Transferring assays between different platforms

artus
the PCR reference

Topics

1. A very brief introduction to artus
2. The diversity of real-time PCR instruments
3. Incompatibility of detection format and instrument
4. PCR multiplexing complicates assay transfer instruments
5. Adaptation of a dual color pathogen detection assays (HSV and Malaria) on three different technological platforms

artus
the PCR reference

A Very Brief Introduction to artus

- ✓ **founded in 1998 as a spin-off from the Bernhard Nocht Institute (BNI) in Hamburg**
- ✓ **established a GMP production facility, one of the largest private owned BSE laboratories in Germany and a R&D department with extensive real-time PCR equipment (first German BSE case confirmed in 2000)**
- ✓ **headquarter in Hamburg, subsidiaries in San Francisco (USA) and Kuala Lumpur (Malaysia)**
- ✓ **focus on the development of real-time PCR based pathogen detection kits (*RealArt*™ kits) for several technological platforms (LightCycler®, Rotor-Gene™, ABI Prism® and SmartCycler®) (first SARS-Coronavirus detection system)**

The Increasing Number of Real-Time PCR Instruments

LightCycler®

Mx4000™

ABI Prism®

Opticon®2

Rotor-Gene™

iCycler

SmartCycler®

Diverse Not Only in Weight...

Product	Company	Heating Mechanism	Reaction Tube	max. # of Samples	Weight
ABI Prism® 7000/7700/7900	Applied Biosystems	Peltier Element	Plates/ Tubes	96 (7900: 384)	34 kg 120 kg 82 kg
iCycler IQ™	Biorad	Peltier	Plates/ Tubes	96/384	17.6 kg
LightCycler®	Roche Diagnostics	Air	Capillaries	32	19.2 kg
Mx4000™	Stratagene	Resisitive/ Peltier hybrid	Plates	96	15 kg
DNA Engine Opticon®2	MJ Research	Peltier	Plates/ Tubes	96	29 kg
Rotor-Gene™	Corbett Research	Resistive heater with air cooling	Tubes	72	17 kg
SmartCycler®	Cepheid	I-CORE®	Tubes	16	10 kg

...but also in Temperature Uniformity...







Product	Max. Heating/ Cooling Rate (°C/sec)	Temperature Accuracy	Temp. Uniformity	Volume (µl)
ABI Prism® 7000/7700/7900	1.5/1.5	+/-0.25°C	+/-0.5°C	up to 100
iCycler IQ™	3.3/2.0	+/-0.3°C	+/-0.4°C	10 - 200
LightCycler®	20.0/20.0	+/-0.3°C	+/-0.2°C	20
Mx4000™	2.2/2.2	+/-0.25°C	+/-0.25°C	10 - 50
DNA Engine Opticon®2	3.0/2.0	+/-0.4°C	+/-0.4°C	10 - 50
Rotor-Gene™	2.5/2.5	+/-0.5°C	+/-0.05°C	10-100 (20 rec.)
SmartCycler®	10.0/2.5	+/-0.5°C	+/-0.5°C	25-100

...and Most Importantly in Optics

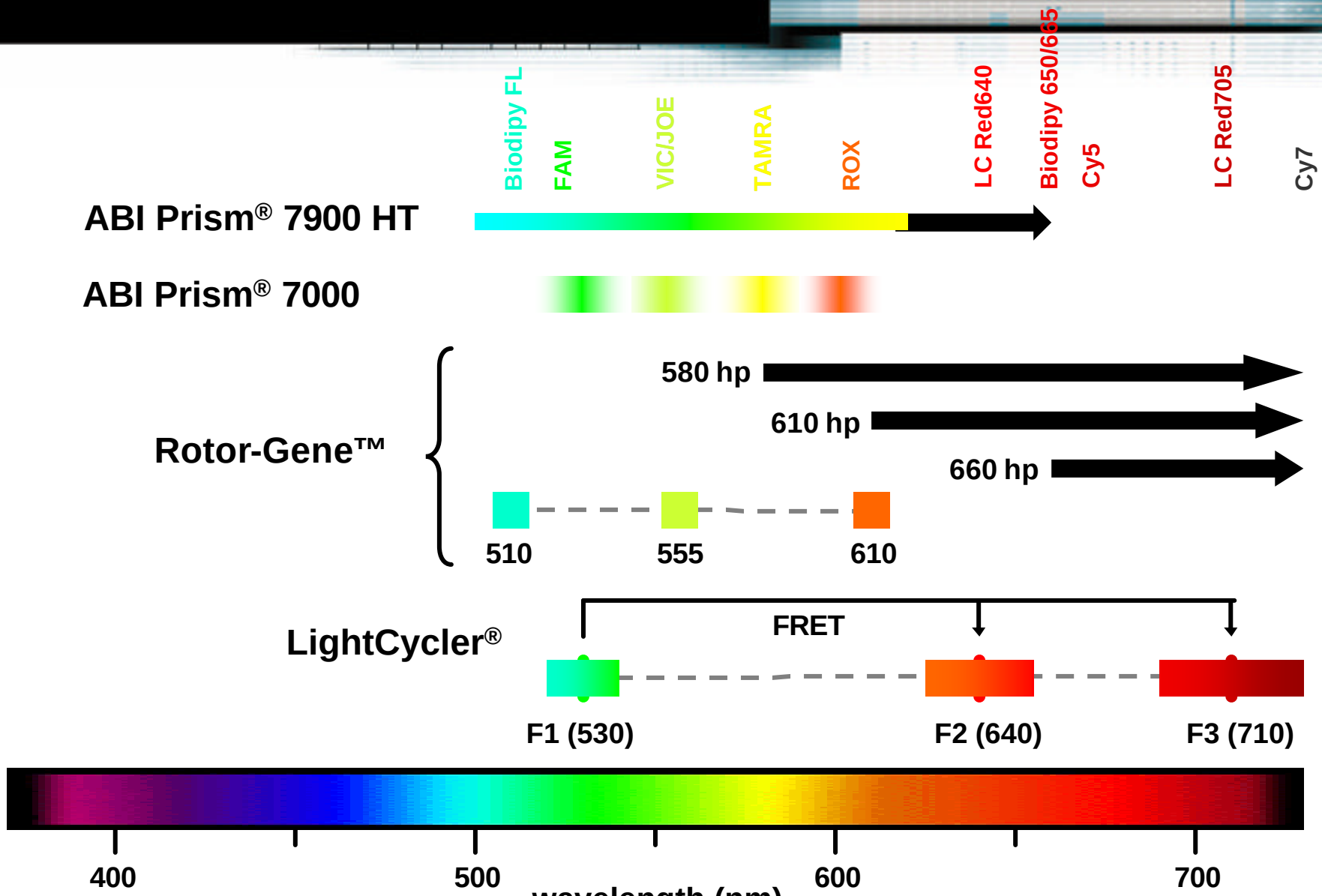






Product	Excitation Source	Excitation Wavel. (nm)	Detection Wavel. (nm)
ABI Prism® 7000/7700/7900	Halogen lamp/ Argon laser	7000: 350 - 750 7900: 488 and 545	7000: Four filter wheel 7900: 500 - 660
iCycler IQ™	Halogen lamp	400 - 700	5 filter positions available (2 provided)
LightCycler®	LED	470	530, 640, 710
Mx4000™	Halogen lamp	350 - 750	350 - 830
DNA Engine Opticon®2	LED	470 - 505	523-543, 540-700
Rotor-Gene™	LED	470, 530, 585, 625	510, 555, 610, 580 hp, 610 hp, 660 hp
SmartCycler®	LED	450-495, 500-550, 565-590, 630-640	510-527, 565-590, 606-650, 670-750

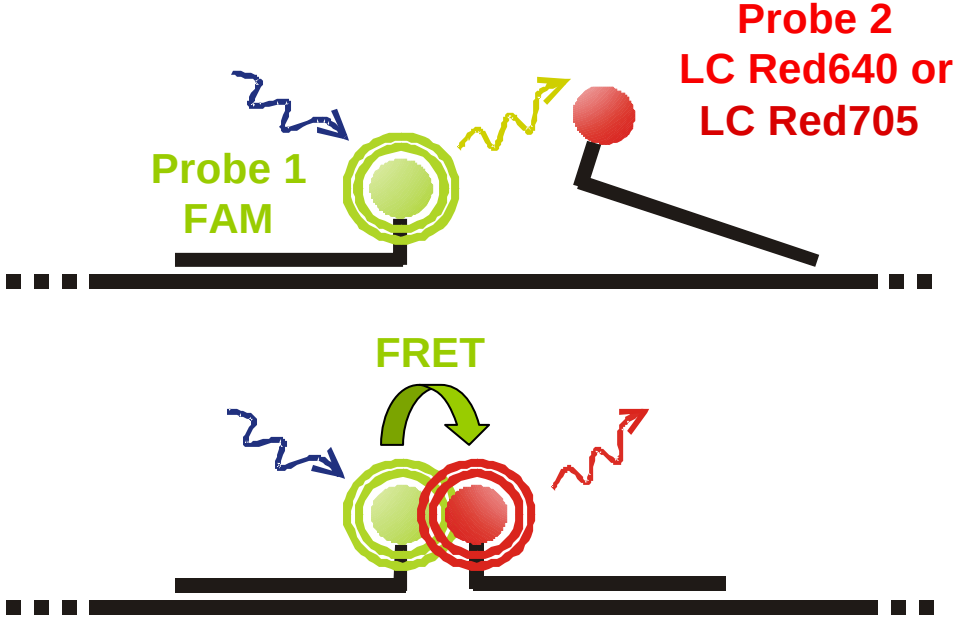
Different Instruments and Their Detection Spectra



The FRET Probe Principle Works Best on the LightCycler®

FRET probes are designed for the LightCycler® instrument

Channel	Detection
F1	FAM: 530 nm
F2	LC Red640: 640 nm
F3	LC Red 705: 710 nm



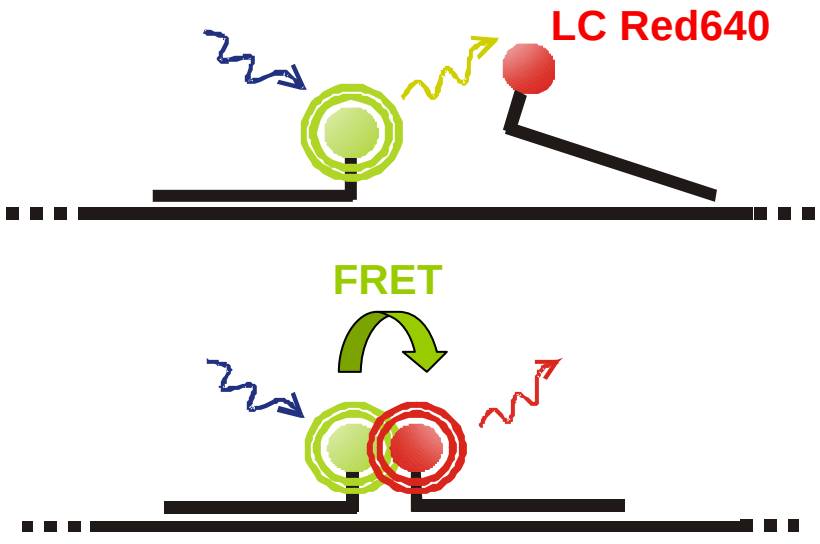
Pathogen Diagnostics Requires Two PCR Reactions in One Tube

Duplex PCR

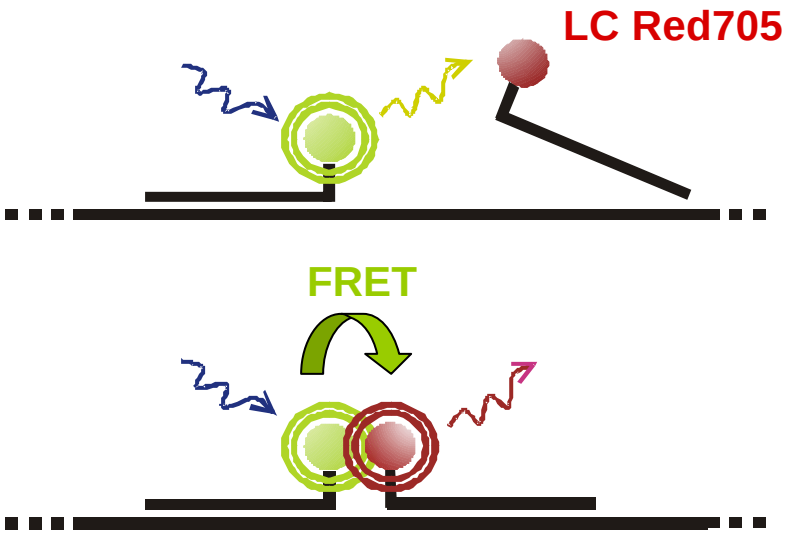
- ✓ **quantitative analytical PCR**
  (determination of pathogen loads)
- ✓ **internal control (IC) PCR**
  - control of PCR inhibition (and extraction efficiency)
  - to verify negative analytical PCR results

Example of a Duplex PCR Using FRET Probes on the LightCycler®

analytical PCR



internal control PCR



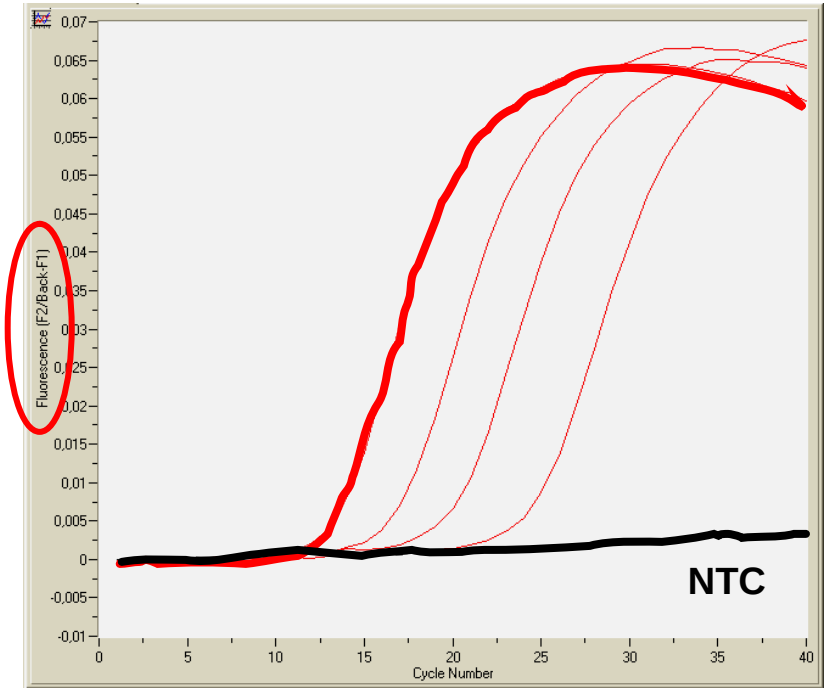
measured in F2

measured in F3

only one excitation wavelength: 470 nm

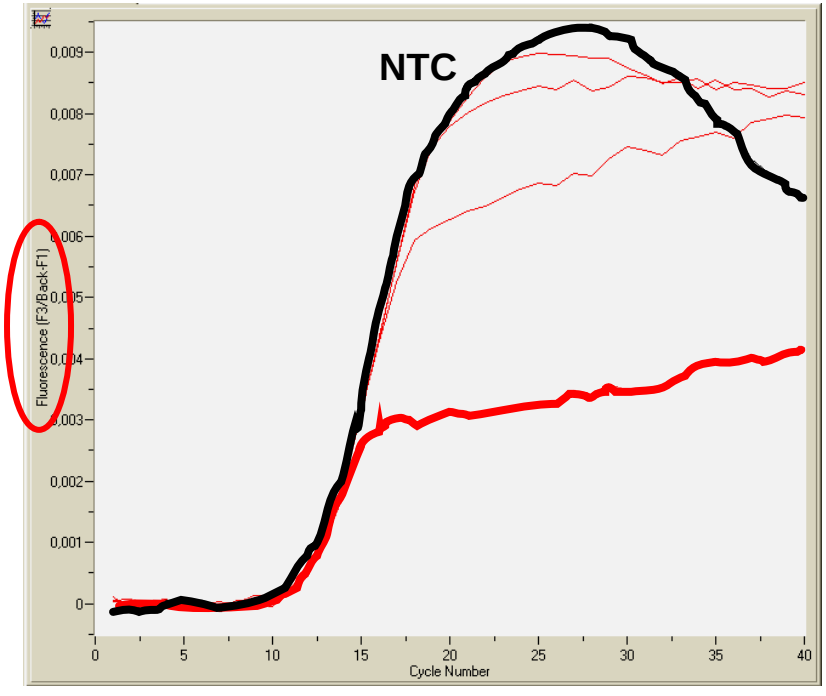
Competition Effect in a Duplex PCR

HSV 1 analytical PCR in F2



HSV 1 quantification standard series of defined concentrations

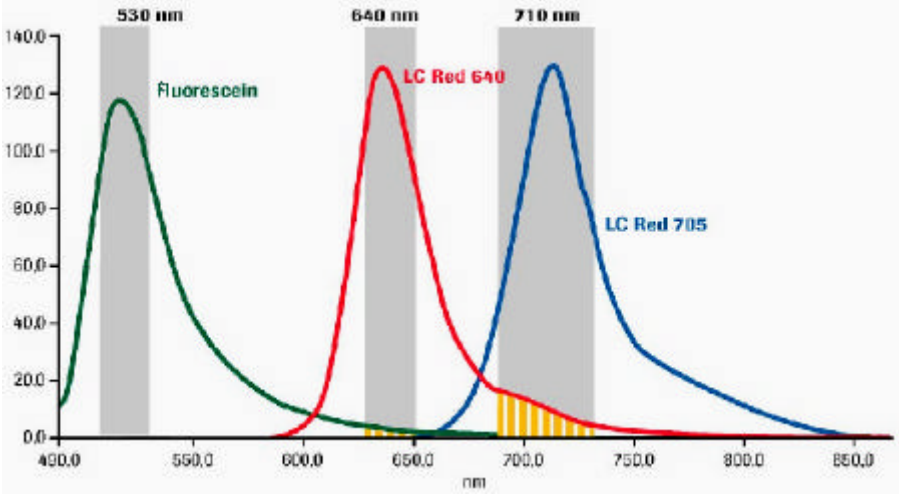
IC PCR in F3



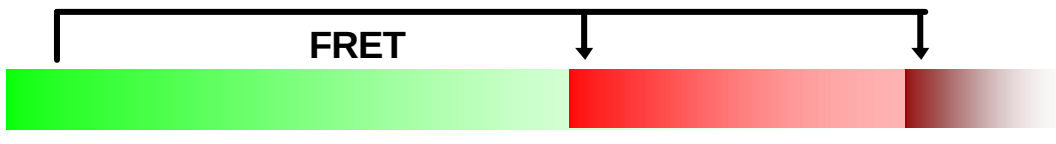
competition between analytical and IC PCR leads to reduced fluorescence intensities

Duplex PCRs in the LightCycler® Require a Color Compensation

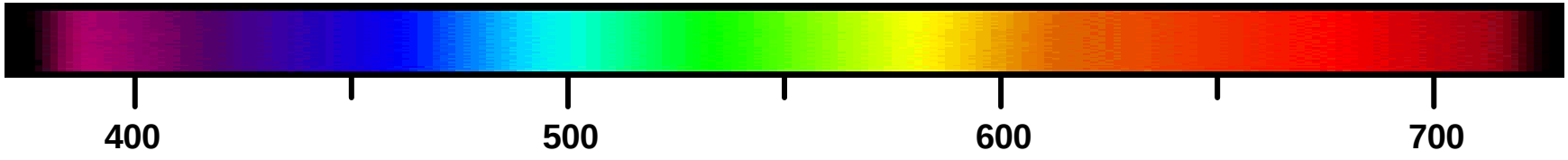
Interferences of fluorescence signals between the channels ("crosstalk")



LightCycler® emission spectra



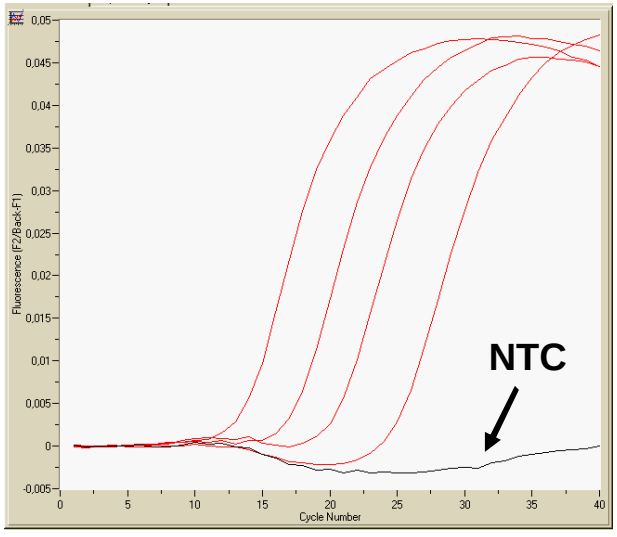
F1 (530) **F2 (640)** **F3 (710)**



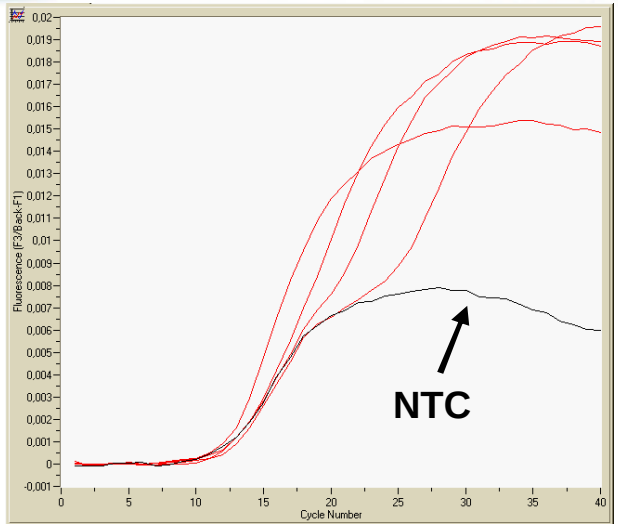
wavelength (nm)

Color Compensation File Subtracts Interfering Fluorescences

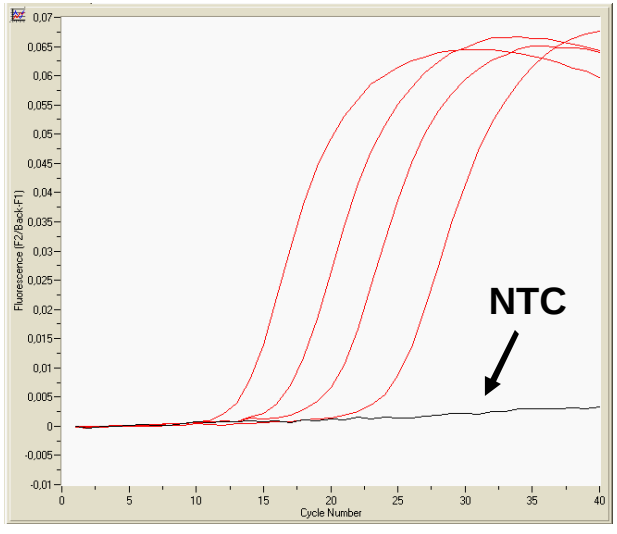
F2



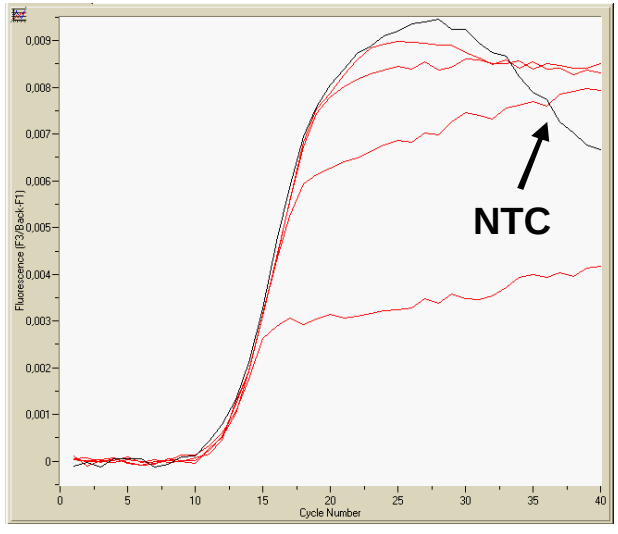
without ccc-file



F3



with ccc-file



Transfer of the HSV Real-Time Assay to the Rotor-Gene™ Instrument

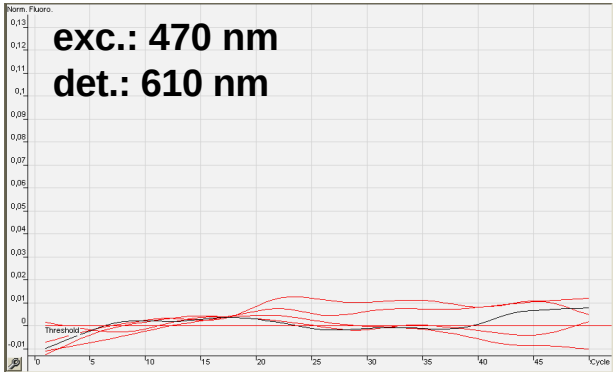
Rotor-Gene™ Channel Setup

Combination of different excitation and detection filters to disriminate between LC Red640 and LC Red705 on the Rotor-Gene™

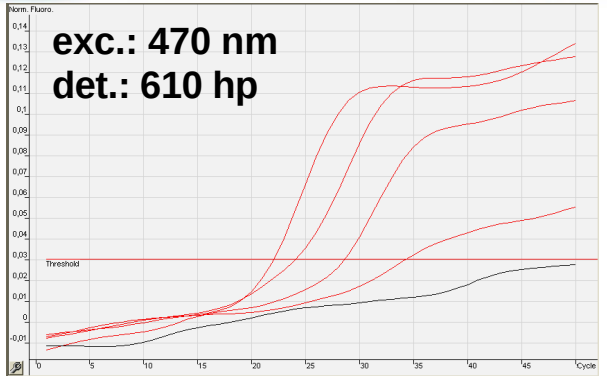
excitation	detection	fluorophore
470 nm	610 nm	LC Red640
470 nm	610 hp	LC Red640/705
470 nm	660 hp	LC Red705
625 nm	660 hp	LC Red705

➡ **No unique channel for LC Red640 available !**

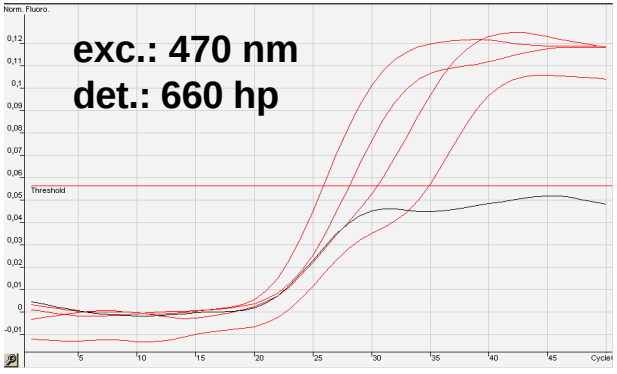
No Discrimination between LC Red640 and LC Red705



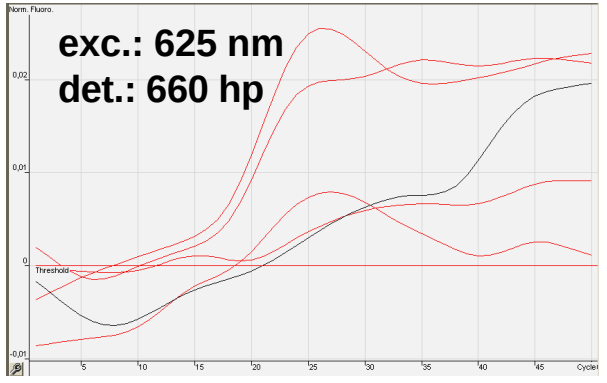
no detectable signal - emission max. of LC Red640 is higher



610 hp detects all emissions of 610 nm and higher - no discrimination between LC Re640 and LC Red705



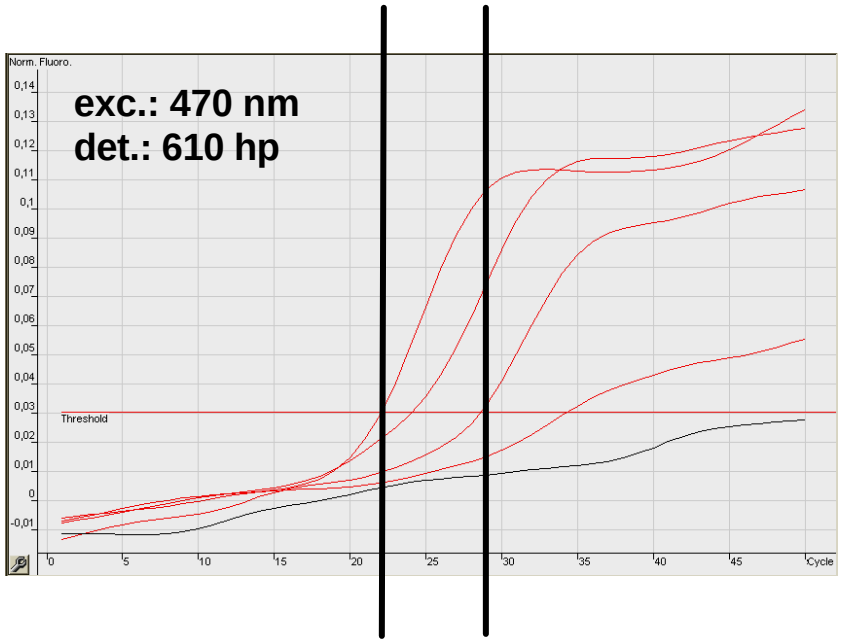
detection of IC (LC Red705) only



absorption max. of LC Red705 is around 680 nm - it can, thus, not efficiently be excited

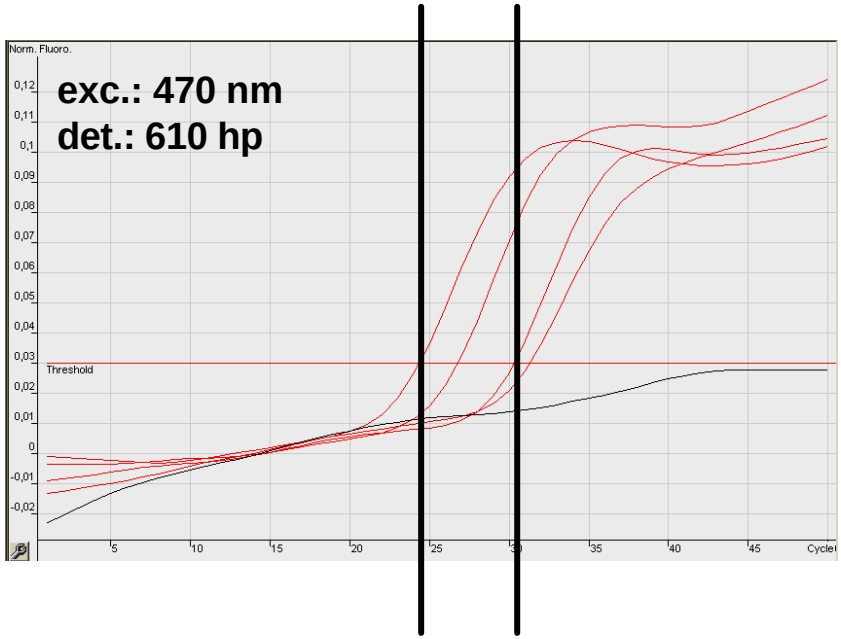
Both PCR Reactions are Detected in One Channel

PCR setup including IC



22.0 28.6

PCR setup excluding IC



24.4 30.3

increase of Ct values due to overall increased detected fluorescence

a LC assay cannot readily be transferred to the Rotor-Gene

Transfer of the HSV Real-Time Assay to the ABI Prism® 7900 Instrument

ABI Prism® 7900 HT

Biodipy FL
FAM
VIC/JOE
TAMRA
ROX
LC Red640
Biodipy 650/665
Cy5
LC Red705
Cy7

calibrated with
pure dyes

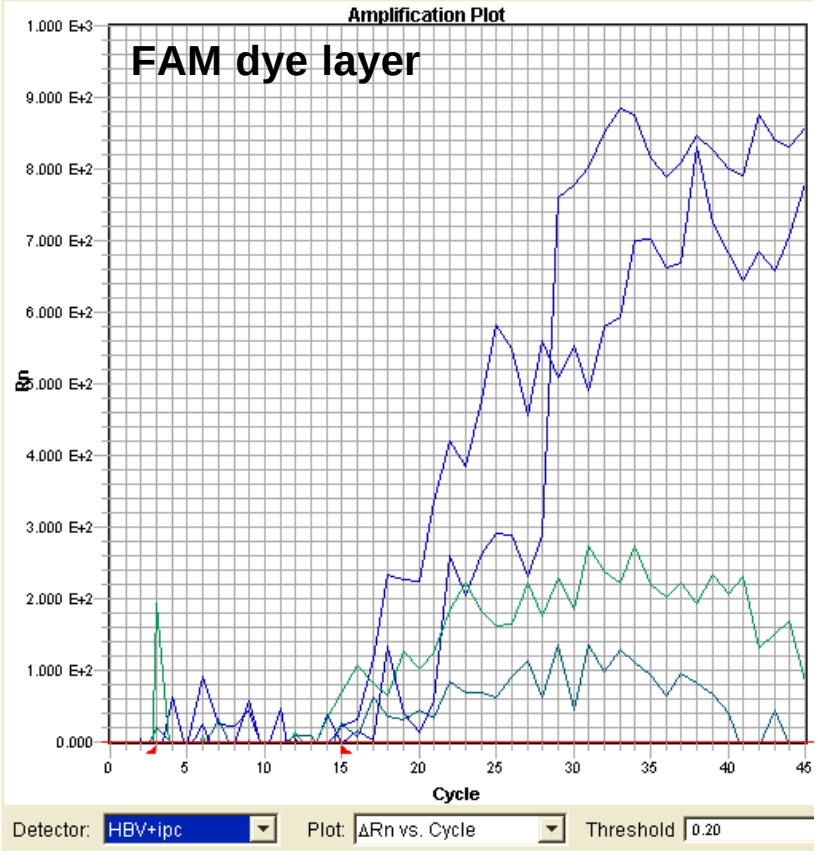
LightCycler®

FRET

F1 (530)
F2 (640)
F3 (710)

400
500
600
700
wavelength (nm)

Transfer of the HSV Real-Time Assay to the ABI Prism® 7900 Instrument



HSV quantification standard series (10^1-10^4 copies/µl)

Detection of fluorescence signals due to FAM-labeled oligo probe 1

Assay transfer Is Further Complicated by Passive Reference Dyes

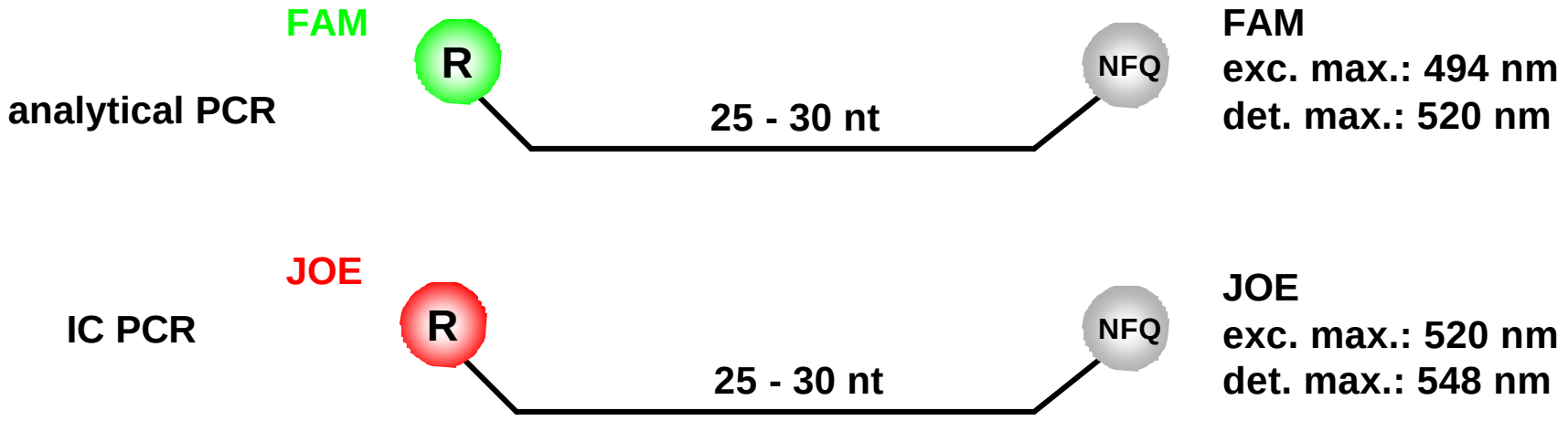
The ABI Prism® instruments require a passive reference dye (usually ROX) which accounts for fluorescent fluctuations due to changes in concentration or volume in the wells.

The software, thus, calculates normalized data, i.e. the ratio of reporter dye fluorescence and the emission of the passive reference (R_n = normalized reporter).

The reference dye is part of the PCR Master and must, hence, be modified.

Alternative Detection Format for Rotor-Gene™ and ABI Prism® Instruments

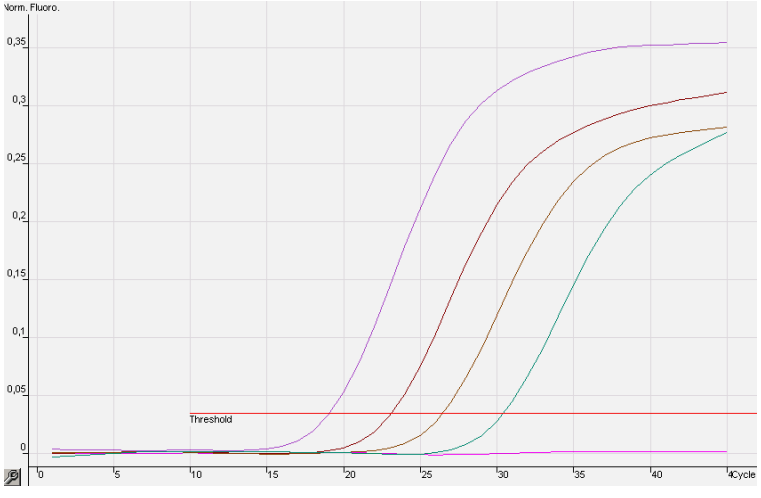
Use of two dual labeled probes: TaqMan probes



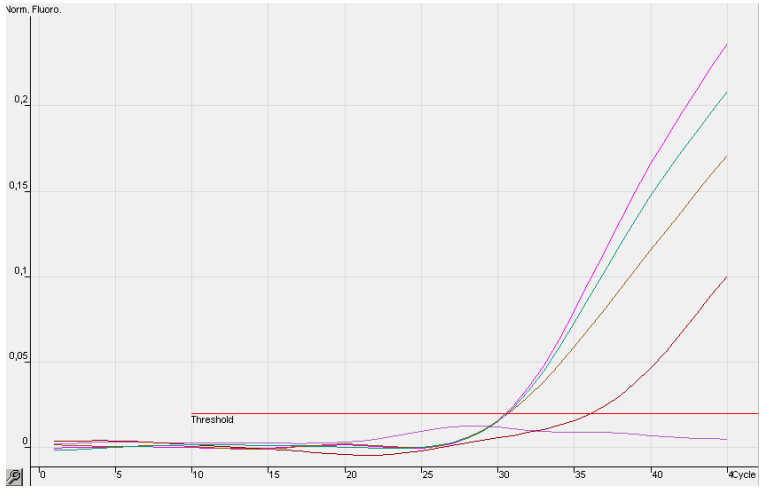
R: reporter fluorophore
NFQ: non-fluorescent quencher

Example of a Rotor-Gene™ Assay Using Dual-Labeled Probes

analytical PCR in FAM channel



IC PCR in JOE channel



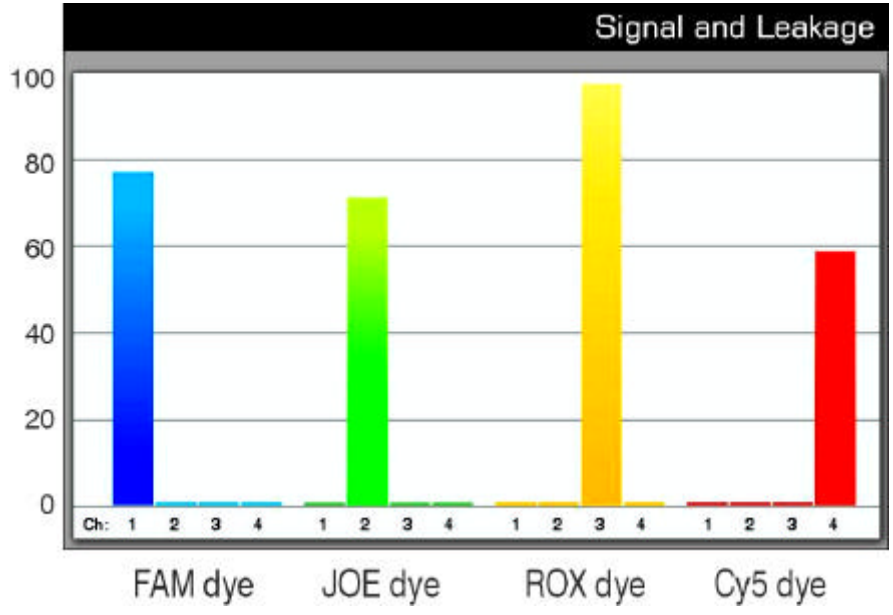
Malaria quantification standard series of defined concentrations

Internal control PCR (competition effect)

good fluorescence signal separation in two channels !!!

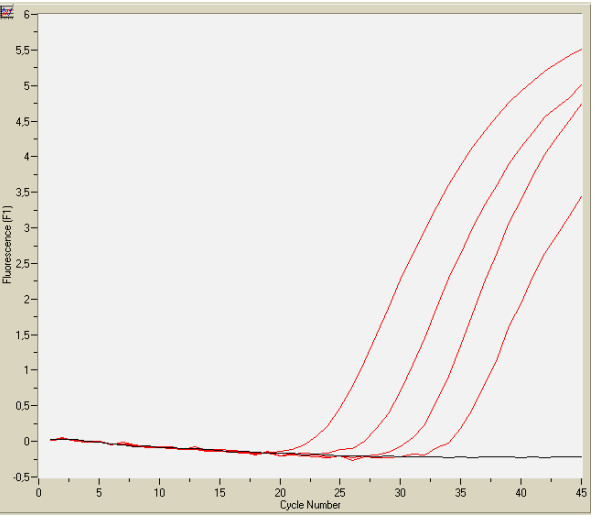
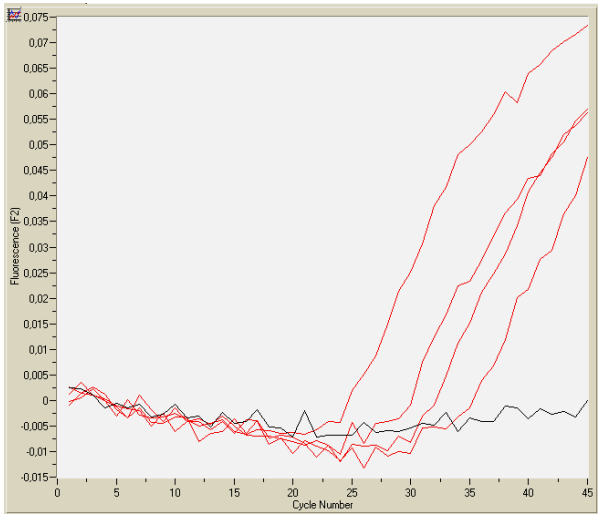
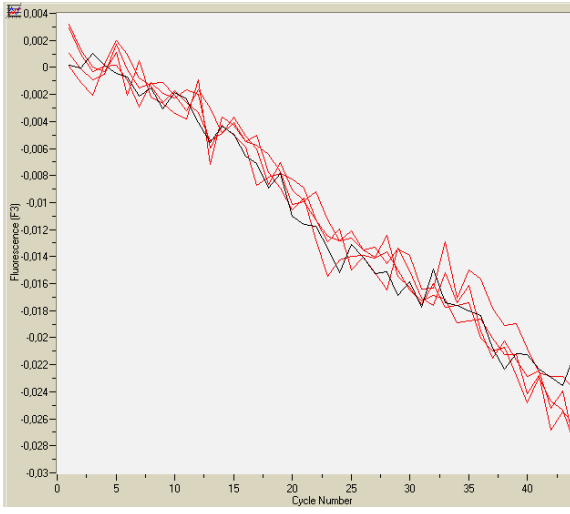
No Color Compensation Required on the Rotor-Gene™

When multiplexing 4 channels, less than 1% of cross-talk is observed between channels.



Transfer of the Malaria Rotor-Gene™ Assay on the LightCycler®

Transfer of the Malaria Rotor-Gene™ Assay on the LightCycler®

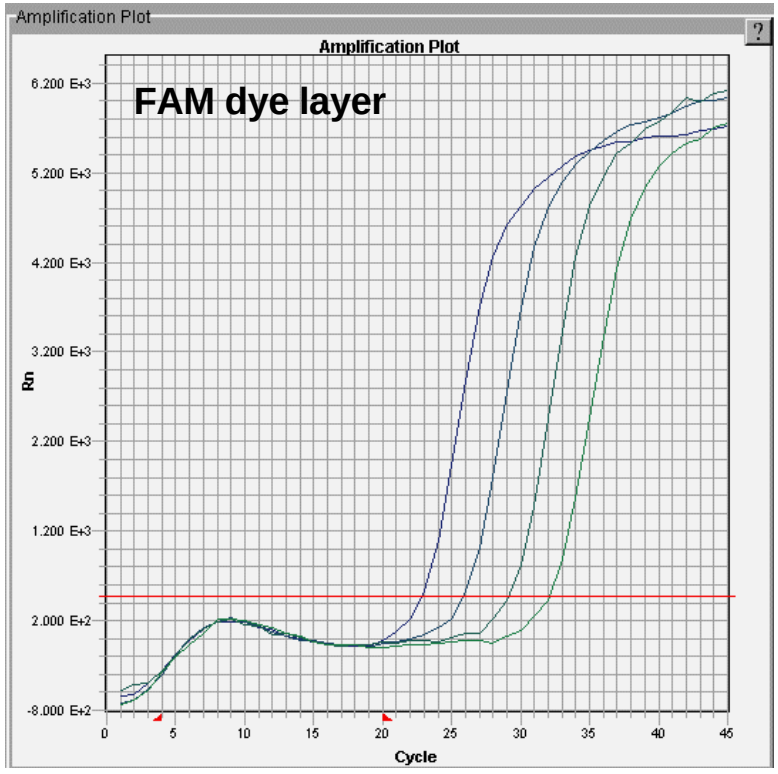
F1	F2	F3
		
analytical Malaria PCR detected in FAM channel	**despite color compensation the F1 signal strikes through into the F2 channel**	**no fluorescences can be measured in the F3 channel**

no IC detection as JOE cannot be excited by the LightCycler®

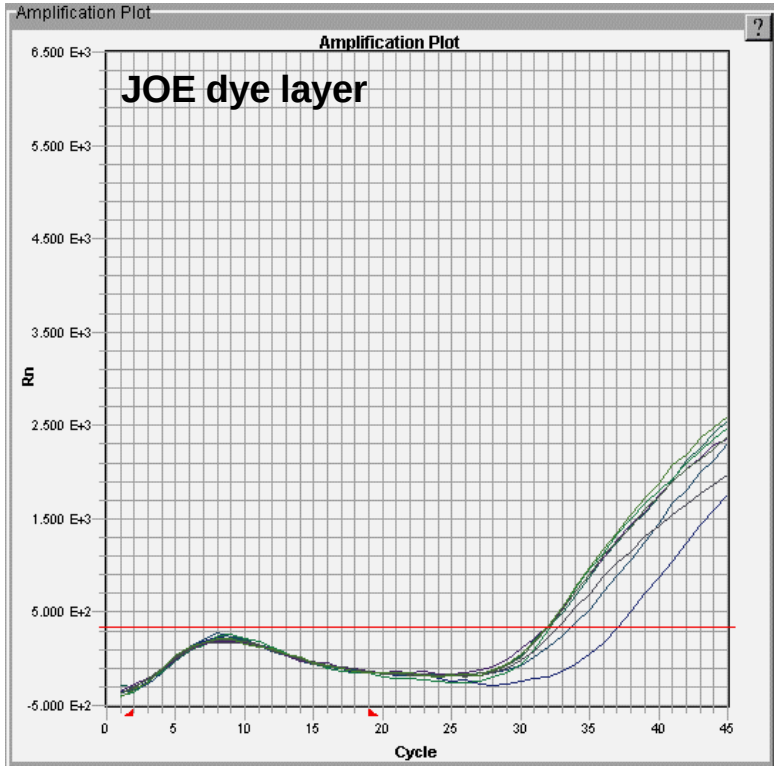
Transfer of the Malaria Rotor-Gene™ Assay on the ABI Prism® 7900

Transfer of the Malaria Rotor-Gene™ Assay on the ABI Prism® 7900

analytical PCR



IC PCR



as ABI Prism instruments require a passive reference, the ROX dye was added to the reaction setup

Other Aspects Important in Transfers of Pathogen Detection Assays

- ✓ **Reaction volume may significantly affect the sensitivity of pathogen detection**

  LC: reaction vol. limited to max. 20 µl
  RG/TM: allow reaction volumes of up to 100 µl

- ➡ **increased total volume allows a larger volume of sample material**

- ✓ **Passive reference dyes**
- ✓ **Temperature profile**
- ✓ **... well, try and see !**