

### Challenge

Performing a six-color hydrolysis probe assay without signal crosstalk as a prerequisite for a multiplex assay with up to 6 targets.

### Solution

qTOWERiris with unique multicolor LED light source and extended wavelength ranges for real 6-plex applications.

### Intended audience

General qPCR users working in the field of molecular biology, genetics, or related areas.

## Performing a Six-Color Probe-based Assay Without Signal Crosstalk and Calibration Effort

### Introduction

In addition to the qualitative detection and quantification of individual targets using real-time PCR, the task of simultaneously detecting multiple targets is becoming increasingly convenient. Setting up a multiplex PCR experiment requires more effort than a singleplex real-time PCR experiment. However, once the setup is established, users can profit from numerous benefits, including:

- Long-term time and cost savings – optimized setups save personnel costs as well as expenses for reagents and consumables
- Reduced impact of pipetting errors due to fewer pipetting steps
- Possibility of data normalization by incorporating an internal reference in each PCR

Currently, most commercial detection assays identify up to four different targets, and rarely up to five genes. A major challenge lies in the precise separation of individual fluorescence signals across different channels. This is one of the main reasons why there are still relatively few assays using more than five different dyes for detection. Thanks to the new technology featuring a unique multicolor LED light source and extended wavelength ranges in qTOWERiris, along with a clever combination of dyes used for probe labeling, real-time PCR with up to six targets, without crosstalk and the need for expensive calibration reagents, is now possible.

## Materials and Methods

### Chemicals and consumables

- 6-Color qPCR Performance Assay from Analytik Jena (Order number: 847-0212000603)
- Components of the assay:
  - 96-well microplate
  - Optical sealing foil PP96
  - Reagent Mix FAM™, lyophilized
  - Reagent Mix JOE™, lyophilized
  - Reagent Mix ATTO550, lyophilized
  - Reagent Mix ROX™, lyophilized
  - Reagent Mix Cy5®, lyophilized
  - Reagent Mix Cy5.5®, lyophilized
  - Standard SRY, lyophilized
  - 2x Master Mix
  - PCR-grade H<sub>2</sub>O
- Components not included in the kit:
  - Reaction tubes
  - Microcentrifuge, if available plate centrifuge
  - Vortex mixer
  - Variable pipettes for 10 µL – 1.000 µL
  - Sterile pipette tips with protection against contamination

Table 1: PCR Mix preparation for each dye (volumes indicated for 4 reactions at 18 µL per dye):

Component	Volume
Reagent Mix	20 µL
Standard SRY (1:100 diluted)	20 µL
2x Master Mix	40 µL
<b>Final volume</b>	<b>80 µL</b>

Dispense 18 µL of PCR mix into 4 wells of the 96-well microplate for each dye. For example, in columns 2, 4, 6, 8, 10 and 12 as indicated in the layout below:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		FAM		JOE		A550		ROX		Cy5		Cy5.5
C		FAM		JOE		A550		ROX		Cy5		Cy5.5
D		FAM		JOE		A550		ROX		Cy5		Cy5.5
E		FAM		JOE		A550		ROX		Cy5		Cy5.5
F												
G												
H												

Fig. 1: Plate layout for the real-time PCR run

### Instrumentation

The real-time PCR thermocycler qTOWERiris including:

- Color module 1, blue (455 nm / 515 nm) was used for FAM™ dye
- Color module 2, green (520 nm / 560 nm) was used for JOE™ dye
- Color module 3, yellow (550 nm / 585 nm) was used for ATTO550 dye
- Color module 4, orange (580 nm / 620 nm) was used for ROX™ dye
- Color module 5, red (625 nm / 670 nm) was used for Cy5® dye
- Color module 6, NIR (660 nm / 710 nm) was used for Cy5.5® dye

Table 2: Temperature and time protocol:

Step	Cycle	Profile	Temperature	Holding time	Ramp rates
1	1	Initial denaturation	95 °C	2 min	max
2	30	Denaturation	95 °C	15 sec	max
		Annealing /Elongation*	60 °C	30 sec	max

\* Data acquisition: color module 1, color module 2, color module 3, color module 4, color module 5 and color module 6 for qTOWERiris Series

## Results and Discussion

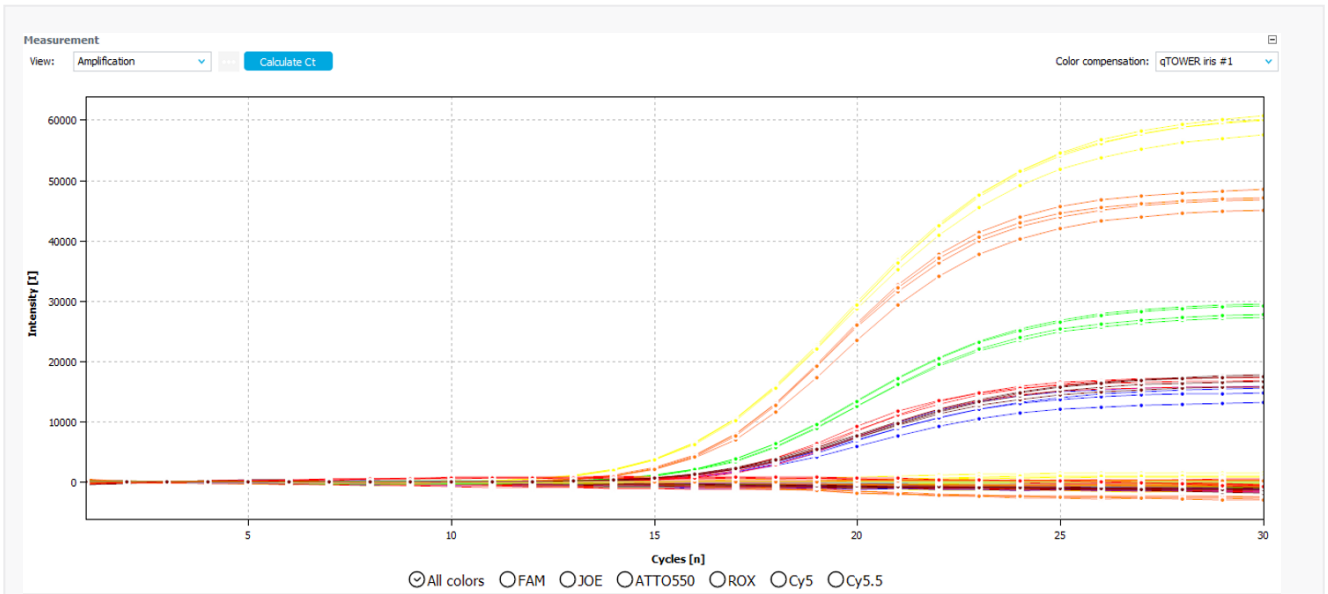


Fig. 2: Results of each channel with Standard Color Compensation qTOWERiris #1

Figure 2 displays the amplification plot for each individual dye (FAM™, JOE™, ATTO550, ROX™, Cy5®, and Cy5.5®) in their respective channels, demonstrating no crosstalk to adjacent channels. This is achieved by applying the spectral color compensation standard qTOWERiris #1, eliminating the need for a complex and expensive device calibration set. Any potential signal crosstalk between certain channel combinations can be further reduced or eliminated using the standard color compensation tool in the qPCRsoft 5.0 software. In the following figures (figure 3-8), the amplification curves and the determination of the Ct value for each color channel are depicted.

### FAM™ signals with Standard Color Compensation qTOWERiris #1

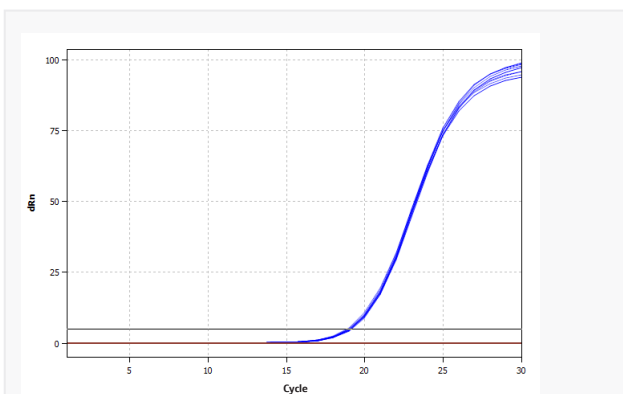


Fig. 3: Amplification curve and Ct value determination of FAM™

Table 3: Ct values of Color Module 1 (FAM™ channel)

Well	Sample type	Dye	Ct	Mean Ct	Std. dev. Ct
C2	Unknown	FAM	16,50	16,53	0,16
D2	Unknown	FAM	16,70	16,53	0,16
E2	Unknown	FAM	16,60	16,53	0,16
F2	Unknown	FAM	16,33	16,53	0,16
C4	Unknown	FAM	No Ct		
D4	Unknown	FAM	No Ct		
E4	Unknown	FAM	No Ct		
F4	Unknown	FAM	No Ct		
C6	Unknown	FAM	No Ct		
D6	Unknown	FAM	No Ct		
E6	Unknown	FAM	No Ct		
F6	Unknown	FAM	No Ct		

### JOE™ signals with Standard Color Compensation qTOWERiris #1

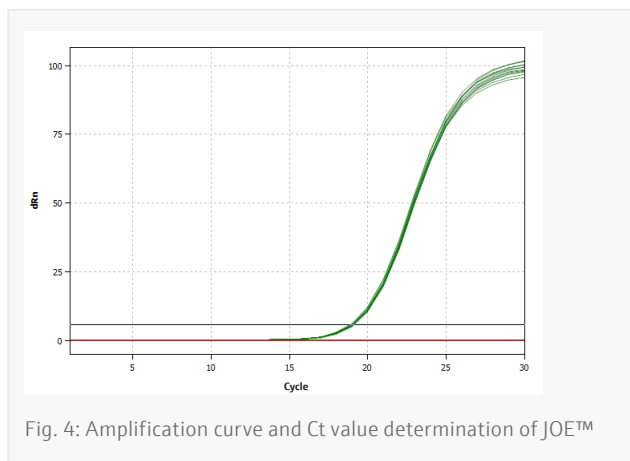


Table 4: Ct values of Color Module 2 (JOE™ channel)

Well	Sample type	Dye	Ct	Mean Ct	Std. dev. Ct
C2	Unknown	JOE	No Ct		
D2	Unknown	JOE	No Ct		
E2	Unknown	JOE	No Ct		
F2	Unknown	JOE	No Ct		
C4	Unknown	JOE	15,31	15,40	0,13
D4	Unknown	JOE	15,41	15,40	0,13
E4	Unknown	JOE	15,57	15,40	0,13
F4	Unknown	JOE	15,30	15,40	0,13
C6	Unknown	JOE	No Ct		
D6	Unknown	JOE	No Ct		
E6	Unknown	JOE	No Ct		
F6	Unknown	JOE	No Ct		

### ATTO550 signals with Standard Color Compensation qTOWERiris #1

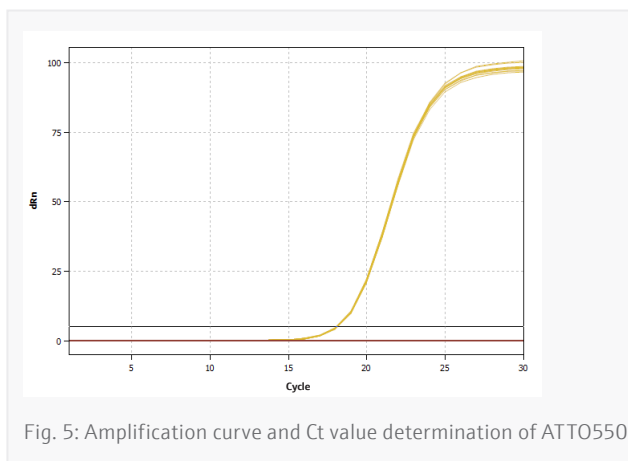


Table 5: Ct values of Color Module 3 (ATTO550 channel)

Well	Sample type	Dye	Ct	Mean Ct	Std. dev. Ct
C4	Unknown	ATTO550	No Ct		
D4	Unknown	ATTO550	No Ct		
E4	Unknown	ATTO550	No Ct		
F4	Unknown	ATTO550	No Ct		
C6	Unknown	ATTO550	14,39	14,47	0,06
D6	Unknown	ATTO550	14,50	14,47	0,06
E6	Unknown	ATTO550	14,48	14,47	0,06
F6	Unknown	ATTO550	14,53	14,47	0,06
C8	Unknown	ATTO550	No Ct		
D8	Unknown	ATTO550	No Ct		
E8	Unknown	ATTO550	No Ct		
F8	Unknown	ATTO550	No Ct		

### ROX™ signals with Standard Color Compensation qTOWERiris #1

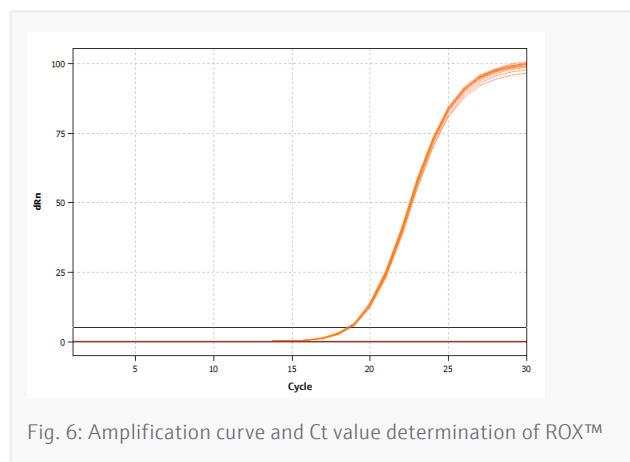


Table 6: Ct values of Color Module 4 (ROX™ channel)

Well	Sample type	Dye	Ct	Mean Ct	Std. dev. Ct
C6	Unknown	ROX	No Ct		
D6	Unknown	ROX	No Ct		
E6	Unknown	ROX	No Ct		
F6	Unknown	ROX	No Ct		
C8	Unknown	ROX	15,70	15,52	0,12
D8	Unknown	ROX	15,49	15,52	0,12
E8	Unknown	ROX	15,42	15,52	0,12
F8	Unknown	ROX	15,48	15,52	0,12
C10	Unknown	ROX	No Ct		
D10	Unknown	ROX	No Ct		
E10	Unknown	ROX	No Ct		
F10	Unknown	ROX	No Ct		

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

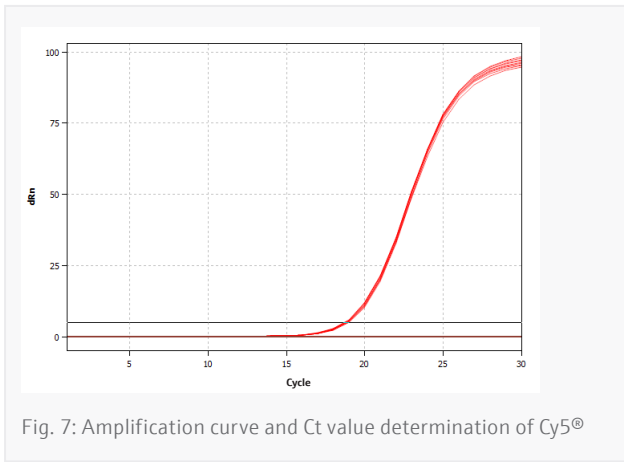


Fig. 7: Amplification curve and Ct value determination of Cy5®

Table 7: Ct values of Color Module 5 (Cy5® channel)

Well	Sample type	Dye	Ct	Mean Ct	Std. dev. Ct
C8	Unknown	Cy5	No Ct		
D8	Unknown	Cy5	No Ct		
E8	Unknown	Cy5	No Ct		
F8	Unknown	Cy5	No Ct		
C10	Unknown	Cy5	16,06	16,30	0,23
D10	Unknown	Cy5	16,53	16,30	0,23
E10	Unknown	Cy5	16,15	16,30	0,23
F10	Unknown	Cy5	16,46	16,30	0,23
C12	Unknown	Cy5	No Ct		
D12	Unknown	Cy5	No Ct		
E12	Unknown	Cy5	No Ct		
F12	Unknown	Cy5	No Ct		

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

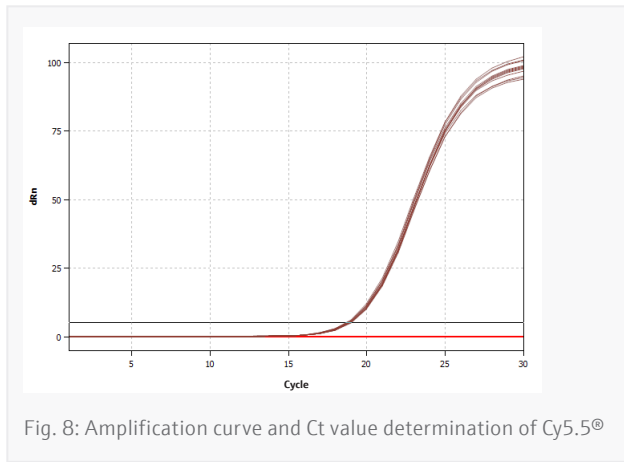


Fig. 8: Amplification curve and Ct value determination of Cy5.5®

Table 8: Ct values of Color Module 6 (Cy5.5® channel)

Well	Sample type	Dye	Ct	Mean Ct	Std. dev. Ct
C8	Unknown	Cy5.5	No Ct		
D8	Unknown	Cy5.5	No Ct		
E8	Unknown	Cy5.5	No Ct		
F8	Unknown	Cy5.5	No Ct		
C10	Unknown	Cy5.5	No Ct		
D10	Unknown	Cy5.5	No Ct		
E10	Unknown	Cy5.5	No Ct		
F10	Unknown	Cy5.5	No Ct		
C12	Unknown	Cy5.5	15,87	15,58	0,29
D12	Unknown	Cy5.5	15,41	15,58	0,29
E12	Unknown	Cy5.5	15,79	15,58	0,29
F12	Unknown	Cy5.5	15,27	15,58	0,29

## Conclusion

The potential for false-positive results, especially in highly multiplexed reactions with crosstalk in adjacent channels, is a significant concern. However, the advanced technology of qTOWERiris, including a unique multicolor LED light source and extended wavelength ranges, combined with a strategic choice of dyes for probe labeling, now facilitates real-time PCR with up to 6 targets. This advancement alleviates concerns of crosstalk and eliminates the need for expensive calibration reagents, thanks to the pre-installed standard color compensation in qPCRsoft software.

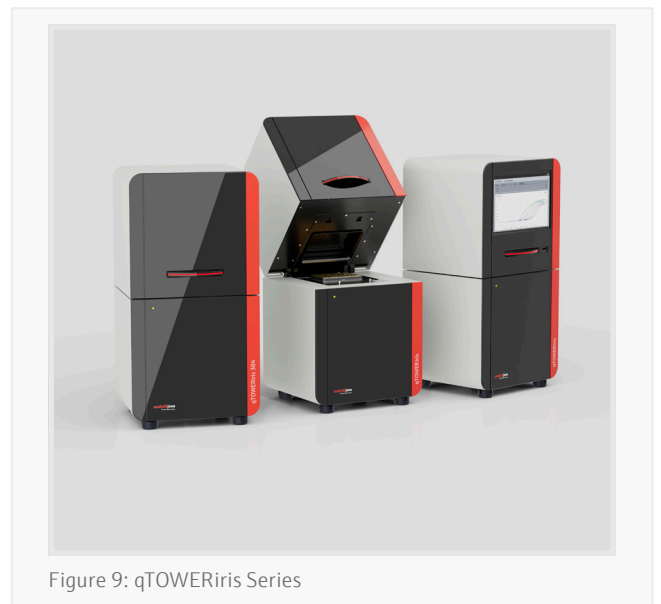


Figure 9: qTOWERiris Series

## Recommended device configuration

Table 9: Overview of required equipment and recommended accessories.

Article	Article number	Description
qTOWERiris incl. Color Module 1	844-00853-x*	Real-time PCR system designed in the standard 96-well format, operable via PC, customizable with up to 6 color modules. Available in 100 V, 115 V, and 230 V version, incl. color module 1 for excitation and detection of fluorescent dyes – FAM™, SYBR®Green, ATTO425 and Cyan500.
Color Module 2 for qTOWERiris series	844-00821-0	Color module for the excitation and emission of fluorescence dyes like JOE™, HEX™, VIC®, YakimaYellow® or TET™.
Color Module 3 for qTOWERiris series	844-00822-0	Color module for the excitation and emission of fluorescence dyes like TAMRA™ or ATTO550.
Color Module 4 for qTOWERiris series	844-00823-0	Color module for the excitation and emission of fluorescence dyes like ROX™, TexasRed®, Cy3.5® or ATTO590.
Color Module 5 for qTOWERiris series	844-00824-0	Color module for the excitation and emission of fluorescence dyes like Cy5® or ATTO633.
Color Module 6 for qTOWERiris series	844-00825-0	Color module for the excitation and emission of fluorescence dyes like Cy5.5® or ATTO665.
6-Color qPCR Performance Assay 96 well	847-0212000603	The 6-Color qPCR Performance Assay is a TaqMan-based assay that allows you to easily verify the performance of your real-time PCR system. The assay can be used for recording a color compensation.

\*x=2 for 230 V, x=4 for 115 V and x=5 for 100 V

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