	28.30		28.09		28.17	
	100	31.49		31.54		31.59	
	100	31.54	31.55	31.45	31.48	31.59	31.59
	100	31.63		31.45		31.58	
	10	35.41		34.96		35.38	
	10	34.76	34.82	36.91	35.40	34.30	34.80
	10	34.28		34.33		34.71	
	1	39.07		38.03		36.59	
	1	38.88	38.68	36.66	37.35	36.56	37.41
	1	38.08		-		39.09	

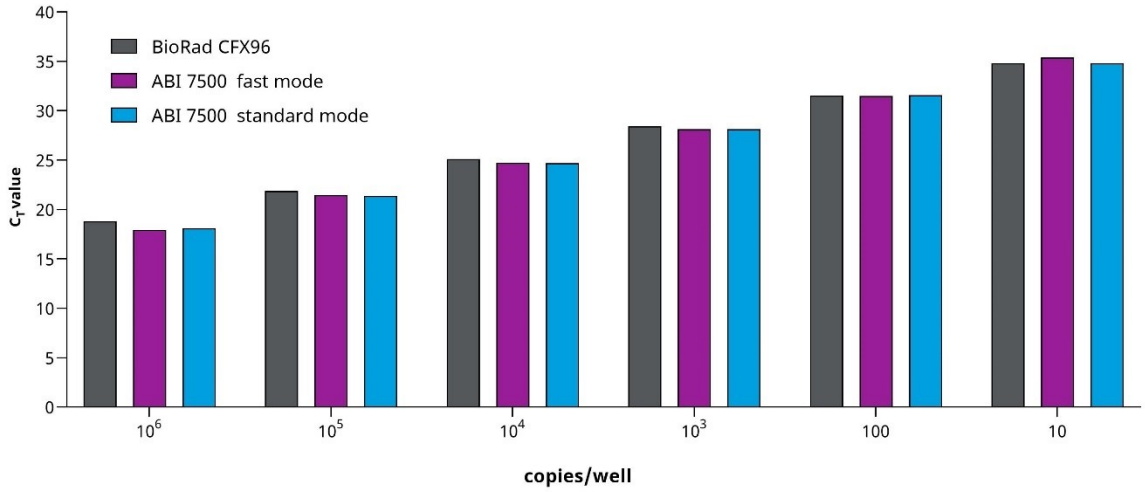

Figure 1. Mean C_T values of a titration series of PRRSV NA *in vitro* RNA tested with virotype PRRSV NA/EU Primers/Probes in combination with IndiMix JOE on BioRad CFX96 and ABI 7500 Fast (fast and standard mode) instruments.

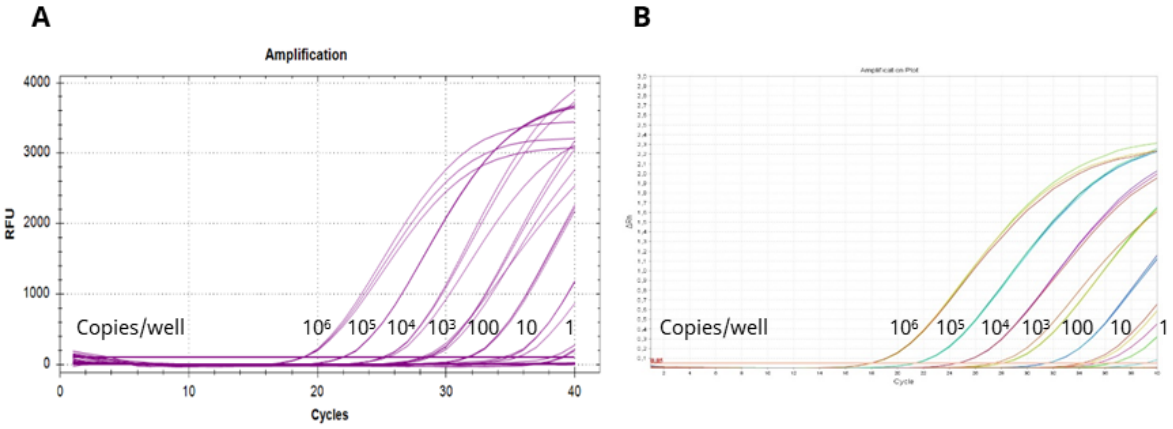

Figure 2. Amplification plots for titration series of PRRSV NA *in vitro* RNA tested with virotype PRRSV NA/EU Primers/Probes in combination with IndiMix JOE on BioRad CFX96 (**A**) and ABI 7500 Fast (**B**) instruments.

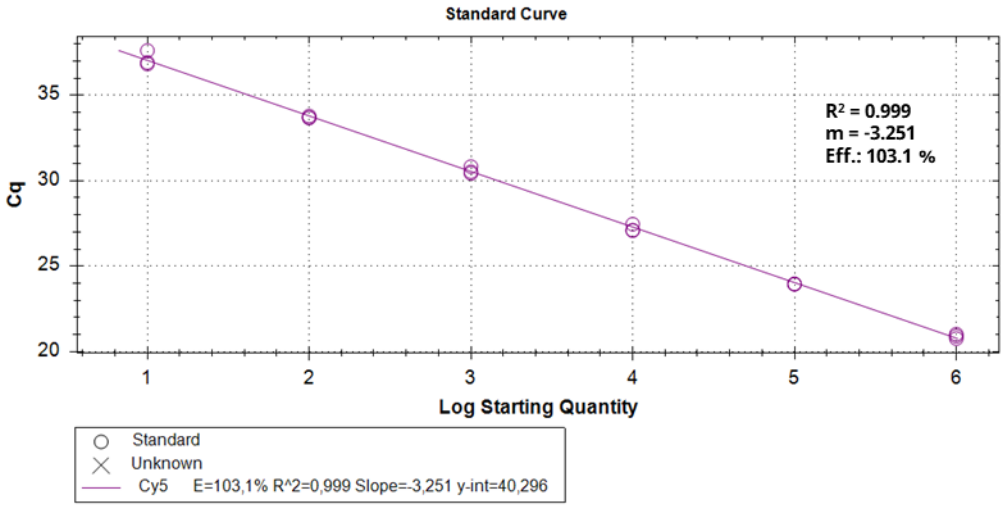

Figure 3. Standard curve of obtained C_T values for PRRSV NA *in vitro* RNA titration series tested in triplicates using virotype PRRSV NA/EU Primers/Probes in combination with IndiMix JOE on the BioRad CFX96 instrument.

2.1.2 Sensitivity testing PRRSV European (EU) genotype positive *in vitro* RNA

The sensitivity of the virotype PRRSV NA/EU Primers/Probes combined with IndiMix JOE was determined by testing two different titration series (EU1, EU2) of PRRSV European (EU) genotype positive *in vitro* RNA. Tests were performed in triplicates of ten-fold dilutions on BioRad CFX96 and ABI 7500 Fast thermocycler (standard and fast mode).

Result/Conclusion

Results are presented in Table 2 and Table 3 as well as Figure 4 - Figure 7. The IndiMix JOE in combination with virotype PRRSV NA/EU Primer/Probes is able to identify down to 1-10 PRRSV EU *in vitro* RNA copies per sample (Table 2, Table 3 and Figure 4 - Figure 6).

Furthermore, a high correlation between RNA copy number and amplification results could be seen with this test setup: A correlation coefficient of 0.999 with an efficiency of 98.0 % for the *in vitro* RNA was calculated (Figure 7).

Table 2. Individual and mean C_T values of PRRSV EU1 *in vitro* RNA amplificates (FAM signal) tested in triplicates. The test was performed on the BioRad CFX96 as well as the ABI 7500 Fast (fast and standard modes) instruments.

		PRRSV EU					
Type	**Copy number**	**BioRad CFX96**		**ABI 7500 (fast)**		**ABI 7500 (standard)**	
		C_T	**C_T mean**	**C_T**	**C_T mean**	**C_T**	**C_T mean**
PRRSV EU1	10^6	20.50		19.35		19.56	
	10^6	20.68	20.72	19.35	19.37	19.66	19.60
	10^6	20.99		19.42		19.59	
	10^5	24.13		23.00		22.98	
	10^5	24.14	24.05	22.88	22.95	23.05	23.01
	10^5	23.87		22.96		23.00	
	10^4	27.43		26.23		26.51	
	10^4	27.55	27.43	26.20	26.22	26.44	26.50
	10^4	27.32		26.23		26.55	
	10^3	30.60	30.69	29.39		29.75	
	10^3	30.82		29.49	29.48	29.95	29.77
	10^3	30.65		29.57		29.62	
	100	34.43		33.08		32.83	
	100	34.13	34.25	33.02	33.10	33.34	33.11
	100	34.19		33.20		33.15	
	10	38.16		36.74		37.29	
	10	37.46	37.65	36.09	36.42	35.53	36.77
	10	37.32		-		37.50	
	1	-		38.28		38.16	
	1	39.95	39.95	36.96	37.62	-	38.57
	1	-		-		38.98	

Table 3. Individual and mean C_T values of PRRSV EU2 *in vitro* RNA amplificates (FAM signal) tested in triplicates. The test was performed on the BioRad CFX96 as well as the ABI 7500 Fast (fast and standard modes) instruments.

		PRRSV EU					
Type	**Copy number**	**BioRad CFX96**		**ABI 7500 (fast)**		**ABI 7500 (standard)**	
		C_T	**C_T mean**	**C_T**	**C_T mean**	**C_T**	**C_T mean**
PRRSV EU2	10^6	19.83		19.03		18.89	
	10^6	19.74	19.81	18.99	19.02	18.90	18.87
	10^6	19.87		19.03		18.83	
	10^5	23.20		22.38		22.08	
	10^5	23.06	23.16	22.36	22.35	22.10	22.09
	10^5	23.23		22.30		22.10	
	10^4	26.50		25.77		25.59	
	10^4	26.56	26.56	25.88	25.89	25.74	25.68
	10^4	26.61		26.01		25.71	
	10^3	29.83		29.05		28.96	
	10^3	29.72	29.82	29.29	29.23	28.92	28.94
	10^3	29.90		29.34		28.94	
	100	32.93		32.93		32.13	
	100	33.24	33.31	33.04	32.82	32.28	32.59
	100	33.77		32.50		33.35	
	10	37.26		38.22		36.48	
	10	38.20	37.65	36.45	36.77	36.15	36.26
	10	37.48		35.64		36.15	
	1	-		36.97		-	
	1	-	37.97	-	37.46	38.08	38.08
	1	37.97		37.94		-	

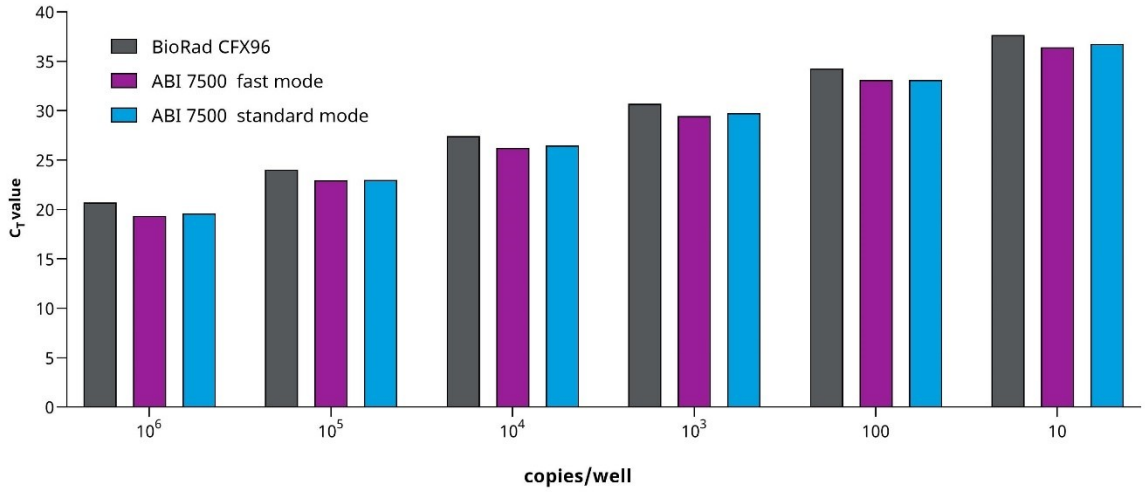

Figure 4. Mean C_T values of a titration series of PRRSV EU1 *in vitro* RNA tested with virotype PRRSV NA/EU Primers/Probes in combination with IndiMix JOE on BioRad CFX96 and ABI 7500 Fast (fast and standard mode) instruments.

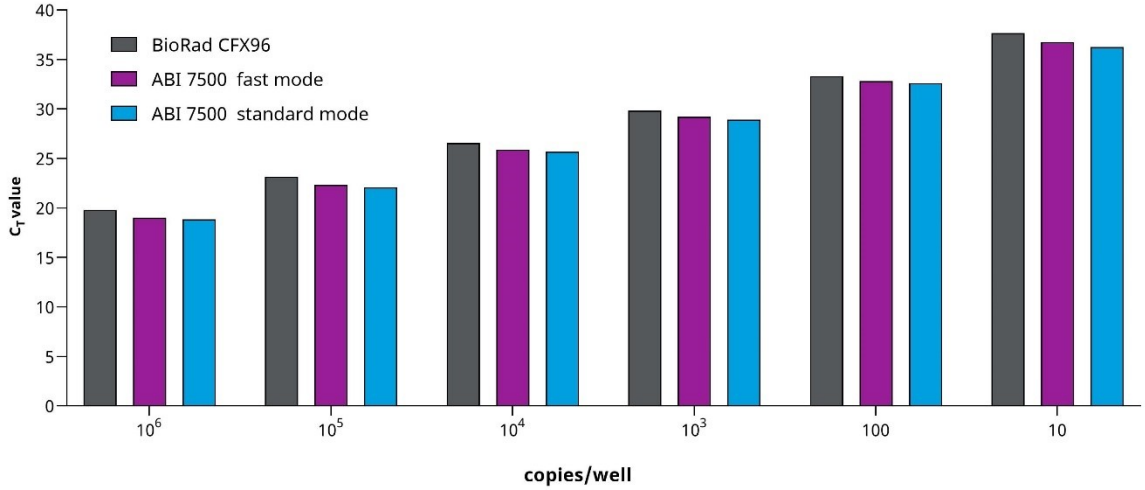

Figure 5. Mean C_T values of a titration series of PRRSV EU2 *in vitro* RNA tested with virotype PRRSV NA/EU Primers/Probes in combination with IndiMix JOE on BioRad CFX96 and ABI 7500 Fast (fast and standard mode) instruments.

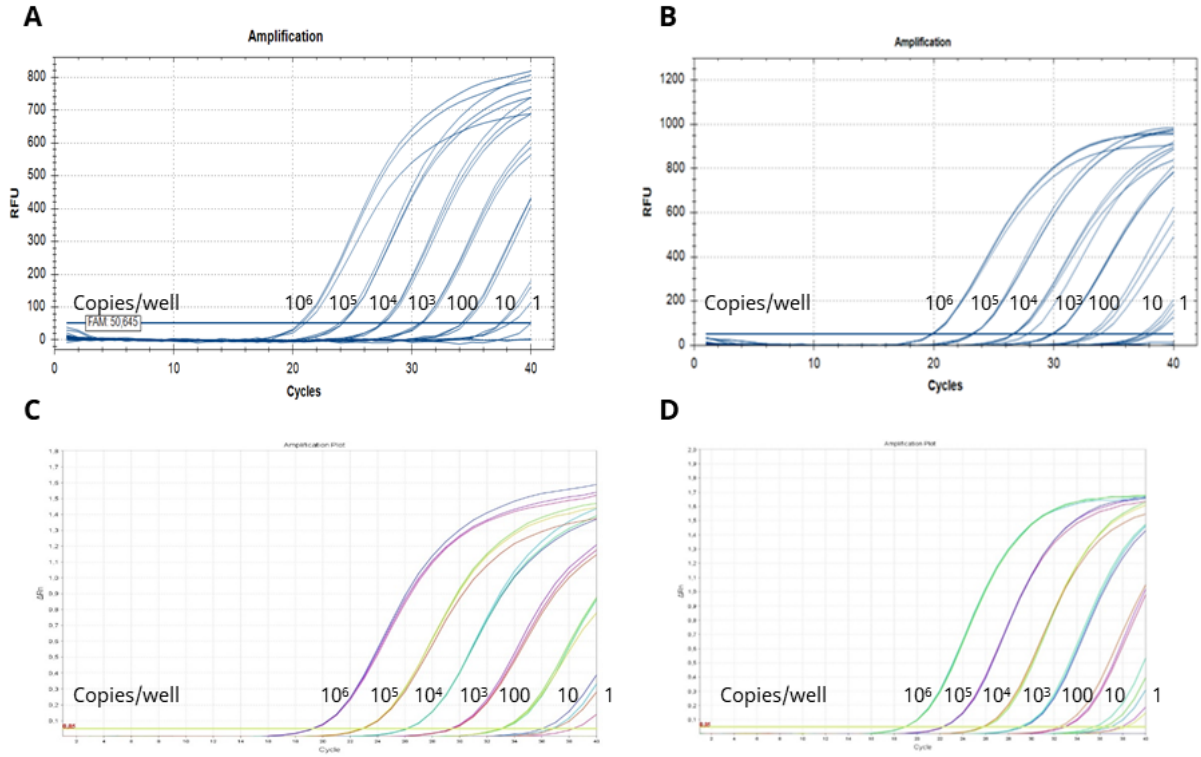

Figure 6. Amplification plots for titration series of PRRSV EU1 and EU2 *in vitro* RNA tested with virotype PRRSV NA/EU Primers/Probes in combination with IndiMix JOE on BioRad CFX96 (**A** for EU1, **B** for EU2) and ABI 7500 Fast (**C** for EU1, **D** for EU2) instruments.

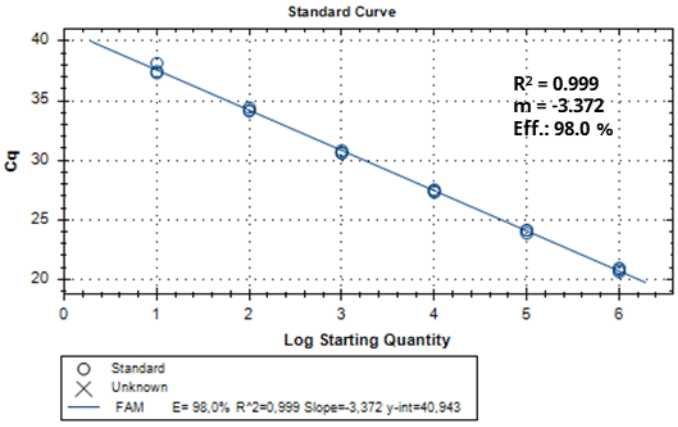

Figure 7. Standard curve of obtained C_T values for PRRSV EU1 *in vitro* RNA titration series tested in triplicates using virotype PRRSV NA/EU Primers/Probes in combination with IndiMix JOE on the BioRad CFX96 instrument.

2.1.3 Sensitivity testing Avian Influenza A Virus (AIV) positive *in vitro* RNA

The sensitivity of the virotype Influenza A Primers/Probes combined with IndiMix JOE was determined by testing individual titration series of Avian Influenza A Virus positive *in vitro* RNA. Tests were performed in triplicates of ten-fold dilutions and performed on BioRad CFX96 and ABI 7500 Fast thermocyclers in fast mode.

Results/Conclusion

Results are shown in Table 4 as well as Figure 8 - Figure 10. The Indimix JOE in combination with virotype Influenza A Primers/Probes is able to identify down to 1 - 10 Avian Influenza A Virus *in vitro* RNA copies per sample (Table 4, Figure 8, and Figure 9).

For this specific study, a high correlation between RNA copy number and amplification results could be seen: When testing the Avian Influenza A Virus *in vitro* RNA titration series on the BioRad CFX96 instrument, a correlation coefficient of 0.997 with an efficiency of 100.9 % for the *in vitro* RNA was determined (Figure 10).

Table 4. Individual and mean C_T values of Avian Influenza A Virus *in vitro* RNA amplificates (FAM signal) tested in triplicates. The test was performed on the BioRad CFX96 as well as the ABI 7500 Fast (fast mode) instruments.

		AIV			
Type	**Copy number**	**BioRad CFX96**		**ABI 7500 (fast)**	
		C_T	**C_T mean**	**C_T**	**C_T mean**
Avian Influenza A Virus	10^6	22.06		19.83	
	10^6	22.01	21.97	19.95	20.05
	10^6	21.83		20.37	
	10^5	25.49		23.17	
	10^5	25.07	25.21	23.13	23.35
	10^5	25.07		23.74	
	10^4	28.93		26.45	
	10^4	28.55	28.60	26.39	26.56
	10^4	28.31		26.83	
	10^3	32.00		29.79	
	10^3	31.67	31.95	29.98	29.89
	10^3	32.19		29.89	
	100	35.41		33.50	
	100	34.58	35.10	32.89	33.10
	100	35.29		32.90	
	10	37.56		-	
	10	39.04	38.30	36.76	36.32
	10	-		35.87	
	1	-		-	
	1	38.70	38.70	-	No mean C_T
	1	-		-	

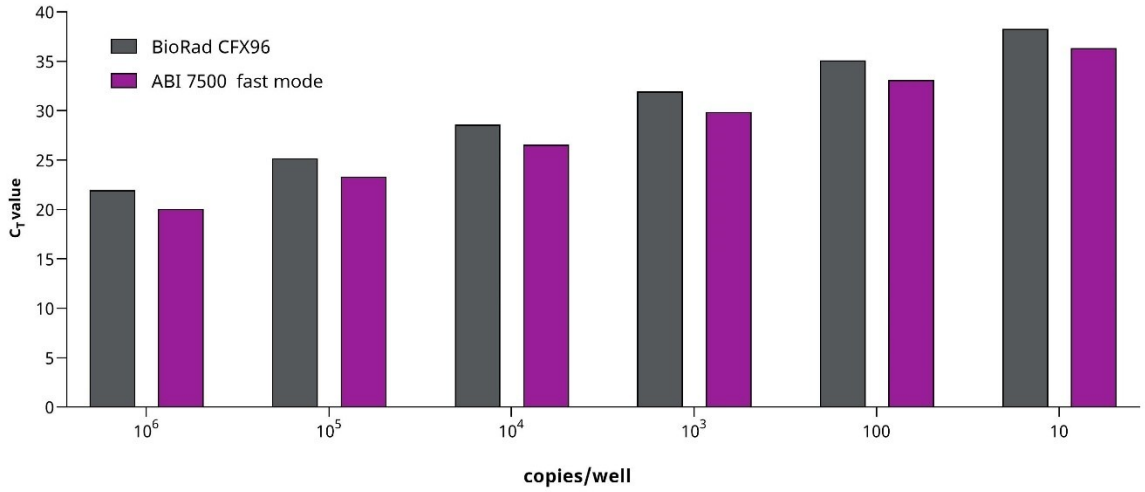

Figure 8. Mean C_T values of a titration series of Avian Influenza A Virus *in vitro* RNA tested with virotype Influenza A Primers/Probes in combination with IndiMix JOE on BioRad CFX96 and ABI 7500 Fast (fast mode) instruments.

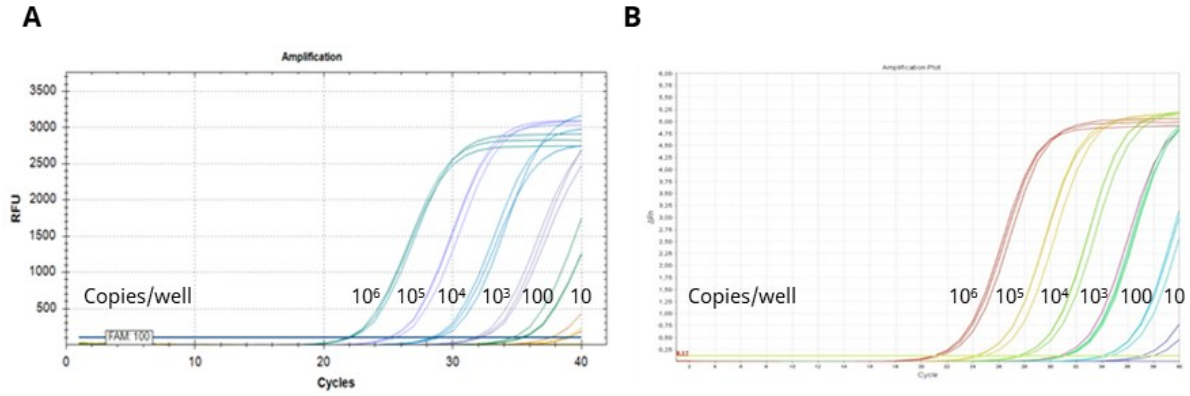

Figure 9. Amplification plots for titration series of Avian Influenza A Virus *in vitro* RNA tested with virotype Influenza A Primers/Probes in combination with IndiMix JOE on BioRad CFX96 (**A**) and ABI 7500 Fast (**B**) instruments.

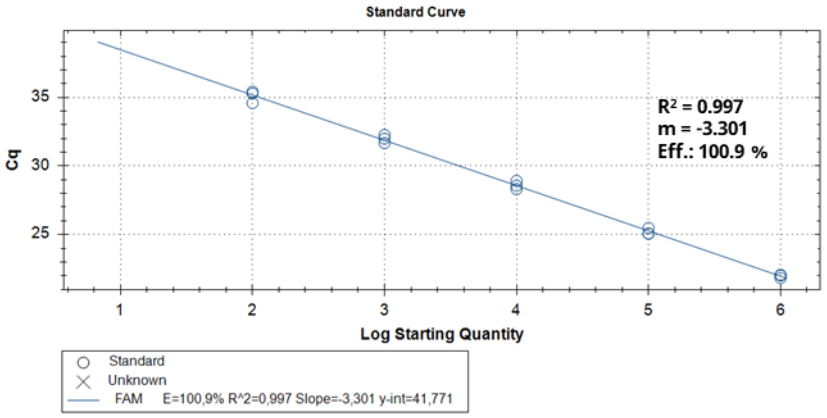

Figure 10. Standard curve of obtained C_T values for Avian Influenza A Virus *in vitro* RNA titration series tested in triplicates using virotype Influenza A Primers/Probes in combination with IndiMix JOE on the BioRad CFX96 instrument.

2.1.4 Sensitivity testing *Porcine Circovirus* 2/3 (PCV2/3) positive *in vitro* DNA

The sensitivity of the virotype PCV2/PCV3 Primers/Probes combined with IndiMix JOE was determined by testing individual titration series of PCV2 and PCV3 positive *in vitro* DNA. Tests were performed in triplicates of ten-fold dilutions and performed on BioRad CFX96 and ABI 7500 Fast thermocyclers in fast mode.

Results/Conclusion

Results are presented in Table 5 and Table 6 as well as Figure 11 - Figure 14. The IndiMix JOE in combination with virotype PCV2/PCV3 Primers/Probes is able to identify down to 1 - 10 copies of PCV2 DNA (Table 5, Figure 11, and Figure 13) and 10 – 100 copies of PCV3 DNA per sample (Table 6, Figure 12, and Figure 13).

Furthermore, a high correlation between DNA copy number and amplification results could be shown in this specific study: When testing the PCV2/3 *in vitro* DNA titration series on the ABI 7500 Fast thermocycler (fast mode), a correlation coefficient of 0.998 with an efficiency of 98.1 % (PCV2) or 103.1 % (PCV3), respectively was calculated (Figure 14).

Table 5. Individual and mean C_T values of PCV2 *in vitro* DNA amplificates (FAM signal) tested in triplicates. The test was performed on the BioRad CFX96 as well as the ABI 7500 Fast (fast mode) instruments.

		PCV2			
Type	**Copy number**	**BioRad CFX96**		**ABI 7500 (fast)**	
		C_T	**C_T mean**	**C_T**	**C_T mean**
PCV2	10^6	22.33	22.38	21.38	21.38
	10^6	22.32		21.35	
	10^6	22.48		21.40	
	10^5	25.65	25.63	24.67	24.71
	10^5	25.48		24.70	
	10^5	25.75		24.76	
	10^4	28.96	28.94	27.93	27.81
	10^4	28.79		27.55	
	10^4	29.08		27.95	
	10^3	32.04	32.06	31.33	31.39
	10^3	32.37		31.47	
	10^3	31.78		31.37	
	100	34.76	35.40	35.29	34.95
	100	36.32		34.94	
	100	35.13		34.62	
	10	-	-	36.98	37.00
	10	-		-	
	10	-		37.01	
	1	-	-	-	37.16
	1	-		-	
	1	-		37.16	

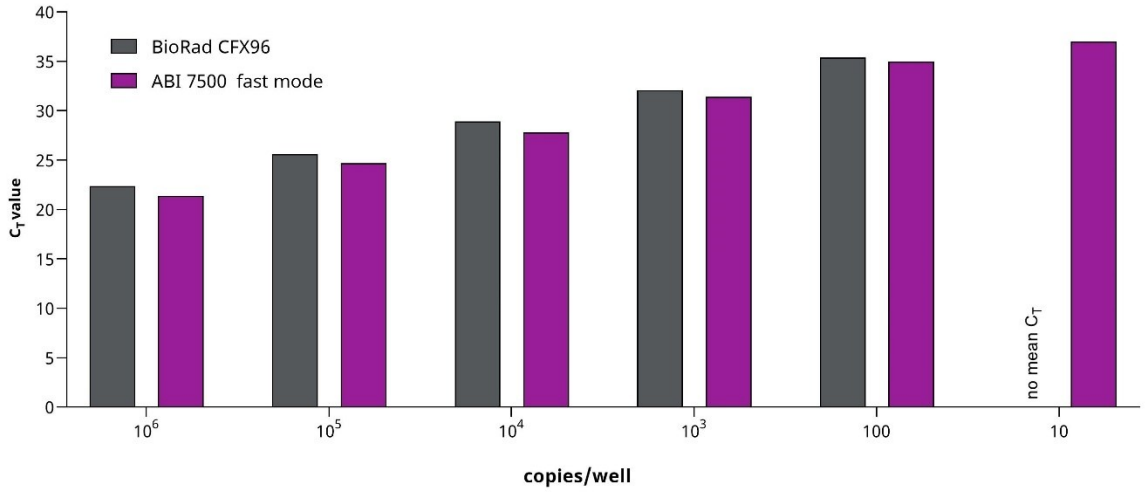

Figure 11. Mean C_T values of a titration series of PCV2 *in vitro* DNA tested with virotype PCV2/PCV3 Primers/Probes in combination with IndiMix JOE on BioRad CFX96 and ABI 7500 Fast (fast mode) instruments.

Table 6. Individual and mean C_T values of PCV3 *in vitro* DNA amplificates (FAM signal) tested in triplicates. The test was performed on the BioRad CFX96 as well as the ABI 7500 Fast (fast mode) instruments.

		PCV3			
Type	**Copy number**	**BioRad CFX96**		**ABI 7500 (fast)**	
		C_T	**C_T mean**	**C_T**	**C_T mean**
PCV3	10^6	23.43		24.64	
	10^6	24.85	24.38	24.52	24.59
	10^6	24.87		24.61	
	10^5	24.23		28.17	
	10^5	27.99	26.69	28.18	28.16
	10^5	27.85		28.14	
	10^4	28.43		31.58	
	10^4	31.37	30.39	31.39	31.42
	10^4	31.36		31.28	
	10^3	37.48		34.05	
	10^3	33.00	33.75	34.61	34.30
	10^3	30.78		34.24	
	100	38.32		37.64	
	100	38.17	38.25	37.87	37.77
	100	-		37.80	

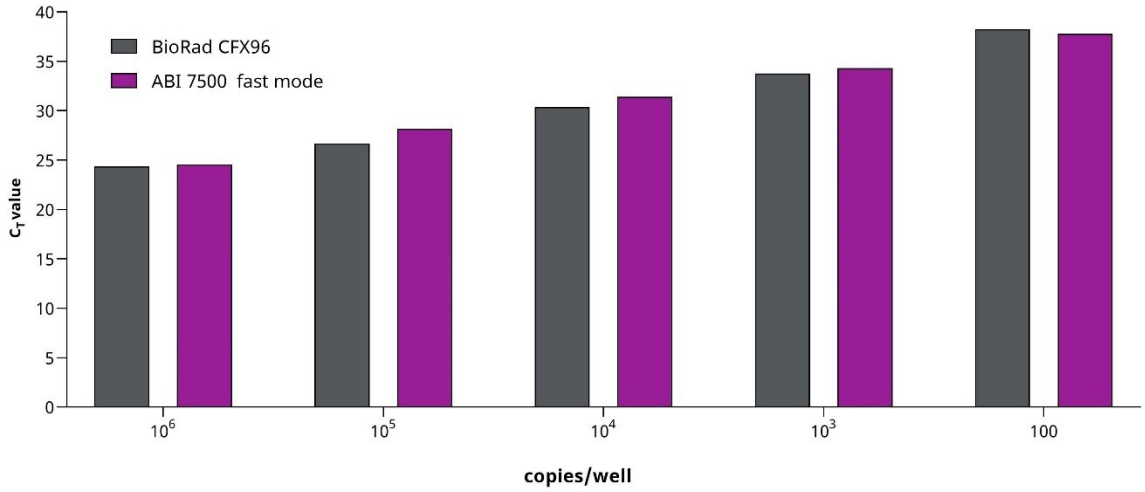

Figure 12. Mean C_T values of a titration series of PCV3 *in vitro* DNA tested with virotype PCV2/PCV3 Primers/Probes in combination with IndiMix JOE on BioRad CFX96 and ABI 7500 Fast (fast mode) instruments.

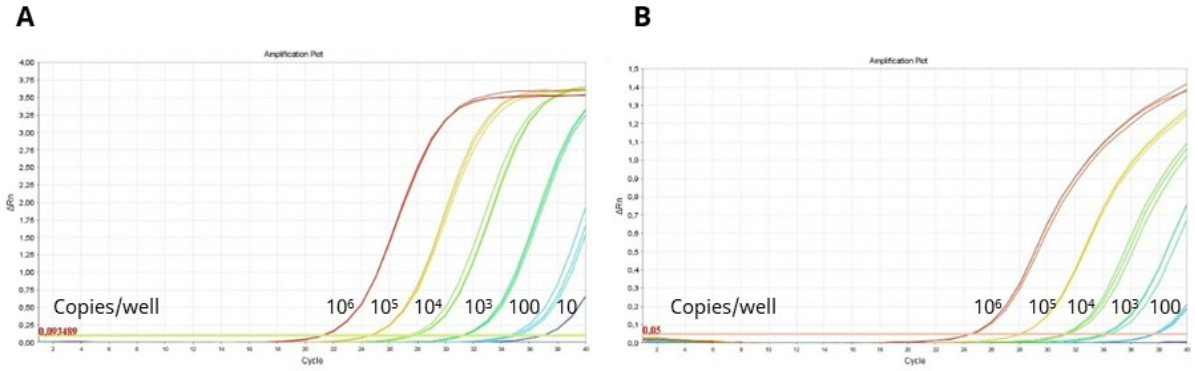

Figure 13. Individual values of amplificates in triplicates. Titration series of PCV2 (**A**) and PCV3 (**B**) *in vitro* DNA tested with virotype PCV2/PCV3 Primers/Probes in combination with IndiMix JOE on ABI 7500 Fast thermocycler (fast mode).

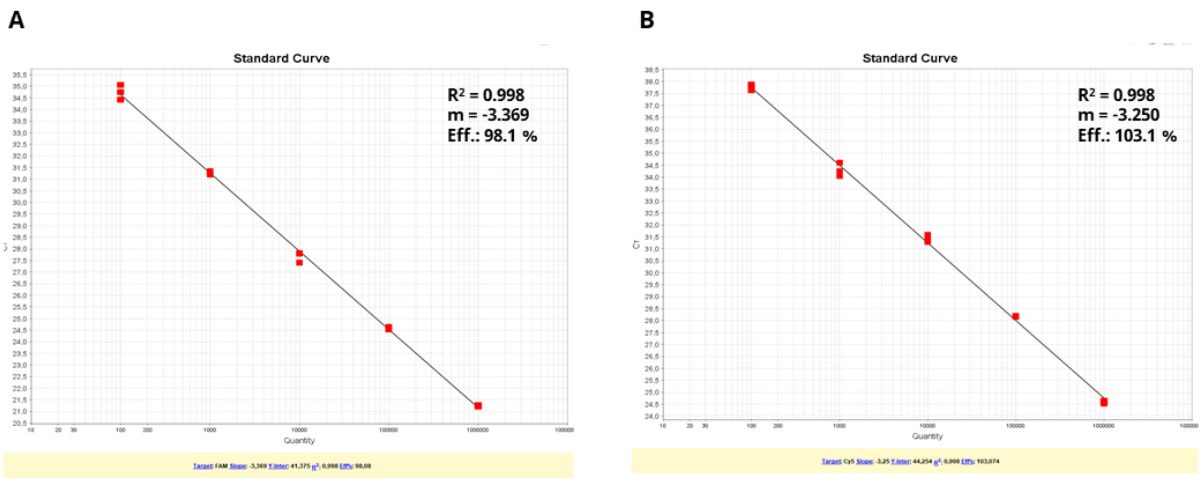

Figure 14. Standard curve of obtained C_T values for PCV2 (**A**) and PCV3 (**B**) *in vitro* DNA titration series tested in triplicates using virotype PCV2/PCV3 Primers/Probes in combination with IndiMix JOE on the ABI 7500 Fast thermocycler (fast mode).

2.2 IndiMix JOE performance, when testing different sample matrices

Altogether *n* = 8 samples (lung, serum, nasal swab) positive or negative for PRRSV as well as *n* = 8 samples (lung, serum, lymph node) positive or negative for PCV2 and/or PCV3 were extracted using the IndiMag Pathogen Kit on KingFisher™ Flex instrument (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Following the extraction, 5 µl of intype IC-RNA (for PRRSV sample set) or 2 µl of intype IC-DNA (for PCV sample set) were added to the samples. The nucleic acid eluates were then tested using virotype PRRSV NA/EU Primers/Probes or PCV2/PCV3 Primers/Probes, respectively, in combination with IndiMix JOE on an ABI 7500 Fast thermocycler (fast mode).

Results/Conclusion:

Extracting nucleic acids from different sample matrices with added Internal control RNA/DNA followed by testing with IndiMix JOE combined with appropriate Primers/Probes leads to reliable identification of pathogen and Internal Control signals (Table 7, Table 8).

Table 7. Testing PRRSV-positive/negative samples with virotype PRRSV NA/EU Primers/Probes in combination with IndiMix JOE on an ABI 7500 Fast thermocycler (fast mode) after extraction using 5 µl of intype IC-RNA.

Pathogen	Sample	PRRSV EU C_T	PRRSV NA C_T	Internal Control C_T
PRRSV-positive	Lung 1720	24.88	18.71	26.48
	Lung 131	32.02	-	26.71
	Serum pool	22.11	-	25.62
	Nasal swab 123	-	32.28	25.39
	Nasal swab 125	-	33.09	25.06
PRRSV-negative	Lung 776	-	-	26.70
	Serum 2565-2	-	-	24.58
	Serum 2624-2	-	-	24.65

Table 8. Testing PCV-positive/negative samples with virotype PCV2/PCV3 Primers/Probes in combination with IndiMix JOE on an ABI 7500 instrument (fast mode) after extraction using 2 µl of intype IC-DNA.

Pathogen	Sample	PCV2 C_T	PCV3 C_T	Internal Control C_T
PCV-positive	Lymph node 135	21.31	29.17	25.03
	Lymph node 125	16.74	-	23.04
	Serum 7	25.99	-	26.48
	Serum 8	20.78	-	25.13
	Serum 10	20.72	-	24.46
	Lung 310	7.49	-	28.08
PCV-neg.	Lymph node 128	-	-	25.99
	Serum 11	-	-	25.12

2.3 IndiMix JOE and Internal Control

The sample yields a signal in the JOE channel if IC-DNA or IC-RNA was used.

If no signal is detected in the JOE (exogenous Internal Control, IC-DNA or IC-RNA) channel, the result is inconclusive. The absence of a signal for IC-DNA or IC-RNA indicates strong PCR inhibition and/or other malfunctions, e.g., during extraction.

To check for inhibition, we recommend 1:5 dilution of the sample DNA/RNA in nuclease free water, to repeat the DNA/RNA extraction, or repeat the whole test procedure starting with new sample material.

The lack of JOE fluorescence signal can be caused by insufficient sample extraction, competition with a strong positive pathogen signal, PCR inhibition or will occur in cases where the IC-DNA or IC-RNA had not been added. Higher C_T values in the JOE channel of a sample compared to the majority of samples may indicate partial inhibition in the sample.

In a first study setup, five PRRSV-positive samples and three PRRSV-negative samples (lung, serum, nasal swabs) and a dilution series of Influenza A Virus culture samples were extracted using the IndiMag Pathogen Kit on a KingFisher Flex instrument. Prior to extraction, 2.5 or 5 µl of IC-RNA per sample were added to the VXL mixture. RNA eluates were tested using virotype PRRSV NA/EU Primers/Probes or Influenza A Primers/Probes in combination with IndiMix JOE on an ABI 7500 Fast thermocycler (fast mode).

Results/Conclusion:

When adding 2.5 or 5 µl of intype IC-RNA to the VXL mixture and extraction using the IndiMag Pathogen kit, the Internal Control can be reliably identified with IndiMix JOE from various sample materials (Table 9, Table 10).

Table 9. Testing PRRSV positive/negative samples with virotype PRRSV NA/EU Primers/Probes in combination with IndiMix JOE on an ABI 7500 Fast thermocycler (fast mode) after extraction using different volumes of intype IC-RNA.

Pathogen	Sample	IC-RNA	PRRSV EU		PRRSV NA		Internal Control	
			2.5 µl	5 µl	2.5 µl	5 µl	2.5 µl	5 µl
			C_T	C_T	C_T	C_T	C_T	C_T
PRRSV-positive	Lung 1720		24.90	24.88	18.60	18.71	27.44	26.48
	Lung 131		32.23	32.02	-	-	28.09	26.71
	Serum pool		22.06	22.11	-	-	26.28	25.62
	Nasal swab 123		-	-	31.96	32.28	27.03	25.39
	Nasal swab 125		-	-	34.40	33.09	26.54	25.06
PRRSV-negative	Lung 776		-	-	-	-	27.85	26.70
	Serum 2565-2		-	-	-	-	25.68	24.58
	Serum 2624-2		-	-	-	-	25.97	24.65
Mean value							26.86	25.65

Table 10. Testing Influenza A Virus positive culture samples with Influenza A Primers/Probes in combination with IndiMix JOE on an ABI 7500 Fast thermocycler (fast mode) after extraction using different volumes of intype IC-RNA.

Pathogen	Sample	IC-RNA	AIV		Internal Control	
			2.5 µl	5 µl	2.5 µl	5 µl
			C_T	C_T	C_T	C_T
Avian Influenza A Virus positive	1645 1:10^2		18.58	18.68	25.22	24.03
	1645 1:10^4		25.36	24.93	24.87	23.75
	1645 1:10^6		32.79	32.67	25.02	23.94
	1645 1:10^7		36.92	35.95	24.92	24.20
	1908 1:10^2		18.43	18.40	23.63	23.13
	1908 1:10^4		25.48	25.32	25.78	24.82
	1908 1:10^6		32.58	32.66	25.69	24.82
	1908 1:10^7		36.30	36.29	25.86	24.42
Mean value					25.12	24.14

In a second study setup, six PCV positive samples and two PCV-negative samples (lung, lymph nodes, serum) were extracted using the IndiMag Pathogen Kit on a KingFisher Flex instrument Prior to extraction, 2µl of IC-DNA per sample were added to the VXL mixture. DNA eluates were tested using virotype PCV2/PCV3 Primers/Probes in combination with IndiMix JOE on an ABI 7500 Fast thermocycler using RT-PCR and PCR protocols.

Results/Conclusion:

Results are shown in Table 11. By adding 2 µl of intype IC-DNA to the VXL mixture and extraction using the IndiMag Pathogen kit, the Internal Control can be reliably and similarly detected from various sample materials and with RT-PCR as well as PCR protocols.

Table 11. Testing PCV-positive/negative samples with virotype PCV2/PCV3 Primers/Probes in combination with IndiMix JOE on an ABI 7500 Fast thermocycler (fast mode) after extraction using 2 µl of intype IC-DNA.

		PCR without RT-step			RT-PCR		
Patho-gen	**Sample**	**PCV2 C_T**	**PCV3 C_T**	**IC C_T**	**PCV2 C_T**	**PCV3 C_T**	**IC C_T**
PCV-positive	Lymph node 135	21.31	29.17	25.03	20.58	28.14	24.25
	Lymph node 125	16.74	-	23.04	16.11	-	21.55
	Serum 7	25.99	-	26.48	25.22	-	25.63
	Serum 8	20.78	-	25.13	20.09	-	24.20
	Serum 10	20.72	-	24.46	20.15	-	23.80
	Lung 310	7.49	-	28.08	6.91	-	27.52
PCV-neg.	Lymph node 128	-	-	25.99	-	-	25.16
	Serum 11	-	-	25.12	-	-	24.26
Mean value		18.84	No mean C_T	25.42	18.18	No mean C_T	24.55

IC = Internal Control
No mean C_T = mean C_T could not be determined due to low number of values

2.4 Repeatability

2.4.1 Intra-assay variance

A titration series of Avian Influenza A Virus (AIV) culture was tested in a 5-fold setup in one run using the virotype Influenza A Primers/Probes in combination with IndiMix JOE on the ABI 7500 Fast thermocycler in fast mode.

Results/Conclusion:

Test results including calculated mean values, standard deviations (SD), and the coefficients of variation (CV) are given in Table 12 and Table 13. Figure 15 depicts the results for the tested samples per channel. The intra-assay variance is on average 0.89 % for AIV (FAM signal) and 2.52 % for the Internal Control (JOE signal). These results show an excellent repeatability with very low intra-assay variance for IndiMix JOE in combination with virotype Influenza A Primers/Probes.

Table 12. Intra-assay variance of C_T values (AIV/FAM signal) for AIV culture sample dilutions tested with virotype Influenza A Primers/Probes combined with IndiMix JOE on the ABI 7500 Fast thermocycler (fast mode).

Samples		Intra-assay variance for AIV Reactions (C_T values)					C_T mean	SD	CV %
		1	2	3	4	5			
1	1645 1:10^2	18.68	18.59	18.63	18.62	18.53	18.61	0.06	0.30
2	1645 1:10^4	24.93	24.94	24.94	24.98	24.96	24.95	0.02	0.09
3	1645 1:10^6	32.86	33.00	32.70	32.89	33.17	32.92	0.18	0.53
4	1645 1:10^7	36.68	37.31	-	36.37	38.58	37.23	0.98	2.63
Mean value									**0.89**

SD = standard deviation, CV = coefficient of variation

Table 13. Intra-assay variance of C_T values (Internal Control/JOE signal) for AIV culture sample dilutions tested with virotype Influenza A Primers/Probes combined with IndiMix JOE on the ABI 7500 Fast thermocycler (fast mode).

Samples		Intra-assay variance for Internal Control Reactions (C_T values)					C_T mean	SD	CV %
		1	2	3	4	5			
1	1645 1:10^2	26.70	24.89	25.29	24.80	25.75	25.49	0.78	3.05
2	1645 1:10^4	26.47	25.25	25.21	26.27	26.51	25.94	0.66	2.53
3	1645 1:10^6	26.46	25.29	25.36	26.16	26.68	25.99	0.63	2.44
4	1645 1:10^7	26.79	25.41	25.81	26.04	26.43	26.10	0.53	2.05
Mean value									**2.52**

SD = standard deviation, CV = coefficient of variation

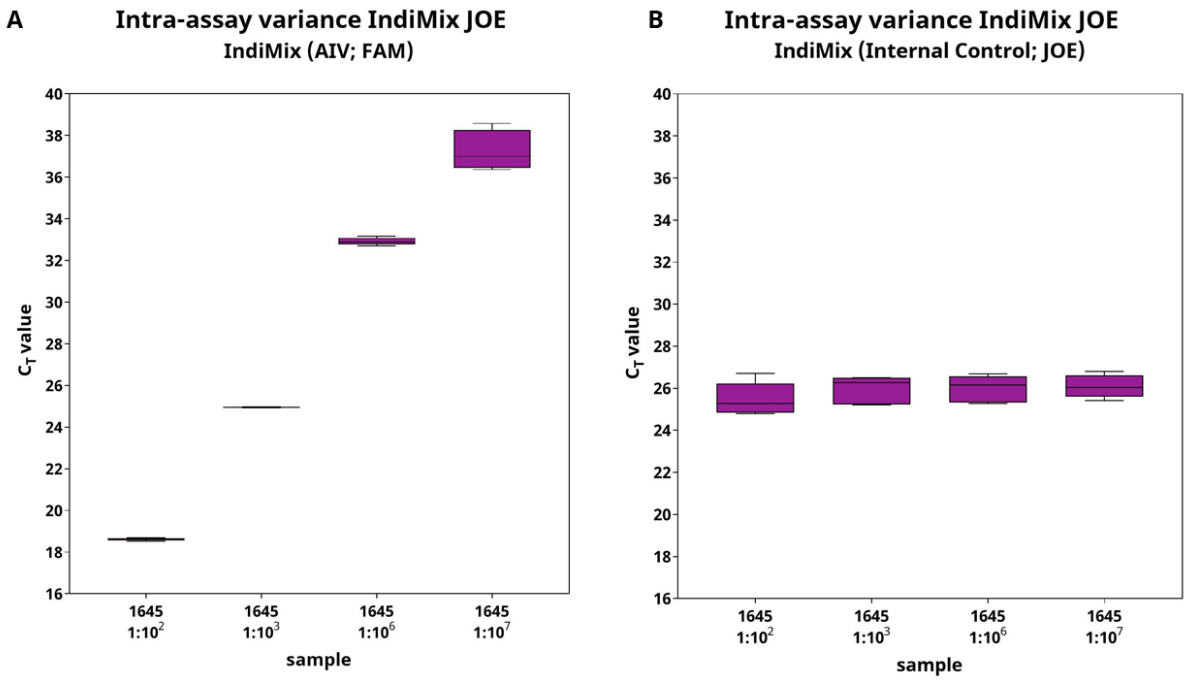

Figure 15. Boxplots showing intra-assay variance for AIV (FAM, **A**), and the Internal Control (JOE, **B**) for AIV culture sample dilutions tested with virotype Influenza A Primers/Probes combined with IndiMix JOE on the ABI 7500 Fast thermocycler (fast mode).

2.4.2 Inter-assay variance

PRRSV EU (samples 1, 2) and PRRSV NA (samples 3, 4)-positive RNA samples, as well as one PRRSV-negative RNA sample (sample 5) were tested in five different RT-PCR runs using the virotype PRRSV NA/EU Primers/Probes in combination with IndiMix JOE on the ABI 7500 Fast thermocycler in fast mode.

Results/Conclusion:

Test results including calculated mean values, standard deviations (SD), and the coefficients of variation (CV) are given in Table 14 - Table 16. Additionally, Figure 16 depicts the results for the tested samples per channel. The intra-assay variance is on average 3.02 % for PRRSV EU (FAM signal), 1.11 % for PRRSV NA (Cy5 signal) and 3.53 % for the Internal Control (JOE signal). These results show an excellent repeatability with very low inter-assay variance for IndiMix JOE in combination with virotype PRRSV NA/EU Primers/Probes.

Table 14. Inter-assay variance of C_T values for PRRSV EU RNA (FAM) tested with virotype PRRSV NA/EU Primers/Probes combined with IndiMix JOE on the ABI 7500 Fast thermocycler (fast mode).

Inter-assay variance for PRRSV EU										
Samples (PRRSV genotype)			**Reactions (C_T values)**					**C_T mean**	**SD**	**CV %**
			1	2	3	4	5			
1	Lung 131	EU	29.60	29.56	30.78	29.12	29.95	29.80	0.62	2.08
2	Serum pool	EU	20.48	20.25	22.17	20.14	20.89	20.79	0.82	3.96
3	Nasal swab 123	NA	-	-	-	-	-	-	-	-
4	Nasal swab 125	NA	-	-	-	-	-	-	-	-
5	Serum 2565-2	Neg	-	-	-	-	-	-	-	-
Mean value										**3.02**

SD = standard deviation, CV = coefficient of variation, EU = European genotype, NA = North American genotype

Table 15. Inter-assay variance of C_T values for PRRSV NA RNA (Cy5) tested with virotype PRRSV NA/EU Primers/Probes combined with IndiMix JOE on the ABI 7500 Fast thermocycler (fast mode).

Inter-assay variance for PRRSV NA										
Samples (PRRSV genotype)			**Reactions (C_T values)**					**C_T mean**	**SD**	**CV %**
			1	2	3	4	5			
1	Lung 131	EU	-	-	-	-	-	-	-	-
2	Serum pool	EU	-	-	-	-	-	-	-	-
3	Nasal swab 123	NA	32.84	32.56	32.58	32.83	33.36	32.83	0.32	0.98
4	Nasal swab 125	NA	33.93	33.79	34.55	33.60	34.45	34.06	0.42	1.23
5	Serum 2565-2	Neg	-	-	-	-	-	-	-	-
Mean value										**1.11**

SD = standard deviation, CV = coefficient of variation, EU = European genotype, NA = North American genotype

Table 16. Inter-assay variance of C_T values for the Internal Control (JOE) tested with virotype PRRSV NA/EU Primers/Probes combined with IndiMix JOE on the ABI 7500 Fast thermocycler (fast mode).

Inter-assay variance for Internal Control										
Samples (PRRSV genotype)			**Reactions (C_T values)**					**C_T mean**	**SD**	**CV %**
			1	2	3	4	5			
1	Lung 131	EU	26.96	26.80	29.13	26.63	27.59	27.42	1.02	3.73
2	Serum pool	EU	26.38	26.26	28.11	26.13	26.98	26.77	0.81	3.04
3	Nasal swab 123	NA	26.62	25.95	28.55	25.89	27.85	26.97	1.18	4.39
4	Nasal swab 125	NA	27.44	26.63	28.63	26.10	28.03	27.37	1.02	3.74
5	Serum 2565-2	Neg	27.69	25.79	26.38	26.58	26.04	26.49	0.73	2.77
Mean value										**3.53**

SD = standard deviation, CV = coefficient of variation, EU = European genotype, NA = North American genotype

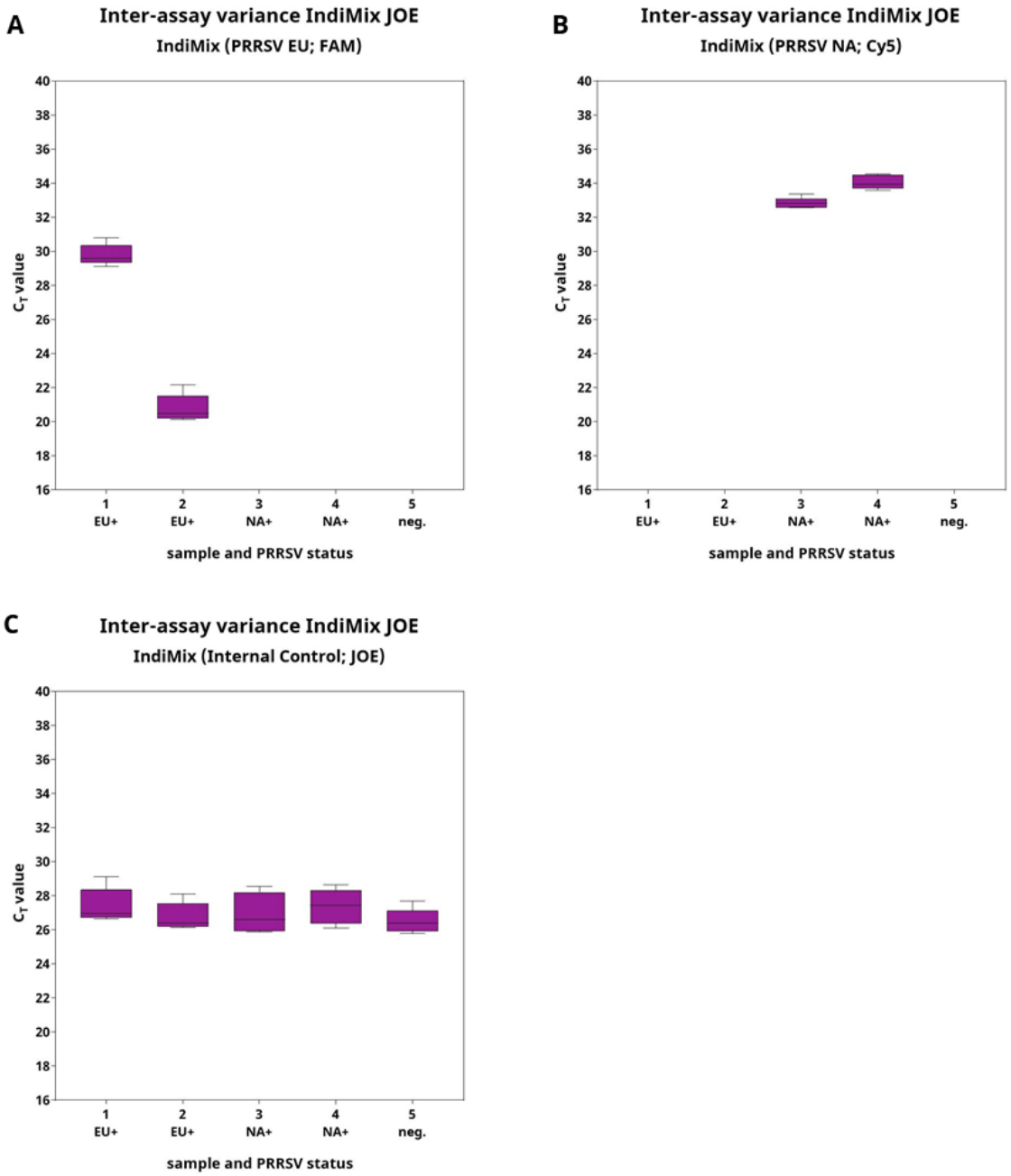

Figure 16. Boxplots showing inter-assay variance for PRRSV EU (FAM, **A**), PRRSV NA (Cy5, **B**) and the Internal Control (JOE, **C**) for the tested sample panel using virotype PRRSV NA/EU Primers/Probes combined with IndiMix JOE on the ABI 7500 Fast thermocycler (fast mode).
+ = positive, neg. = negative

2.5 Robustness

2.5.1 Stability testing (freeze/thaw cycles, storage at higher temperature)

The stability of the IndiMix JOE was assessed by testing different PRRSV-positive RNA samples using IndiMix JOE in combination with virotype PRRSV NA/EU Primers/Probes recently produced and after multiple freeze/thaw cycles. In addition, the IndiMix JOE was stored at 2 - 8°C and room temperature for 6 and 24 hours, respectively, followed by testing of the samples.

Results/Conclusion:

Table 17 shows the results of the recently produced (“standard”) test compared to the different considered conditions. Individual ΔC_T for all samples, respectively as well as mean ΔC_T per channel were calculated for the different tested conditions compared to the results obtained with the standard test. The variance of test results was very low for all channels in all tested conditions with mean ΔC_T values ranging between -1.17 to +0.72. With this, the IndiMix JOE shows very good stability and robustness with no significant impact on performance after multiple freeze/thaw cycles, storage for several hours at 2-8°C or even at room temperature.

Table 17. Stability testing at different conditions with a panel comprising PRRSV EU or NA-positive lung, serum pool or nasal swab samples using virotype PRRSV NA/EU Primers/Probes combined with IndiMix JOE on the ABI 7500 Fast thermocycler (fast mode).

	C_T values at different conditions							
Condition	**PRRSV EU**		**PRRSV NA**		**Internal Control**			
	Lung	**Serum pool**	**Nasal swab 1**	**Nasal swab 2**	**Lung**	**Serum pool**	**Nasal swab 1**	**Nasal swab 2**
Fresh/standard	30.33	21.27	35.51	36.23	27.48	26.79	27.73	27.95
multiple freeze/thaw	31.03	21.60	35.01	35.56	28.59	27.00	27.81	28.10
Δ C_T vs. standard	0.70	0.33	-0.50	-0.67	1.11	0.21	0.08	0.15
Mean Δ C_T vs. standard	**0.52**		**-0.58**		**-0.39**			
6 h 2-8°C	30.19	20.36	34.80	36.45	27.41	25.56	26.14	26.17
Δ C_T vs. standard	-0.14	-0.91	-0.71	0.22	-0.07	-1.23	-1.59	-1.78
Mean Δ C_T vs. standard	**-0.52**		**-0.24**		**-1.17**			
24 h 2-8°C	30.32	20.49	34.85	35.46	27.55	25.97	26.18	26.22
Δ C_T vs. standard	-0.01	-0.78	-0.66	-0.77	0.07	-0.82	-1.55	-1.73
Mean Δ C_T vs. standard	**-0.40**		**-0.71**		**-1.01**			
6 h RT	30.90	21.85	34.70	36.19	28.49	27.33	28.46	28.54
Δ C_T vs. standard	0.57	0.58	-0.81	-0.04	1.01	0.54	0.73	0.59
Mean Δ C_T vs. standard	**0.58**		**-0.42**		**0.72**			
24 h RT	30.32	20.71	35.21	37.18	27.84	25.83	26.24	26.30
Δ C_T vs. standard	-0.01	-0.56	-0.30	0.95	0.36	-0.96	-1.49	-1.65
Mean Δ C_T vs. standard	**-0.28**		**0.33**		**-0.94**			

RT = room temperature

2.5.2 Inhibitor tolerance

To test the inhibitor tolerance of the IndiMix JOE, sample inhibition was simulated by treating one Avian Influenza A Virus-positive RNA sample with an increasing concentration of heparin (0.9 – 34.0 U/µl).

Results/Conclusion:

Table 18 shows the results for inhibitor tolerance testing of the IndiMix JOE. Increasing concentration of heparin added to the reaction mix leads to inhibition of the RT-PCR reaction and can be seen for both, the pathogen and the Internal Control signal. The inhibition study using heparin shows that the Internal Control signal cannot be detected anymore at 34 U/µl heparin, whereas the pathogen signal is still detectable, but decreased at this heparin concentration.

Table 18. Testing the IndiMix JOE for inhibitor tolerance by treatment of an Avian Influenza A Virus RNA-positive sample with an increasing concentration of heparin.

Pathogen	Heparin concentration (U/µl)	AIV C_T	Internal Control C_T
Avian Influenza A Virus sample 1645 (1:10^4)	-	28.59	30.81
	0.9	28.70	30.83
	1.7	29.48	31.98
	8.5	32.58	34.07
	17.0	33.67	37.81
	34.0	36.16	-