Measuring Protocol
qTOWERiris

Achieving Optimal Homogeneity and Multiplex Capability with qTOWERiris and highQu Master Mix

Introduction

Real-time PCR or qPCR has become an essential tool in molecular biology for both detection and quantification of nucleic acids. The method is indispensable in various areas including research and development, the pharmaceutical sector, quality assurance of food and animal feed, forensics. The qPCR devices available in the market must meet increasingly stringent quality and performance standards. Alongside the ability to employ diverse analysis methods and simultaneously detect multiple targets in a multiplex assay, achieving high sensitivity and reproducibility of measurement results is crucial. This is primarily accomplished through uniform excitation and detection across the entire sample block. The qTOWERiris, as demonstrated in this Measuring Protocol, meets these high standards by offering optimal homogeneity and multiplex capability in measurement results using 5 different qPCR MasterMix of highQu.

Your Benefits

- Patented high performance optical system of qTOWERiris series
- Optimal homogeneous excitation and detection in each of the 96 wells
- High reproducibility and Sensitivity

Application

For the SYBR Green application, two different Mastermix were used, one colorless and one colored with an inert blue dye for a convenient sample handling. The standard real-time PCR experiment was performed with 24 samples in 3 different columns, using the real-time PCR thermal cycler qTOWERiris. For the two tested probe Mastermix, a 6-color assay with specific dye-labeled probes was performed in 4 replicates per channel. In the analysis, Ct values were considered, the homogeneity of the measurements based on the standard deviation of the Ct values (and melting point values in the SYBR Green Assays), and the deviation of the final fluorescence of all samples were considered. In addition, a 1 Step RT qPCR Probe Kit was tested with human RNA. The gradient function of qTOWERiris was used to determine the ideal annealing temperature for the primers used as well as suitable temperatures for the reverse transcription part of the assay.

Table 1: Overview of tested Master Mix from highQu

	ORA™ qPCR Green ROX L Mix, 2x	ORA™ SEE qPCR Green ROX L Mix, 2x
Order number highQu	QPD0101	QPD0501

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Author: JoMo

Starting material	Bacterial DNA	Bacterial DNA

	ORA™ qPCR Probe Mix, 2x	**ORA™ SEE qPCR Probe Mix, 2x**
Order number highQu	QPP0101	QPP0401
Starting material	Human DNA	Human DNA

	4x 1Step RT qPCR Probe Kit
Order number highQu	QOP1401
Starting material	Human RNA

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ORA™ qPCR Green ROX L Mix, 2x and ORA™ qPCR SEE Green ROX L Mix, 2x

Table 2: Pipetting scheme of 24 replicates bacterial DNA with ORA™ qPCR Green ROX L Mix, 2x and ORA™ SEE qPCR Green ROX L Mix, 2x

Volume per Reaction:	20
repeat of determination	25

Date					
Template		E.*coli*			
Primer		K12			
Size		100 bp			
Device		qTOWERiris			
Master	**Description**	**Stock-solution**	**Final Concentration**	**Volume: [µl]**	**500,00**
MasterMIx	ORA™ (SEE) qPCR Green ROX L Mix, 2x	2 x	1 x	**250,0 µl**	
Water				**235,0 µl**	
Template	E.*coli*	10 ng/µl	0,1 ng/µl	**5,0 µl**	
Primer 1A	K12 Fwd	50 µM	0,5 µM	**5,0 µl**	
Primer 2A	K12 rev	50 µM	0,5 µM	**5,0 µl**	

Final Volume per Reaction: (Control)	**500,0 µl**

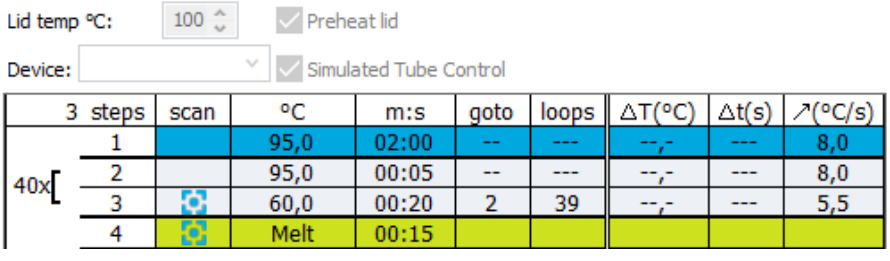

Figure 1: Temperature-time protocol of detection of bacterial DNA with ORA™ qPCR Green ROX L Mix, 2x and ORA™ SEE qPCR Green ROX L Mix, 2x

Results

ORA™ qPCR Green ROX L Mix, 2x

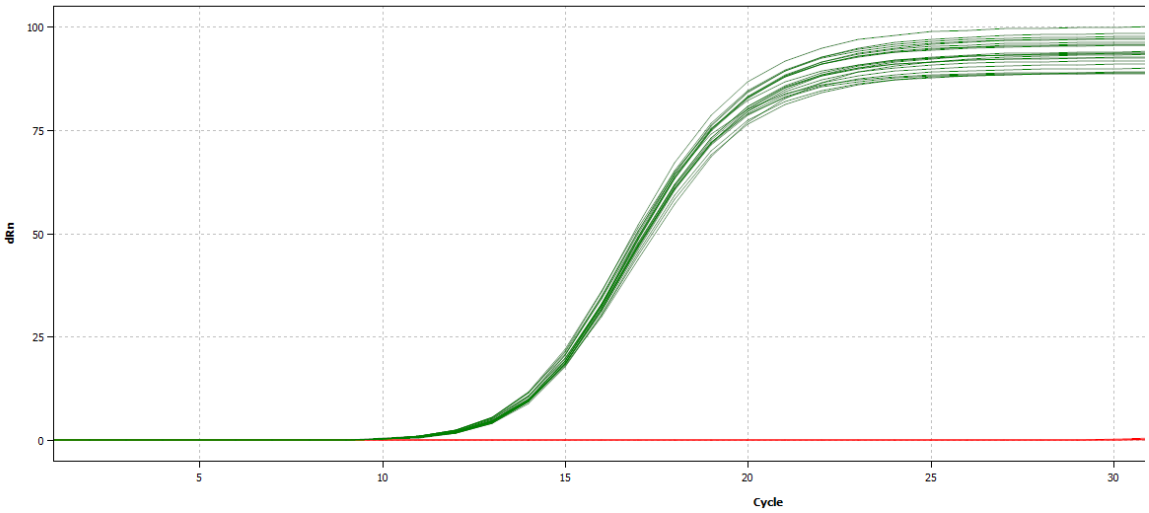

Figure 2: qPCR amplification curves of standard qPCR experiment over 24 samples of bacterial DNA with ORA™ qPCR Green ROX L Mix, 2x

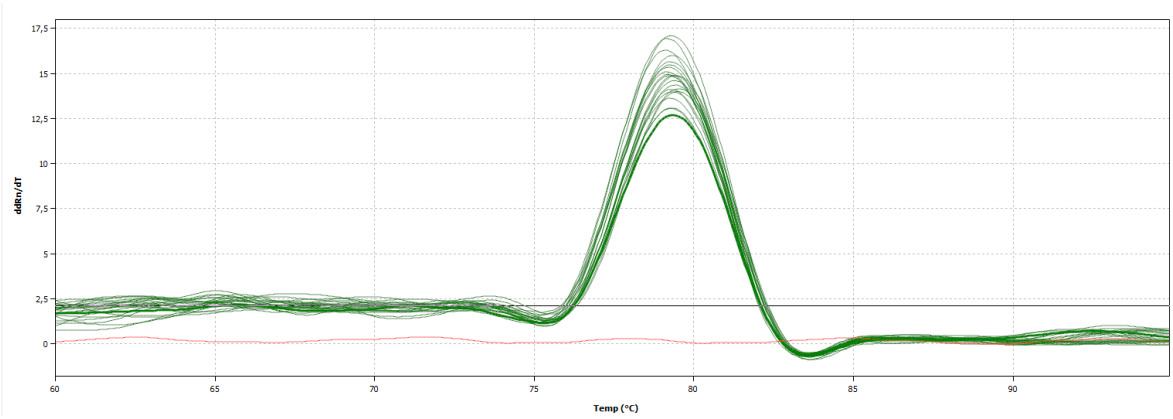

Figure 3: qPCR melting curves of standard qPCR experiment over 24 samples of bacterial DNA with ORA™ qPCR Green ROX L Mix, 2x

Table 3: Analysis data of qPCR amplification- and melting curve. [Ct: Cycle threshold. Std dev. Ct: Standard deviation of Ct, Tm: Melting point, Std dev. Melt: Standard deviation of melting point]

Ct value	Std dev. Ct	Min. Tm	Max. Tm	Diff. Tm	Std dev. Tm
11.92	0.16	79.1 °C	79.6 °C	0.5 °C	0.12

Min. fluorescence	Max. fluorescence	Diff. fluorescence	Deviation
77758	86107	8349	2.8 %

ORA™ SEE qPCR Green ROX L Mix, 2x

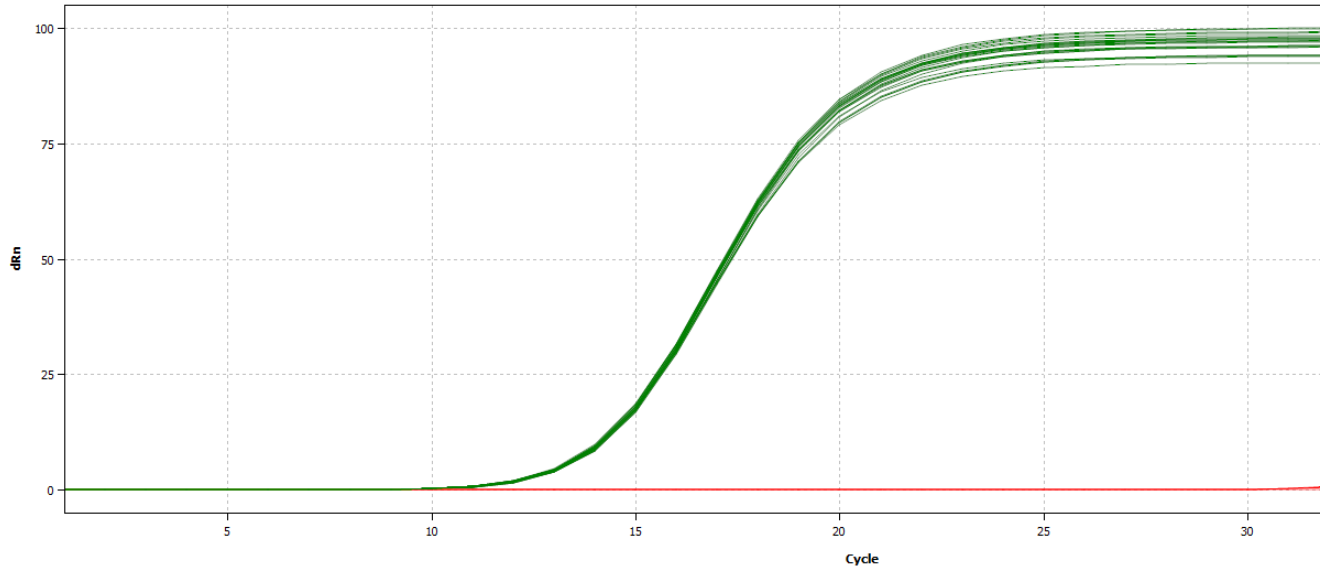

Figure 4: qPCR amplification curves of standard qPCR experiment over 24 samples of bacterial DNA with ORA™ SEE qPCR Green ROX L Mix, 2x

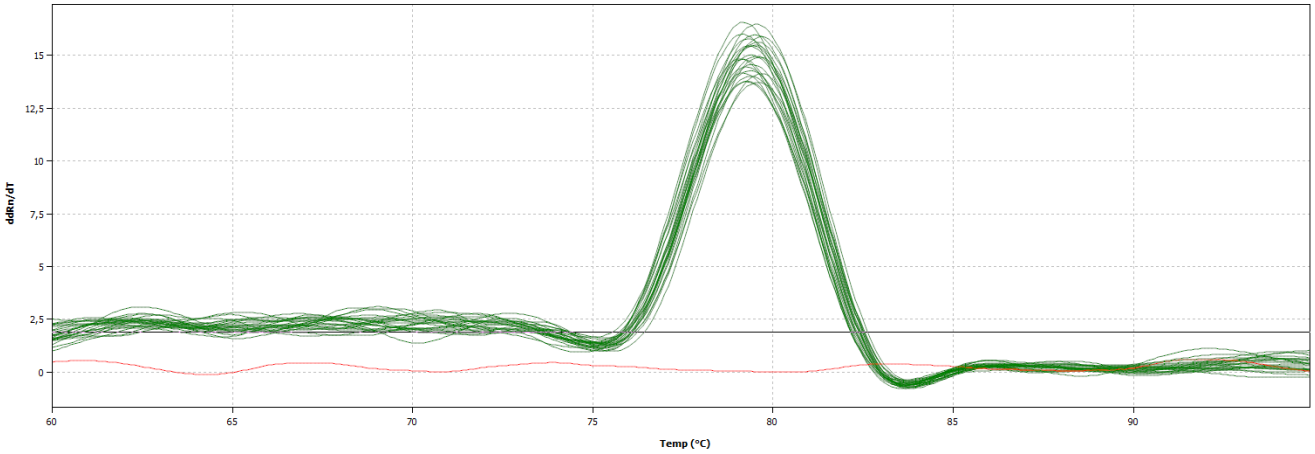

Figure 5: qPCR melting curves of standard qPCR experiment over 24 samples of bacterial DNA with ORA™ SEE qPCR Green ROX L Mix, 2x

Table 4: Analysis data of qPCR amplification- and melting curve. [Ct: Cycle threshold. Std dev. Ct: Standard deviation of Ct, Tm: Melting point, Std dev. Melt: Standard deviation of melting point]

Ct value	Std dev. Ct	Min. Tm	Max. Tm	Diff. Tm	Std dev. Tm
11.92	0.16	79.1 °C	79.7 °C	0.6 °C	0.17

Min. fluorescence	Max. fluorescence	Diff. fluorescence	Deviation
75896	83848	7952	2.4 %

Conclusion

Figures 2 to 5 clearly show the very homogeneous distribution of the curves in both the amplification plot as well as the melting curve plot of the standard qPCR experiment over 24 samples, using the ORA™ qPCR Green ROX L Mix, 2x and ORA™ qPCR SEE Green ROX L Mix, 2x Master Mix to detect a bacterial DNA template. With a standard deviation of 0.12 / 0.17, the Ct values are within a very precise range. Furthermore, the deviation in the final fluorescence over the 24 samples was 2.8 / 2.4%, respectively.

ORA™ qPCR Probe Mix, 2x and ORA™ SEE qPCR Probe Mix, 2x

Table 5: Pipetting scheme of 24 replicates human DNA with ORA™ qPCR Probe Mix, 2x and ORA™ SEE qPCR Probe Mix, 2x (with surplus).

Volume per Reaction:	20
repeat of determination	30

Template		human genomic DNA			
Primer		SRY			
Size		100 bp			
Device		qTOWERiris			
Master	**Description**	**Stock-solution**	**Final Concentration**	**Volume: [µl]**	**600,00**
MasterMIx	ORA™ (SEE)qPCR Probe Mix, 2x	2 x	1 x	**300,00 µl**	
Water				**286,80 µl**	
Template	human DNA	10 ng/µl	0,1 ng/µl	**6,00 µl**	
Primer 1A	SRY Fwd	50 µM	0,3 µM	**3,60 µl**	
Primer 2A	SRY rev	50 µM	0,3 µM	**3,60 µl**	
		Final Volume per Reaction: (Control)		**600,00 µl**	

6 tubes á 100,00 µl

Probe	**FAM**	5	µM	0,1	µM	**2,00**	**µl**
Probe	**JOE**	5	µM	0,1	µM	**2,00**	**µl**
Probe	**Atto550**	5	µM	0,1	µM	**2,00**	**µl**
Probe	**ROX**	5	µM	0,1	µM	**2,00**	**µl**
Probe	**Cy5**	5	µM	0,1	µM	**2,00**	**µl**
Probe	**Cy5.5**	5	µM	0,1	µM	**2,00**	**µl**

	3 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
50x[	1		95,0	02:00	--	---	--,-	---	8,0
	2		95,0	00:05	--	---	--,-	---	8,0
	3		60,0	00:30	2	49	--,-	---	5,5

Figure 6: Temperature-time protocol of detection of human DNA with ORA™ qPCR Probe Mix, 2x and ORA™ SEE qPCR Probe Mix, 2x

Results

ORA™ qPCR Probe Mix, 2x

FAM™ signals with Standard Color Compensation qTOWERiris #1

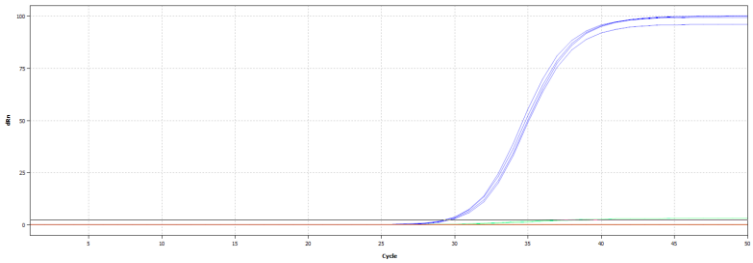

Figure 7: Amplification curve and Ct value determination of FAM™

Table 6: Ct values of Color Module 1 (FAM™ channel)

Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B2	FAM	FAM	29,35	29,50	0,19
C2	FAM	FAM	29,36	29,50	0,19
D2	FAM	FAM	29,53	29,50	0,19
E2	FAM	FAM	29,76	29,50	0,19

JOE™ signals with Standard Color Compensation qTOWERiris #1

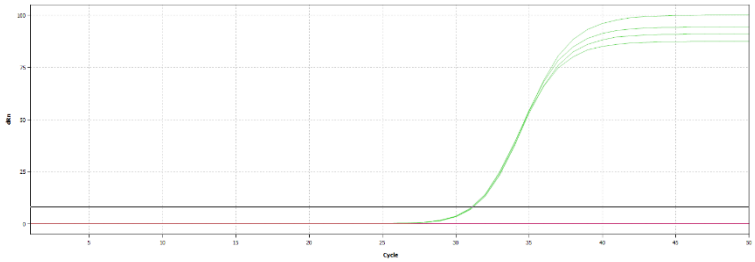

Figure 8: Amplification curve and Ct value determination of JOE™

Table 7: Ct values of Color Module 1 (FAM™ channel

Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B4	JOE	JOE	31,15	31,17	0,04
C4	JOE	JOE	31,21	31,17	0,04
D4	JOE	JOE	31,20	31,17	0,04
E4	JOE	JOE	31,12	31,17	0,04

Atto550 signals with Standard Color Compensation qTOWERiris #1

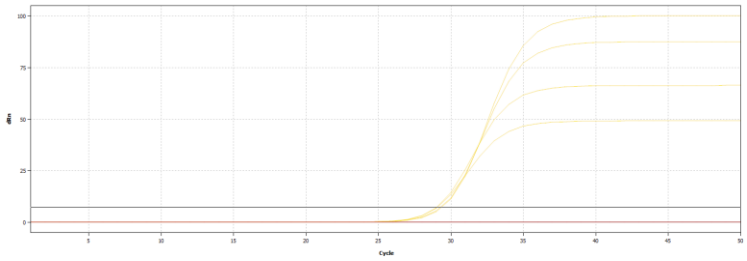

Figure 9: Amplification curve and Ct value determination of Atto550

Table 8: Ct values of Color Module 3 (Atto550 channel)

Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B6	ATTO550	ATTO550	29,38	29,21	0,16
C6	ATTO550	ATTO550	29,31	29,21	0,16
D6	ATTO550	ATTO550	29,06	29,21	0,16
E6	ATTO550	ATTO550	29,10	29,21	0,16

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ROX™ signals with Standard Color Compensation qTOWERiris #1

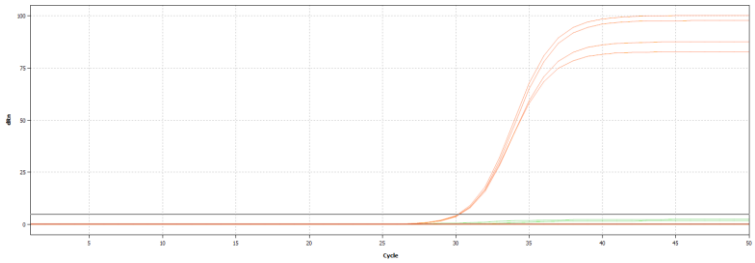

Figure 10: Amplification curve and Ct value determination of ROX™

Table 9: Ct values of Color Module 4 (ROX™ channel)

Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B8	ROX	ROX	30,13	30,20	0,05
C8	ROX	ROX	30,21	30,20	0,05
D8	ROX	ROX	30,27	30,20	0,05
E8	ROX	ROX	30,21	30,20	0,05

Cy5® signals with Standard Color Compensation qTOWERiris #1

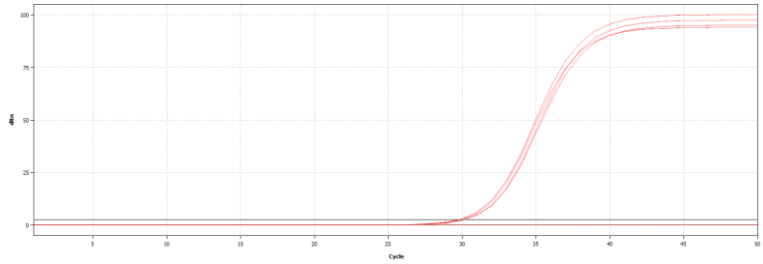

Figure 11: Amplification curve and Ct value determination of Cy5®

Table 10: Ct values of Color Module 5 (Cy5® channel)

Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B10	Cy5	Cy5	30,10	29,97	0,19
C10	Cy5	Cy5	29,90	29,97	0,19
D10	Cy5	Cy5	30,15	29,97	0,19
E10	Cy5	Cy5	29,73	29,97	0,19

Cy5.5® signals with Standard Color Compensation qTOWERiris #1

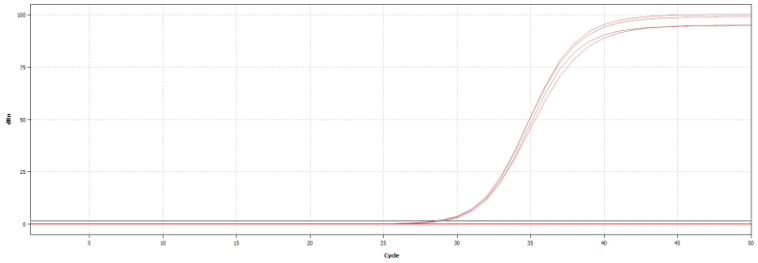

Figure 12: Amplification curve and Ct value determination of Cy5.5®

Table 11: Ct values of Color Module 6 (Cy5.5® channel)

Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B12	Cy5.5	Cy5.5	28,78	28,93	0,18
C12	Cy5.5	Cy5.5	29,15	28,93	0,18
D12	Cy5.5	Cy5.5	29,01	28,93	0,18
E12	Cy5.5	Cy5.5	28,78	28,93	0,18

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ORA™ SEE qPCR Probe Mix, 2x

FAM™ signals with Standard Color Compensation qTOWERiris #1

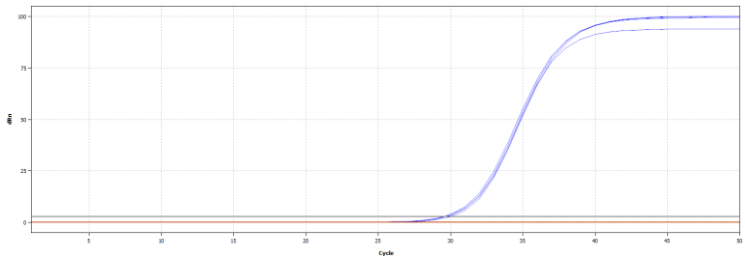


Figure 13: Amplification curve and Ct value determination of FAM™

Table 12: Ct values of Color Module 1 (FAM™ channel)

Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B2	FAM	FAM	29,38	29,59	0,23
C2	FAM	FAM	29,93	29,59	0,23
D2	FAM	FAM	29,54	29,59	0,23
E2	FAM	FAM	29,53	29,59	0,23

JOE™ signals with Standard Color Compensation qTOWERiris #1

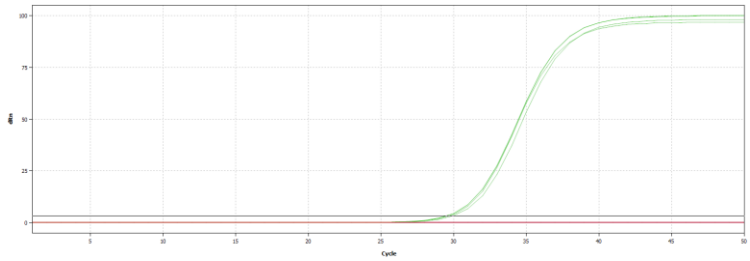


Figure 14: Amplification curve and Ct value determination of JOE™

Table 13: Ct values of Color Module 1 (FAM™ channel

Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B4	JOE	JOE	29,98	29,69	0,22
C4	JOE	JOE	29,71	29,69	0,22
D4	JOE	JOE	29,51	29,69	0,22
E4	JOE	JOE	29,54	29,69	0,22

Atto550 signals with Standard Color Compensation qTOWERiris #1

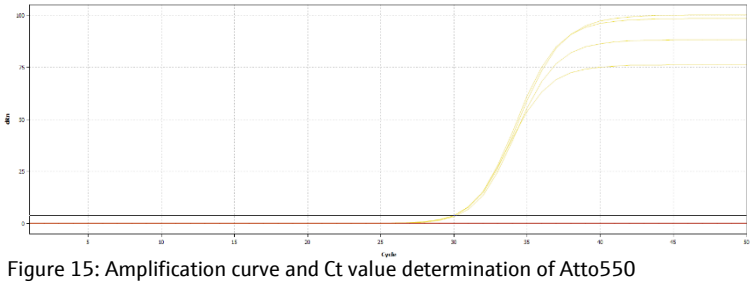


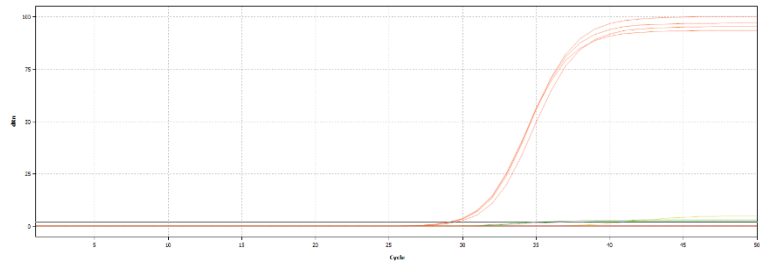
Figure 15: Amplification curve and Ct value determination of Atto550

Table 14: Ct values of Color Module 3 (Atto550 channel)

Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B6	Atto550	ATTO550	30,06	30,10	0,09
C6	Atto550	ATTO550	30,05	30,10	0,09
D6	Atto550	ATTO550	30,24	30,10	0,09
E6	Atto550	ATTO550	30,06	30,10	0,09

ROX™ signals with Standard Color Compensation qTOWERiris #1

Table 15: Ct values of Color Module 4 (ROX™ channel)

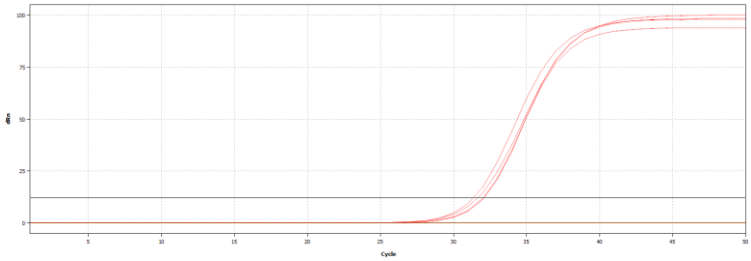


Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B8	ROX	ROX	29,17	29,29	0,21
C8	ROX	ROX	29,23	29,29	0,21
D8	ROX	ROX	29,60	29,29	0,21
E8	ROX	ROX	29,17	29,29	0,21

Figure 16: Amplification curve and Ct value determination of ROX™

Cy5® signals with Standard Color Compensation qTOWERiris #1

Table 16: Ct values of Color Module 5 (Cy5® channel)

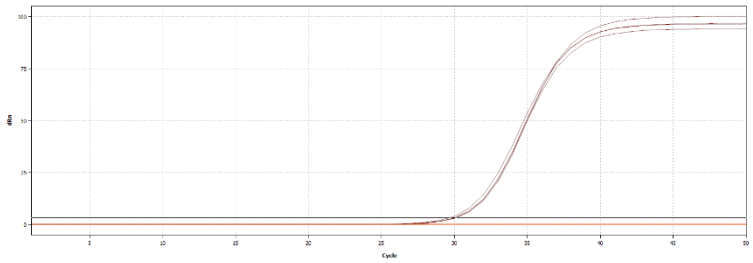


Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B10	Cy5	Cy5	32,48	32,57	0,31
C10	Cy5	Cy5	32,78	32,57	0,31
D10	Cy5	Cy5	32,17	32,57	0,31
E10	Cy5	Cy5	32,83	32,57	0,31

Figure 17: Amplification curve and Ct value determination of Cy5®

Cy5.5® signals with Standard Color Compensation qTOWERiris #1

Table 17: Ct values of Color Module 6 (Cy5.5® channel)



Well	Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
B12	Cy5.5	Cy5.5	30,13	30,02	0,21
C12	Cy5.5	Cy5.5	30,16	30,02	0,21
D12	Cy5.5	Cy5.5	29,71	30,02	0,21
E12	Cy5.5	Cy5.5	30,06	30,02	0,21

Figure 18: Amplification curve and Ct value determination of Cy5.5®

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Conclusion

In addition to intercalating dye-based applications, specific probe-based detection also delivers precise results using the ORA™ qPCR Probe Mix, 2x and ORA™ SEE qPCR Probe Mix, 2x together with the real-time PCR thermal cycler qTOWER iris.
After adapting the pre-installed color compensation, we received clear signals in each specific channel regarding signal heights, Ct values and standard deviations.
In summary, these results show a very precise and homogenous performance of the qTOWER iris using the qPCR probe Master Mixes from highQu.

4x 1Step RT qPCR Probe Kit Annealing/Elongation Temperature Gradient Test

Table 18: Pipetting scheme of 20 replicates human RNA with 1Step RT qPCR Mix, 4x (with surplus).

Volume per Reaction:	20
repeat of determination	22

Date							
Template		human RNA					
Primer		GAPDH					
Size		97 bp					
Device		qTOWERiris					
Master	**Description**	**Stock concentration**		**Final Concentration**		**Volume µl**	**440,00**
MasterMix	1Step RT qPCR Mix, 4x	4 x		1 x		**110,00**	**µl**
RT 7 Mix	highQu	20 x		1 x		**22,00**	**µl**
Water						**298,32**	**µl**
Template	human RNA	100	ng/µl	1,0	ng/µl	**4,40**	**µl**
Primer 1A	fwd	50	µM	0,3	µM	**2,64**	**µl**
Primer 2A	rev	50	µM	0,3	µM	**2,64**	**µl**
Probe	GAPDH-Yakima Yellow	5	µM	0,1	µM	**8,80**	**µl**
				Final Volume per Reaction: (Control)		**440,00**	**µl**

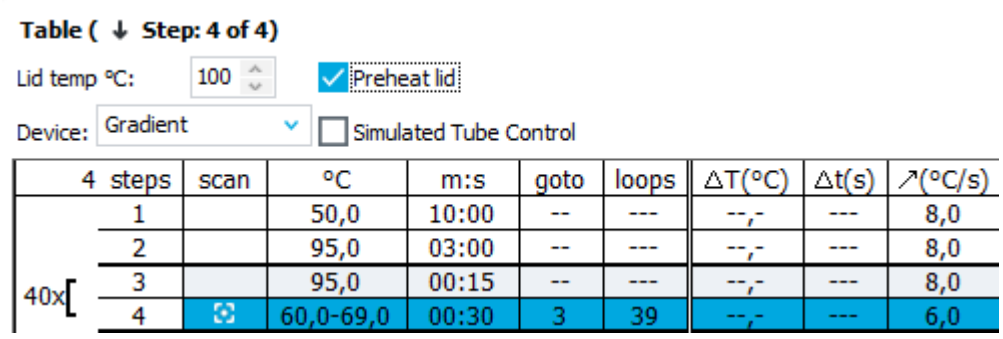

Figure 19: Temperature-time protocol of detection of human RNA with 4x 1Step RT qPCR Probe Kit

Results

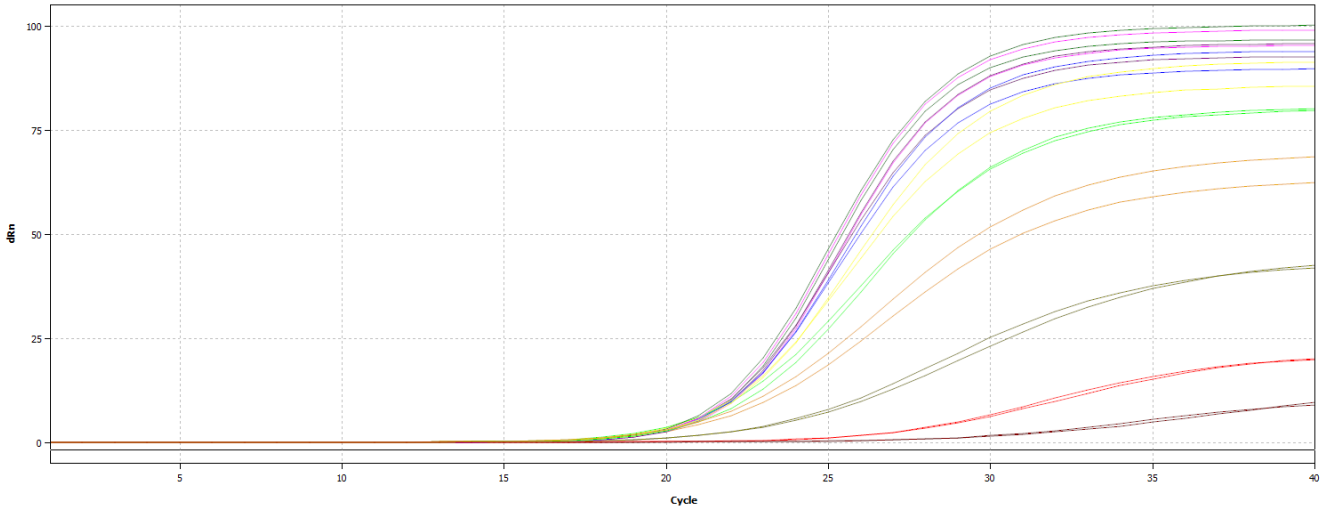

Figure 20: Amplification curve and Ct value determination of Annealing/Elongation Temperature Gradient Test

Table 19: Ct values and standard deviations of Annealing/Elongation Temperature Gradient Test

Well		Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
A1		60 °C	YakimaYellow	19,41	19,28	0,18
B1		60 °C	YakimaYellow	19,15	19,28	0,18
A3		61 °C	YakimaYellow	19,55	19,42	0,18
B3		61 °C	YakimaYellow	19,29	19,42	0,18
A4		62 °C	YakimaYellow	19,24	19,34	0,14
B4		62 °C	YakimaYellow	19,44	19,34	0,14
A5		63 °C	YakimaYellow	19,25	19,30	0,07
B5		63 °C	YakimaYellow	19,35	19,30	0,07
A6		64 °C	YakimaYellow	19,03	19,13	0,14
B6		64 °C	YakimaYellow	19,22	19,13	0,14
A7		65 °C	YakimaYellow	18,76	19,01	0,35
B7		65 °C	YakimaYellow	19,25	19,01	0,35
A8		66 °C	YakimaYellow	19,25	19,12	0,18
B8		66 °C	YakimaYellow	19,00	19,12	0,18
A9		67 °C	YakimaYellow	21,19	21,19	0,01
B9		67 °C	YakimaYellow	21,18	21,19	0,01
A10		68 °C	YakimaYellow	26,31	26,29	0,03
B10		68 °C	YakimaYellow	26,26	26,29	0,03
A12		69 °C	YakimaYellow	30,97	30,73	0,34
B12		69 °C	YakimaYellow	30,49	30,73	0,34

Conclusion

The 4x concentrated probe master mix for the application of a 1 step rt qPCR assay also showed homogeneous results in the evaluation, as shown by the Ct values and standard deviations of the individual replicates (Table). The gradient test of the annealing/elongation temperature showed a clear dependency between temperature and fluorescence height of the amplified DNA on the one hand (Figure…) and the Ct values on the other (Table…) Although the Ct values remained relatively constant with increasing temperatures up to a temperature of 66°C, there was a clear drop in the fluorescence height and thus the efficiency of the reaction, especially at temperatures of 65°C and higher. This can be explained by the decreasing ability of the primers to bind to the target as the temperature increases. It is therefore recommended to test the optimal annealing/elongation temperature with a gradient function in advance of each qPCR assay.

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4x 1Step RT qPCR Probe Kit RT-Temperature Gradient Test

Volume per Reaction:	20
repeat of determination	18

Date	
Template	human RNA
Primer	GAPDH
Size	97 bp
Device	qTOWERiris

Master	Description	Stock concentration		Final Concentration		Volume µl	360,00
MasterMix	1Step RT qPCR Mix, 4x	4	x	1	x	**90,00**	**µl**
RT 7 Mix	highQu	20	x	1	x	**18,00**	**µl**
Water						**244,08**	**µl**
Template	human RNA	100	ng/µl	1,0	ng/µl	**3,60**	**µl**
Primer 1A	fwd	50	µM	0,3	µM	**2,16**	**µl**
Primer 2A	rev	50	µM	0,3	µM	**2,16**	**µl**
Probe	GAPDH-Yakima Yellow	5	µM	0,1	µM	**7,20**	**µl**

Final Volume per Reaction: (Control)	**360,00**	**µl**

Table (↓ Step: 1 of 4)

Lid temp °C: 100 Preheat lid

Device: Gradient Simulated Tube Control

	4 steps	scan	°C	m:s	goto	loops	ΔT(°C)	Δt(s)	↗(°C/s)
	1		40,0-55,0	10:00	--	---	--,-	---	8,0
	2		95,0	03:00	--	---	--,-	---	5,5
40x[	3		95,0	00:15	--	---	--,-	---	8,0
	4		60,0	00:30	3	39	--,-	---	5,5

Figure 20: Time-temperature protocol of detection of human RNA with 4x 1Step RT qPCR Probe Kit

Results

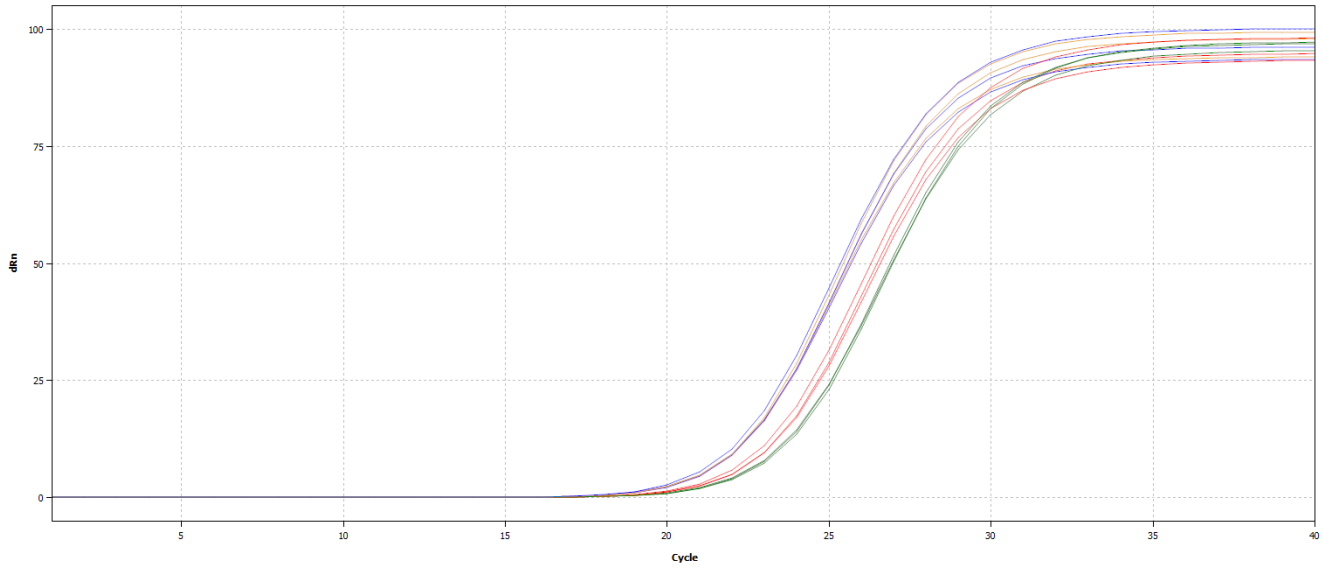

Figure 21: Amplification curve and Ct value determination of **RT-Temperature Gradient Test**

Table 20: Ct values and standard deviations of RT-Temperature Gradient Test

Well		Sample name	Dye	Ct	Mean Ct	Std. dev. Ct
A1		40°C	YakimaYellow	19,99	19,86	0,21
B1		40°C	YakimaYellow	19,97	19,86	0,21
C1		40°C	YakimaYellow	19,62	19,86	0,21
A5		45 °C	YakimaYellow	19,02	19,06	0,03
B5		45 °C	YakimaYellow	19,08	19,06	0,03
C5		45 °C	YakimaYellow	19,08	19,06	0,03
A8		50 °C	YakimaYellow	19,01	18,97	0,15
B8		50 °C	YakimaYellow	19,09	18,97	0,15
C8		50 °C	YakimaYellow	18,80	18,97	0,15
A12		55°C	YakimaYellow	20,12	20,20	0,07
B12		55°C	YakimaYellow	20,22	20,20	0,07
C12		55°C	YakimaYellow	20,26	20,20	0,07

Conclusion

Figure 21 and Table 20 clearly show that the temperature of reverse transcription in the 40-55°C range has no significant influence on the results of the Ct values, standard deviation and fluorescence levels and thus the efficiency of the reaction. The reverse transcriptase of 4x 1Step RT qPCR Probe Kit can therefore be used very flexibly in a wide variety of assay setups.

Reference: MeasProt_qTOWERiris_ MasterMix_highQu_0001_en

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