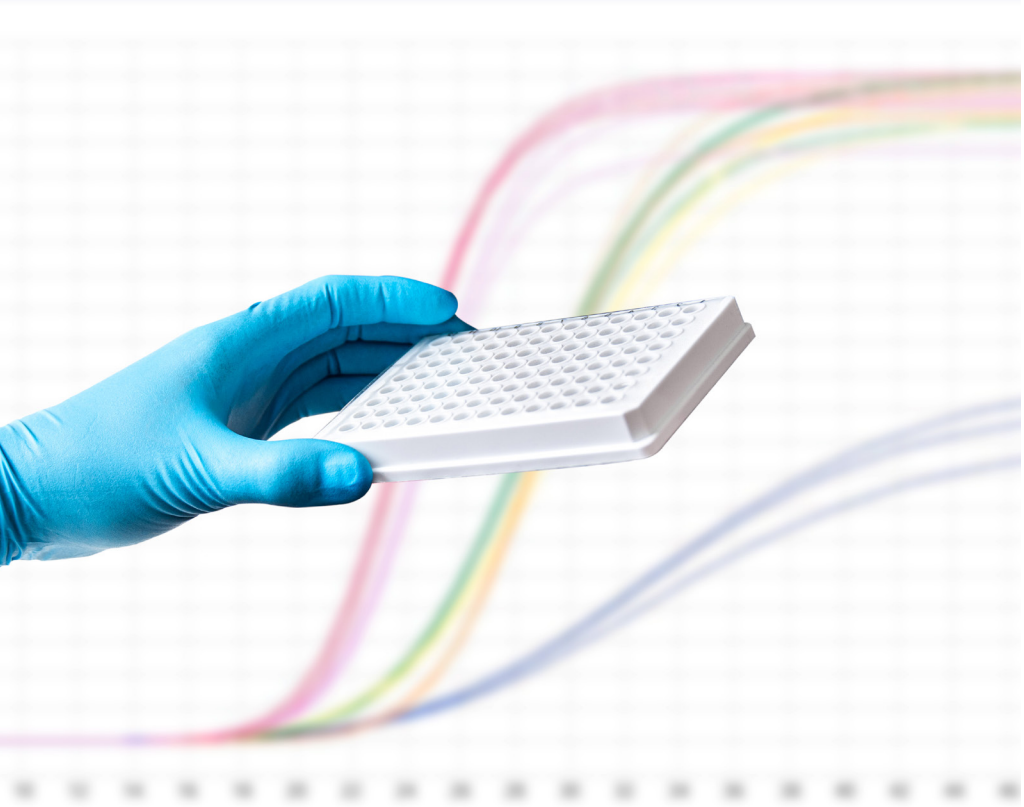

Challenge
Detecting six distinct targets in a single sample using real-time PCR.

Solution
qTOWERiris with improved color modules enabling applications at an extended wavelength range.

Intended audience
Experienced real-time PCR users in fields of molecular biology and genetics.

Enabling 6-Target Multiplex Real-Time PCR on qTOWERiris

Introduction

In addition to qualitative detection and quantification of individual targets using real-time PCR, parallel measurement of as many different targets as possible in one reaction is becoming more frequent to reduce assay costs and time. Currently, commercial multiplex assays are designed to detect four different targets, including an internal control. Multiplex assays for the detection of five targets are scarce with a major challenge being the clean separation of the individual fluorescent signals between the targets. The careful selection of fluorescence dyes is crucial, considering potential crosstalk in their emission spectra; this challenge can be addressed by using filters with optimized excitation and emission wavelengths and an extended spectrum into the ultraviolet range, as is the case with the qTOWERiris.

The development of a multiplex qPCR assay becomes progressively more challenging as the complexity increases with the number of parallel targets. In the beginning, the design of primers and probes needs to be optimized for the different target sequences in a single qPCR reaction. This application note outlines the three essential steps for developing a functional multiplex qPCR assay capable of detecting six different gene targets from human genomic DNA:

1. Determination of the suitability and specificity of PCR primers for the gene targets
2. Establishment of the six individual reactions with hydrolysis probes
3. Performing a 6-target multiplex real-time PCR

Six genes from the NCBI sequence database were selected, for conducting a 6-target multiplex real-time PCR using qTOWERiris (Table 1).

Table 1: Overview of the target genes for the multiplex assay designed by Analytik Jena.

Target	Description
SRY gene	Sex-determining region Y
GAPDH gene	Human specific glyceraldehyde 3-phosphate dehydrogenase
LIPE gene	Hormone-sensitive lipase, role in lipid metabolism and the synthesis of steroid hormones
RhesusD gene	RHD gene which encodes the RhD protein with the D antigen (→ Rh+)
ACTB gene	Actin beta gene involved in cell motility, structure and integrity
c-MYC gene	Proto-oncogene, transcription factor, role in cell cycle progression, apoptosis and cellular transformation

1. Determination of the suitability and specificity of PCR primers for the gene targets

Materials and Methods

Chemicals and consumables

- innuMIX qPCR SyGreen Sensitive (IST InnuScreen AG)
- Human genomic DNA (Promega Corporation)
- Specific primers for:
  - Sex-determining region Y (SRY) gene
  - Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene
  - Lipase E (LIPE) gene
  - RhesusD gene
  - Actin beta (ACTB) gene
  - Cellular myelocytomatosis proto-oncogene (c-MYC) gene
- Molecular grade water
- 96-well PCR Plate (0.2 mL; low profile), full-skirted, white, 844-70038-S (Analytik Jena GmbH+Co. KG)
- Optical sealing foil, 844-70046-0 (Analytik Jena GmbH+Co. KG)

Instrumentation

The real-time PCR thermocycler qTOWERiris including Color module 1, blue (455 nm / 515 nm) for FAM™ dye (Analytik Jena GmbH+Co. KG).

As a first step, gene target specific primers were determined in silico using the open-source platform Primer3Plus with target sequences imported from the NCBI database. Primer suitability and specificity for all targets was checked with a SYBR™Green assay including a melting curve. The reaction mix for five replicates of each target gene is shown in Table 2, following the PCR conditions in qPCRsoft for the qTOWERiris displayed in Table 3.

Table 2: Preparation of the qPCR master mix for the primer specificity test with one primer set for each of the gene targets and five replicates.

Components	Stock concentration	Final concentration	Volume [20 µL/reaction]
innuMIX qPCR SyGreen Sensitive (IST InnuScreen AG)	2x	1x	50 µL
Human genomic DNA	10 ng/µL	0.2 ng/µL	2.0 µL
Human specific primer FWD (e.g SRY)	50 µM	0.3 µM	0.6 µL
Human specific primer REV (e.g. SRY)	50 µM	0.3 µM	0.6 µL
Molecular grade water			48 µL

Table 3: Temperature and time protocol.

Step	Cycle	Profile	Temperature	Holding time	Ramp rates
1	1	Initial denaturation	95 °C	3 min	8 °C/s
2	50	Denaturation	95 °C	10 sec	8 °C/s
		Annealing	58 °C	10 sec	5.5 °C/s
		Elongation*	72 °C	20 sec	8 °C/s
3	1	Melting curve*	60 °C - 95 °C	15 sec	5.5 °C/s

*Data acquisition with color module 1 for qTOWERiris Series.

Results and Discussion

Amplification products of the SYBRGreen assay for each target gene were consistently identified across replicates (Figure 1), displaying a low standard deviation (SD) of less than 0.2 (Table 4).

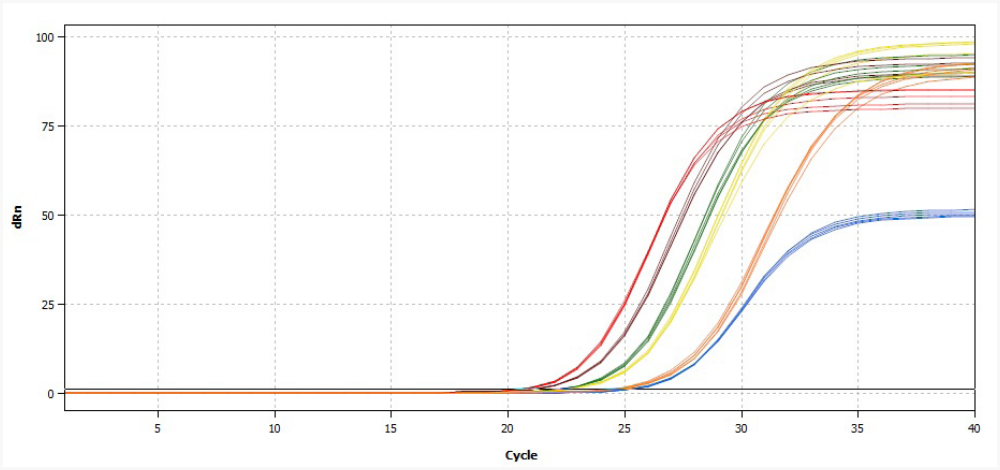

Figure 1: SYBR®Green amplification curves of six different target genes with a threshold of 1.194.

Table 4: Mean Ct and standard deviation of the six different target genes.

Target	Mean Ct	Standard deviation
SRY gene	25.4	0.03
GAPDH gene	22.48	0.06
LIPE gene	22.81	0.07
RhesusD gene	24.82	0.17
ACTB gene	20.84	0.11
c-MYC gene	21.21	0.15

Subsequently, a melting curve analysis was performed to determine the specificity of the amplification curves and to examine potential primer dimers or non-specific products. Figure 2 shows that the amplification products were specific for the individual primer pairs, and no primer dimers or by-products were detected.

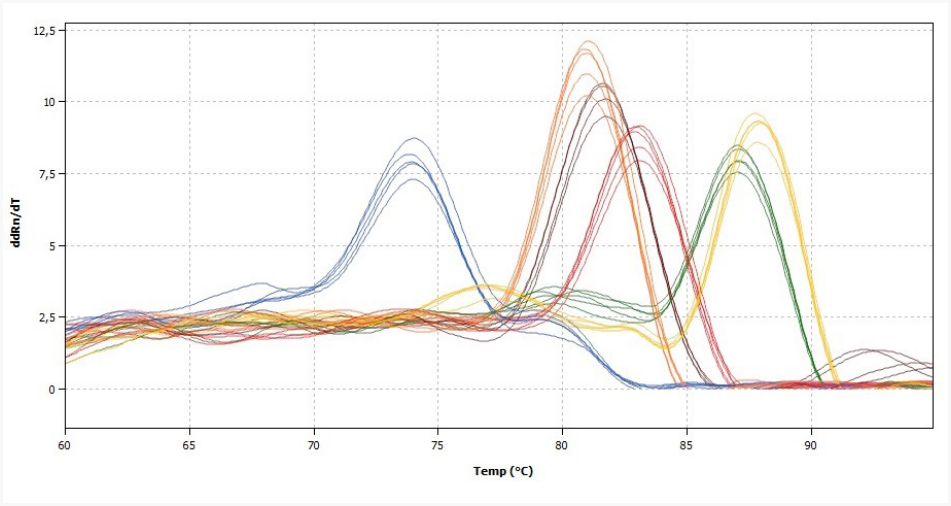

Figure 2: Melting curves of six different genes using SYBR®Green measured with color module 1.

2. Establishment of the six individual reactions with hydrolysis probes

Materials and Methods

Chemicals and consumables

- Multiplex PCR Mix Probe 4x (A&A Biotechnology)
- Human genomic DNA (Promega Corporation)
- Specific primers and probes (Table 5)
- 96-well PCR Plate (0.2 ml; low profile), full-skirted, white, 844-70038-S (Analytik Jena GmbH+Co. KG)
- Optical sealing foil, 844-70046-0 (Analytik Jena GmbH+Co. KG)

Instrumentation

- Real-time PCR thermal cycler qTOWERiris (Analytik Jena GmbH+Co. KG) including:
  - Color module 1, blue (455 nm / 515 nm) for FAM™ dye
  - Color module 2, green (520 nm / 560 nm) for Yakima Yellow® dye
  - Color module 3, yellow (550 nm / 585 nm) for ATTO550 dye
  - Color module 4, orange (580 nm / 620 nm) for ROX™ dye
  - Color module 5, red (625 nm / 670 nm) for Cy5® dye
  - Color module 6, NIR (660 nm / 710 nm) for Cy5.5® dye

After confirming primer specificity, hydrolysis probes were designed similarly for each target gene using Primer3Plus, with a distinctive reporter dye and quencher assigned to each gene (Table 5).

Table 5: Target genes and the corresponding reporter dye and quencher.

Target	Reporter	Quencher
SRY gene	FAM™	BHQ-1
GAPDH gene	Yakima Yellow®	BHQ-1
LIPE gene	ATTO550	BHQ-2
RhesusD gene	ROX™	BHQ-2
ACTB gene	Cy5®	BBQ-650
c-MYC gene	Cy5.5®	BBQ-650

In order to determine potential crosstalk among the spectra of the six dyes, separate qPCR reactions for each target gene (five replicates) were performed using one probe and a primer pair (Table 6), following the thermal cycler conditions outlined in Table 7. An adjusted color compensation for all dyes was recorded using qPCRsoft to eliminate signal crosstalk among the color channels.

Table 6: Preparation of the qPCR master mix for the probe specificity test, with one primer and probe set and five replicates for each target gene.

Components	Stock concentration	Final concentration	Volume [20 µL/reaction]
Multiplex PCR Mix Probe 4x (A&A Biotechnology)	4x	1x	25 µL
Human genomic DNA	10 ng/µL	0.2 ng/µL	2 µL
Human specific primer FWD (e.g., SRY)	50 µM	0.3 µM	0.6 µL
Human specific primer REV (e.g., SRY)	50 µM	0.3 µM	0.6 µL
Human specific probe (e.g., FAM™)	5 µM	0.1 µM	2.0 µL
Molecular grade water			73 µL

Table 7: Temperature and time protocol.

Step	Cycle	Profile	Temperature	Holding time	Ramp rates
1	1	Initial denaturation	95 °C	5 min	8 °C/s
2	45	Denaturation	95 °C	15 sec	8 °C/s
		Annealing / Elongation*	60 °C	45 sec	5.5 °C/s

*Data acquisition with color module 1-6 for qTOWERiris series.

Results and Discussion

Amplification of the single target genes using hydrolysis probes were examined for potential crosstalk of the dyes between the color channels. Figures 3a to 8a show the signals of the respective target gene probes along with the associated dyes including minor crosstalk between the dyes. By using the standard color compensation, the remaining signal overlap was completely removed (Figures 3b - 8b), which can be applied to other assays with the same dye combination.

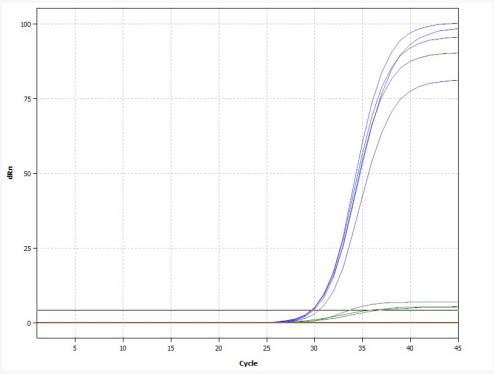

Figure 3a: SRY amplification curve for FAM™ with crosstalk from Yakima Yellow®.

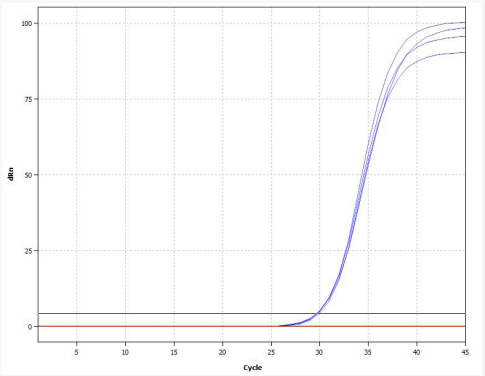

Figure 3b: SRY amplification curve for FAM™ without crosstalk.

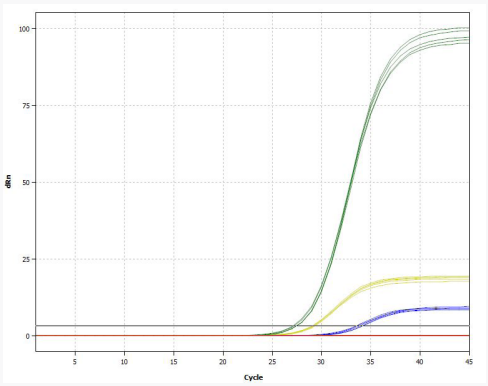

Figure 4a: GAPDH amplification curve for Yakima Yellow® with crosstalk from FAM and ATTO550.

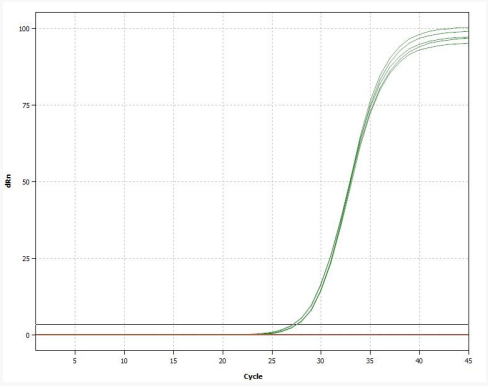

Figure 4b: GAPDH amplification curve for Yakima Yellow® without crosstalk.

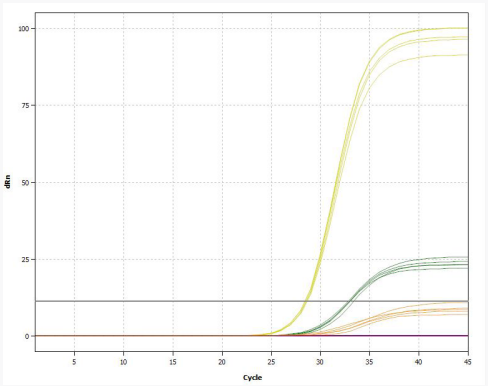

Figure 5a: LIPE amplification curve for ATTO550 with crosstalk from Yakima Yellow® and ROX ™.

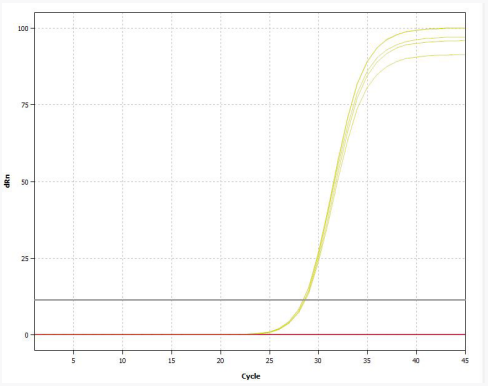

Figure 5b: LIPE amplification curve for ATTO550 without crosstalk.

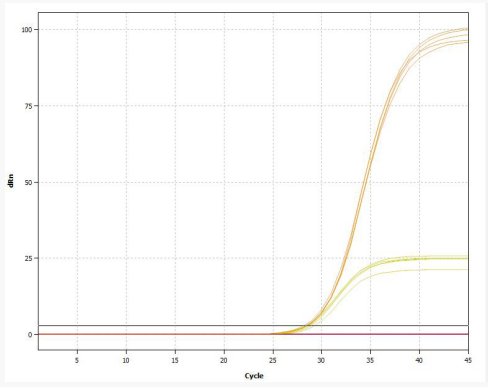

Figure 6a: RhesusD amplification curve for ROX™ with crosstalk from ATTO550.

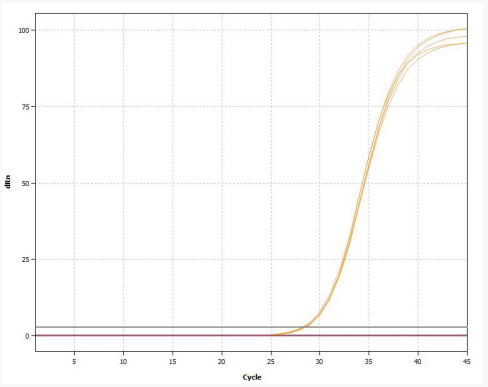

Figure 6b: RhesusD amplification curve for ROX™ without crosstalk.

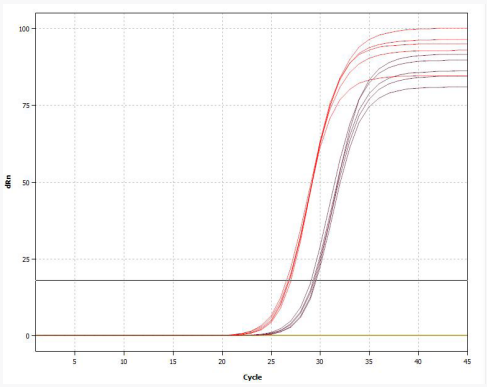

Figure 7a: ACTB amplification curve for Cy5® with crosstalk from Cy5.5®.

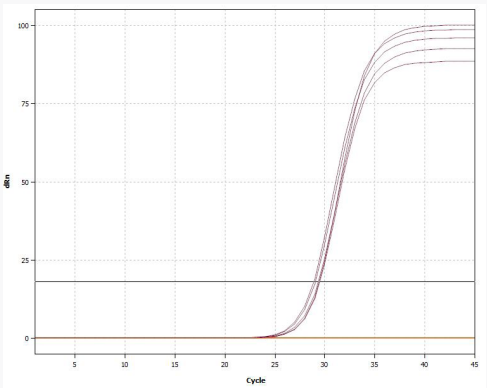

Figure 7b: ACTB amplification curve for Cy5® without crosstalk.

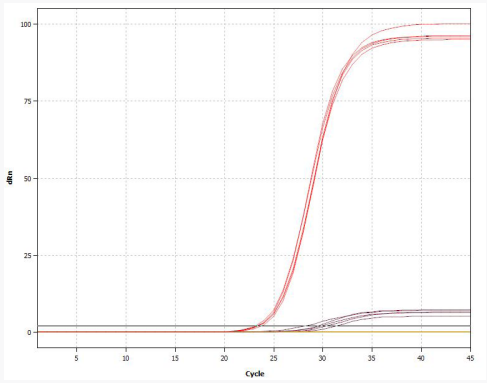

Figure 8a: c-MYC amplification curve for Cy5.5® with crosstalk from Cy5®.

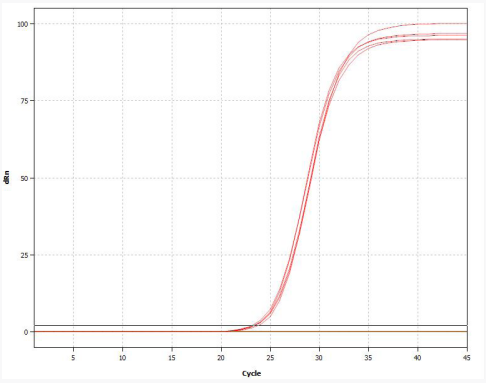

Figure 8b: c-MYC amplification curve for Cy5.5® without crosstalk.

3. Performing a 6-target multiplex real-time PCR

Materials and Methods

Chemicals and consumables

- Multiplex PCR Mix Probe 4x (A&A Biotechnology)
- Human genomic DNA (Promega Corporation)
- Gene specific primers and probes (Table 5)
- 96-well PCR Plate (0.2 mL; low profile), full-skirted, white, 844-70038-S (Analytik Jena GmbH+Co. KG)
- Optical sealing foil, 844-70046-0 (Analytik Jena GmbH+Co. KG)

Instrumentation

- Real-time PCR thermal cycler qTOWERiris (Analytik Jena GmbH+Co. KG) including:
  - Color module 1, blue (455 nm / 515 nm) for FAM™ dye
  - Color module 2, green (520 nm / 560 nm) for Yakima Yellow® dye
  - Color module 3, yellow (550 nm / 585 nm) for ATTO550 dye
  - Color module 4, orange (580 nm / 620 nm) for ROX™ dye
  - Color module 5, red (625 nm / 670 nm) for Cy5® dye
  - Color module 6, NIR (660 nm / 710 nm) for Cy5.5® dye

To perform a proper multiplex qPCR assay, all primer pairs and the corresponding probes for the six target genes were mixed in one reaction with three replicates and human DNA (Table 8) following the thermal cycler conditions outlined in Table 9. The previously generated color compensation was applied after the run to remove the signal overlap.

Table 8: Preparation of the 6-color multiplex reaction for three replicates.

Components	Stock concentration	Final concentration	Volume [20 µL/reaction]
A&A Biotechnology Multiplex PCR Mix Probe 4x	4x	1x	25 µL
Human mixed genomic DNA	10 ng/µL	0.2 ng/µL	2 µL
Human specific primer FWD, for each gene	50 µM	0.3 µM	6 * 0.6 µL
Human specific primer REV, for each gene	50 µM	0.3 µM	6 * 0.6 µL
Human specific probe, for each gene	5 µM	0.1 µM	6 * 2.0 µL
Molecular grade water			55.8 µL

Table 9: Temperature and time protocol.

Step	Cycle	Profile	Temperature	Holding time	Ramp rates
1	1	Initial denaturation	95 °C	5 min	8 °C/s
2	50	Denaturation	95 °C	15 sec	8 °C/s
		Annealing / Elongation*	60 °C	45 sec	5.5 °C/s

*Data acquisition with color module 1-6 for qTOWERiris series.

Results and Discussion

Each target gene was amplified in all the samples as shown in Figure 9, with a SD below 0.4 across the triplicate reactions (Table 10). The spectral overlap between the dyes was removed by applying the previosly established color compensation which resulted in clear signal separation.

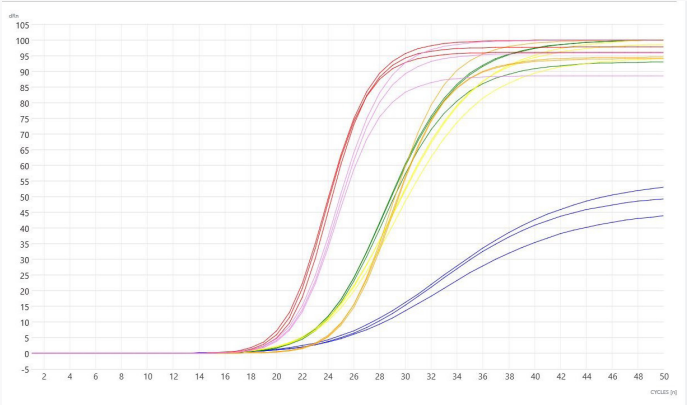

Figure 9: Amplification curves of all target genes using six dyes with three times replication each (generated via qPCRsoft touch).

Table 10: Mean Ct values (triplicates) and standard deviations for all six target genes.

Target	Dye	Mean Ct	Standard deviation
SRY gene	FAM™	24.54	0.37
GAPDH gene	Yakima Yellow®	21.27	0.10
LIPE gene	ATTO550	22.51	0.13
RhesusD gene	ROX™	22.57	0.10
ACTB gene	Cy5®	19.43	0.29
c-MYC gene	Cy5.5®	33.33	0.12

Conclusion

This application note demonstrates the approach of detecting six different target genes in one sample using the novel LED light source and adjusted color modules of the qTOWERiris to increase and separate the fluorescent signals between individual dyes. First, ensuring proper primer and probe design is crucial to establish a successful multiplex assay, avoid artifacts and verify primer specificity before using valuable sample material. In addition, potential signal overlap of the dyes between the color channels needs to be evaluated before the actual multiplex qPCR. The crosstalk can be reduced or removed using the qTOWERiris software package, which offers a default color compensation and the feasibility to generate assay-specific custom compensations. The qTOWERiris real-time PCR instrument is capable of reliable detection of up to six targets in a single qPCR reaction with consistent and excellent results. The instrument provides high flexibility in dye selection, featuring an extended signal spectrum, including UV, with minimal crosstalk between color channels and without any calibration effort.

Figure 10: qTOWERiris series.

Recommended device configuration

Table 10: Overview of required equipment and recommended accessories and consumables.

Article	Article number	Description
qTOWERiris incl. Color Module 1	844-00853-x*	Real-time PCR system designed in the 96-well format, operable via PC, customizable with up to six color modules.
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.
96-well PCR plate	844-70038-S	96 well full-skirted PCR plate, volume 0.2 mL, low profile, white
Optical sealing foil	844-70046-0	Highly transparent, adhesive sealing foils suitable for standard 96 or 384 well (q)PCR plates (79 x 142 mm, peelable, free of DNA, ATP, PCR inhibitors and endotoxins, free of DNases and RNases).

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

References

[1] NCBI Database: https://www.ncbi.nlm.nih.gov/
[2] Primer3Plus: https://www.primer3plus.com/index.html

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