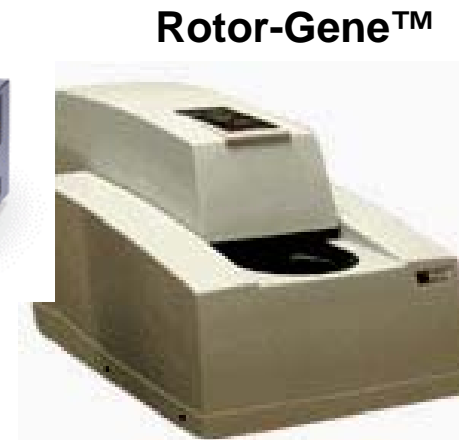




*Pitfalls in Transfer of Diagnostic Duplex qPCR Assays
between Technological Platforms*

The Increasing Number of Real-Time PCR Instruments



Diverse Not Only in Weight...



Instrument	Company	Launch Date	Heating Mechanism	Reaction Tube	max. # of Samples	Weight
ABI PRISM® 7000/7700/7900HT	Applied Biosystems	2001/1996/ 2000	Peltier Element	Plates/ Tubes	96 (7900: 384)	34 kg 120 kg 82 kg
iCycler IQ™	Biorad	Oct. 1999	Peltier	Plates/ Tubes	96/384	17.6 kg
LightCycler®	Roche Diagnostics	1998	Air	Capillaries	32	19.2 kg
LightCycler® 2.0	Roche Diagnostics	end 2003	Air	Capillaries	32	22 kg
Mx3000P™	Stratagene	200?	Solid-state/ Peltier hybrid	Plates	96	20 kg
DNA Engine Opticon®2	MJ Research	Oct. 2003	Peltier	Plates/ Tubes	96	29 kg
Rotor-Gene™ 3000	Corbett Research	RG 3000: June 2002	Resistive heater with air cooling	Tubes	72	17 kg
SmartCycler®	Cepheid	Jan. 2003	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®	3.7/2.3	+/-0.3°C	+/-0.2°C	10 - 20
LightCycler® 2.0	3.3/3.0 (20 µl) 2.0/1.9 (100 µl)	+/-0.3°C	+/-0.15°C	10 - 100
Mx3000P™	up to 2.5/2.5	+/-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™ 3000	2.5/2.5	+/-0.5°C	+/-0.01°C	10-100 (20 rec.)
SmartCycler®	10.0/2.5	+/-0.5°C	+/-0.5°C	25-100

...and Most Importantly in Optics

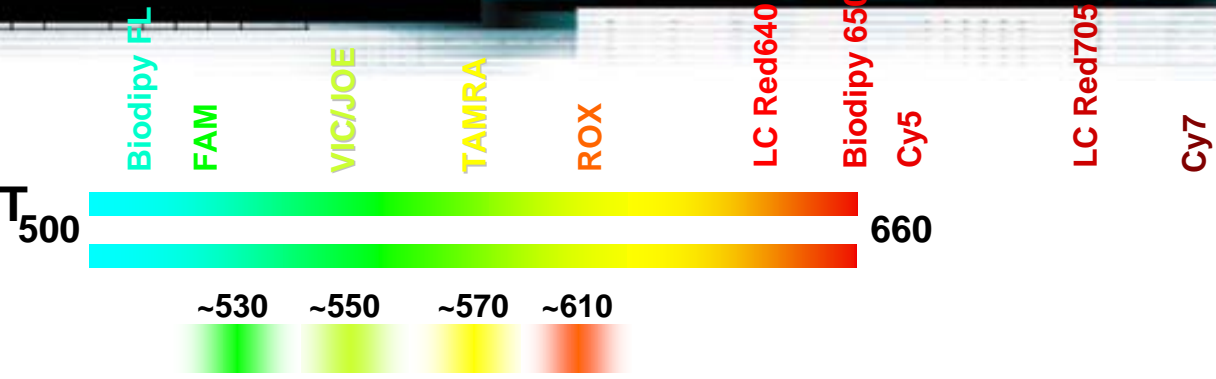


Product	Excitation Source	Excitation Wavel. (nm)	Detection Wavel. (nm)
ABI PRISM® 7000/7700/7900	Halogen lamp/ Argon laser	7000: 350 – 750 7700: 488 7900: 488 and 545	7000: Four filter wheel 7900: 500 - 660
iCycler IQ™	Halogen lamp	400 - 700	5 filter positions available (2 provided)
LightCycler®	LED	470	530, 640, 710
LightCycler® 2.0	LED	470	530, 555, 610hp, 640, 670hp, 710
Mx3000P™	Halogen lamp	350 - 750	Filter wheel: 350 - 700
DNA Engine Opticon®2	LED	470 - 505	523-543, 540-700
Rotor-Gene™ 3000	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

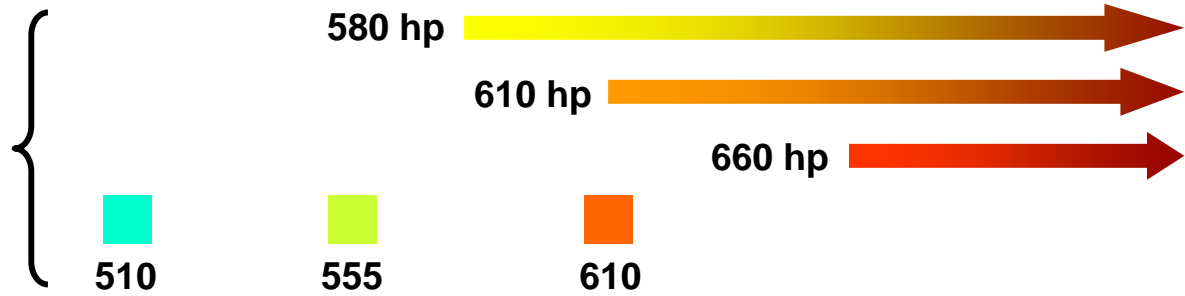
Excitation
[nm]

488+545 **ABI PRISM® 7900 HT**
 488 **ABI PRISM® 7700**
 350-750 **ABI PRISM® 7000**
 eff. ~488

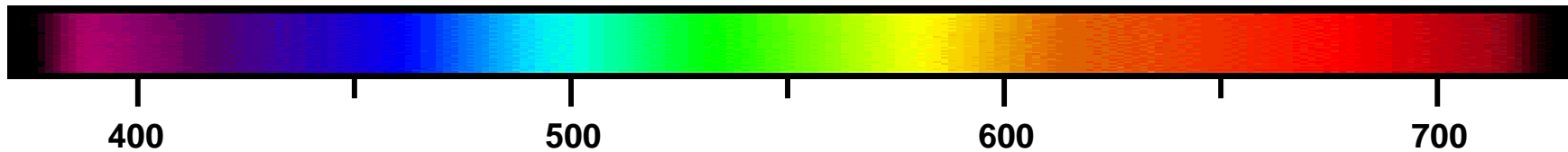


470
530
585
625

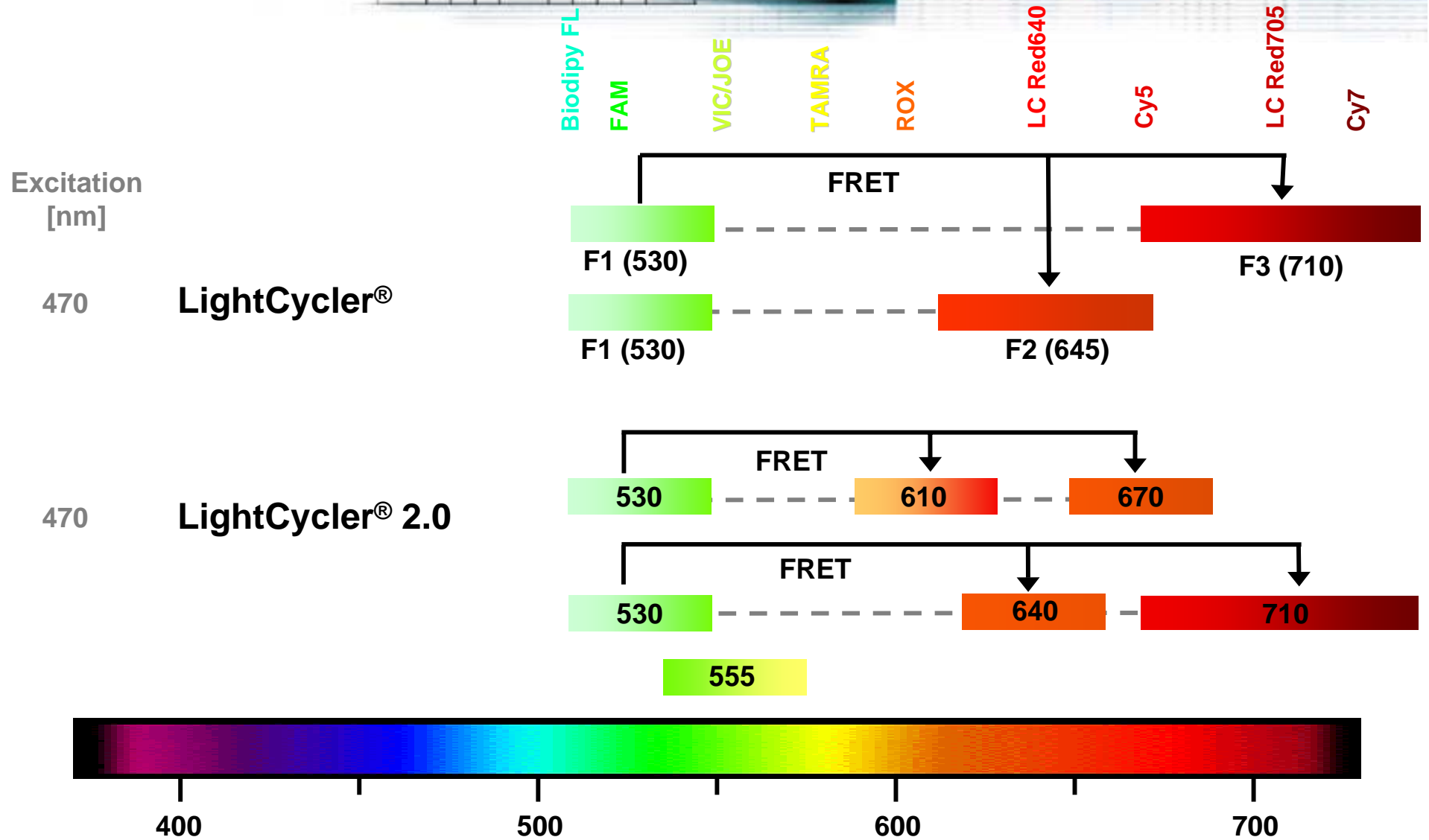
Rotor-Gene™



350-750 **Mx3000P™**



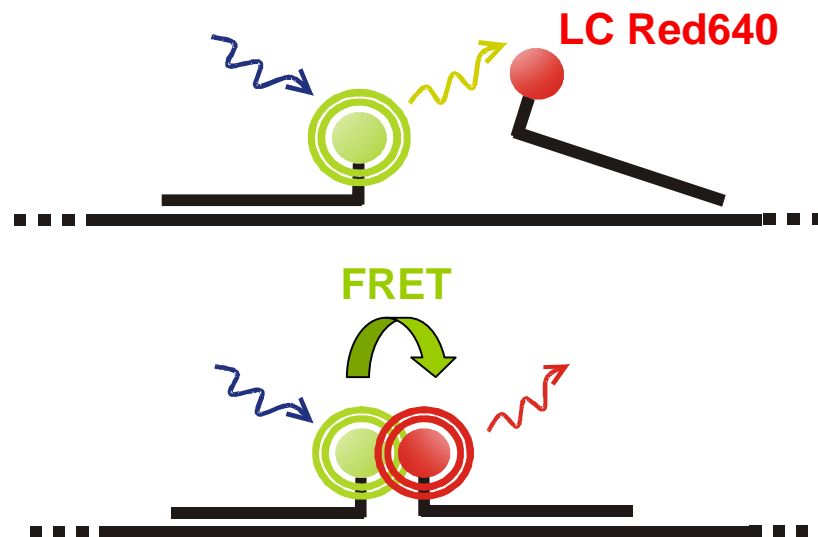
Different Instruments and Their Detection Spectra



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

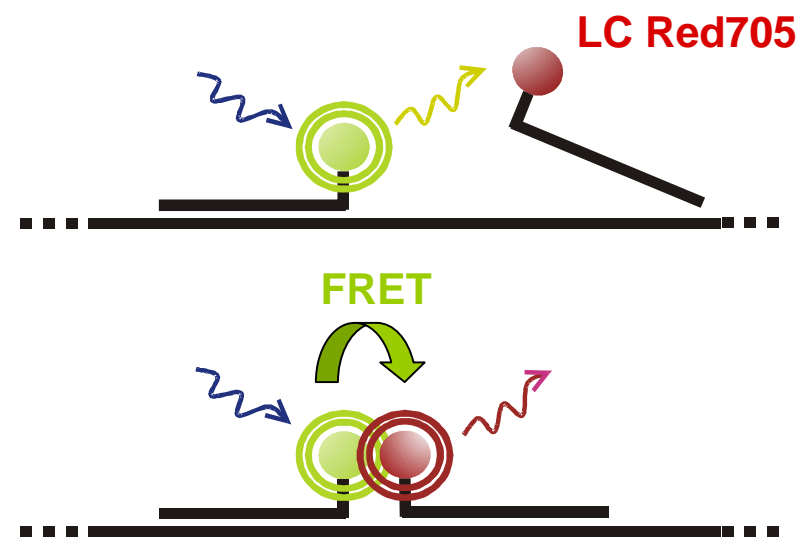
Duplex diagnostic PCR assays consist of a quantitative analytical PCR and an internal control PCR (monitoring PCR inhibition, extraction success

analytical PCR



measured in F2

internal control PCR

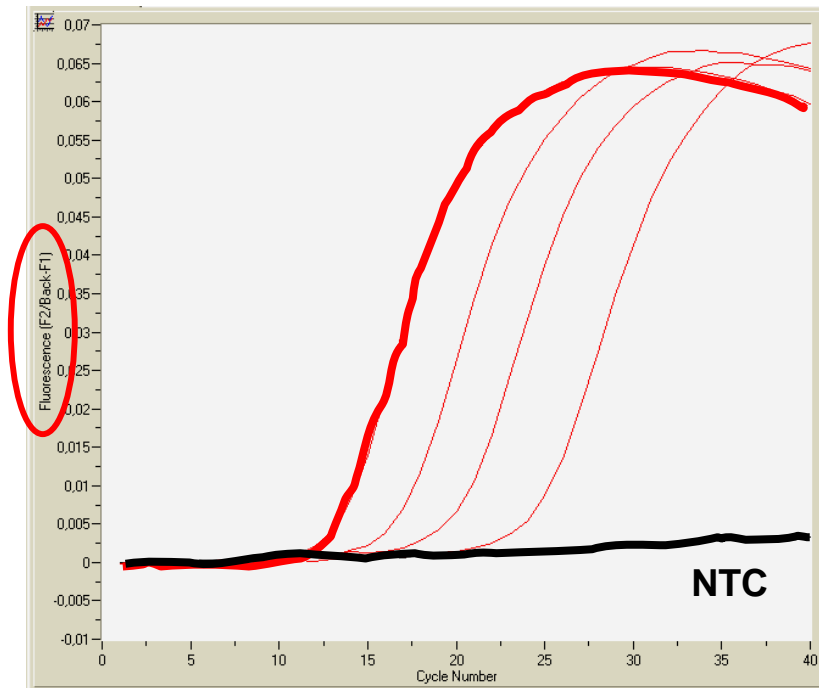


measured in F3

only one excitation wavelength: 470 nm

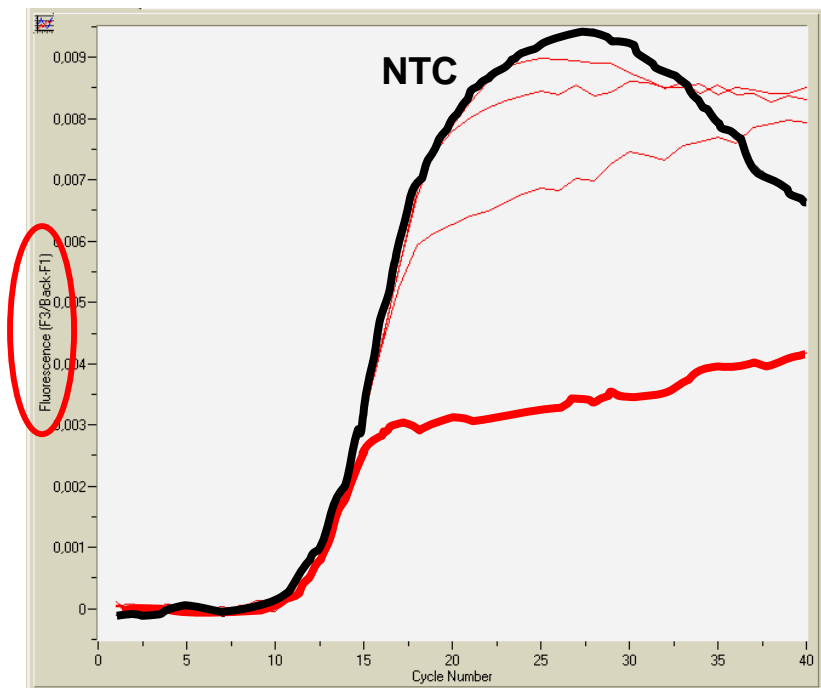
Competition Effect in a Duplex PCR

HSV 1 analytical PCR in F2



HSV 1 quantification standard series of defined concentrations

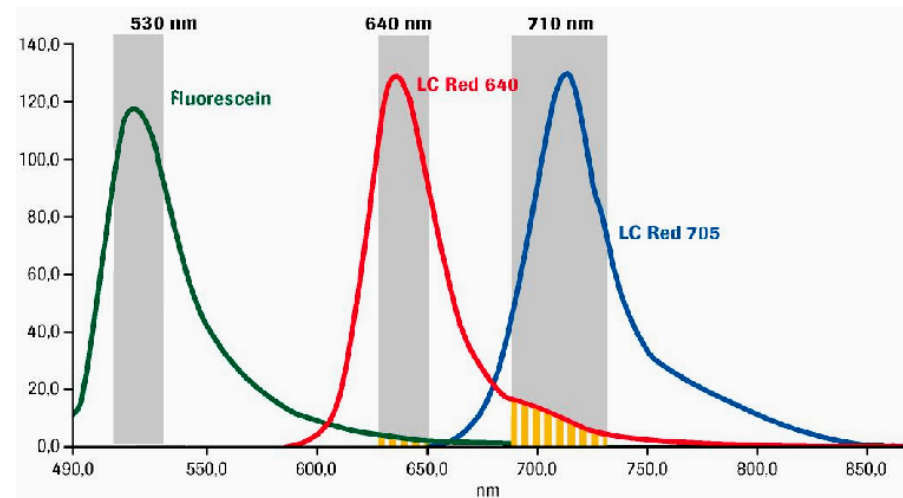
IC PCR in F3



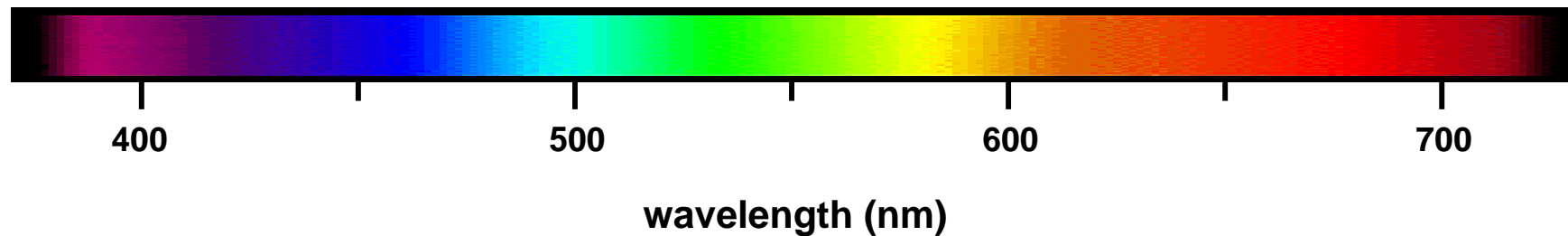
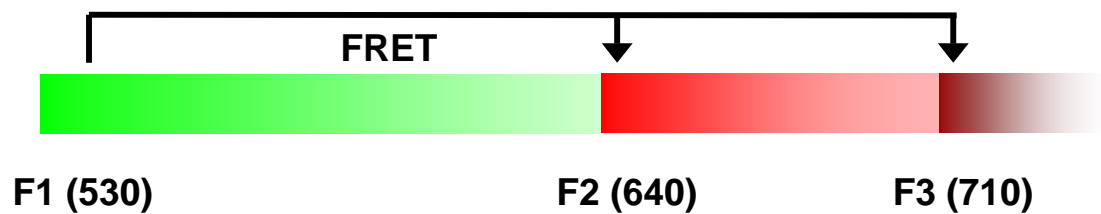
competition between analytical and IC PCR leads to reduced fluorescence intensities

Duplex PCRs in the LightCycler® Require a Color Compensation

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

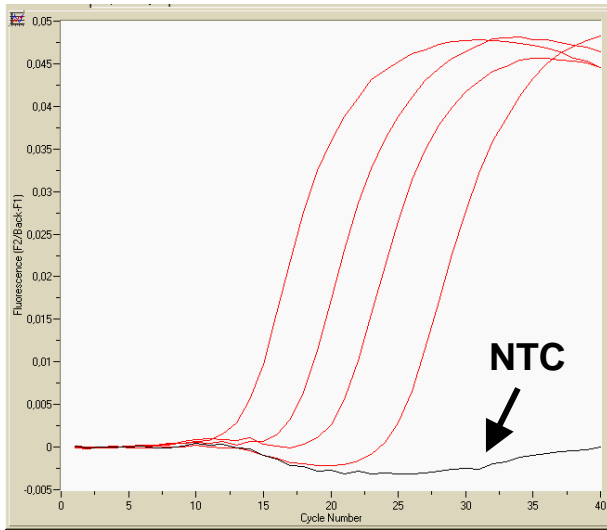


LightCycler®
emission spectra

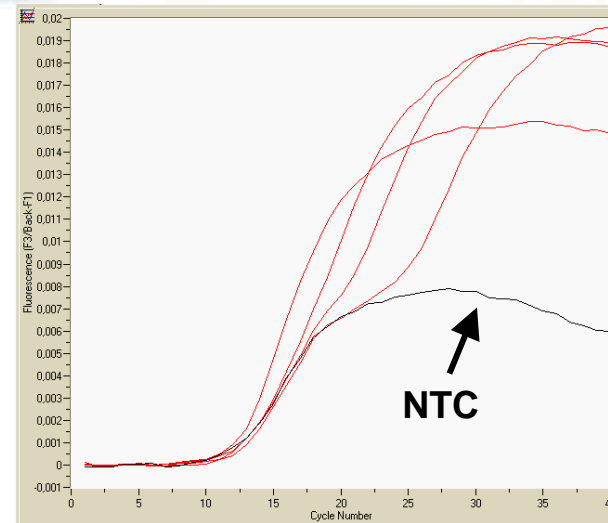


Color Compensation File Subtracts Interfering Fluorescences

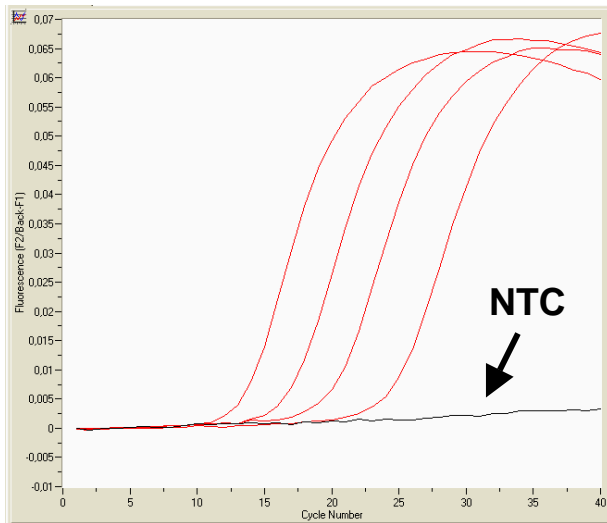
F2



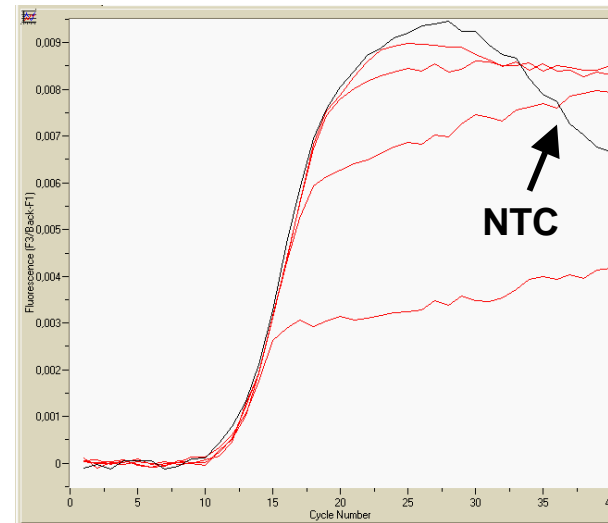
without
ccc-file



F3



with
ccc-file

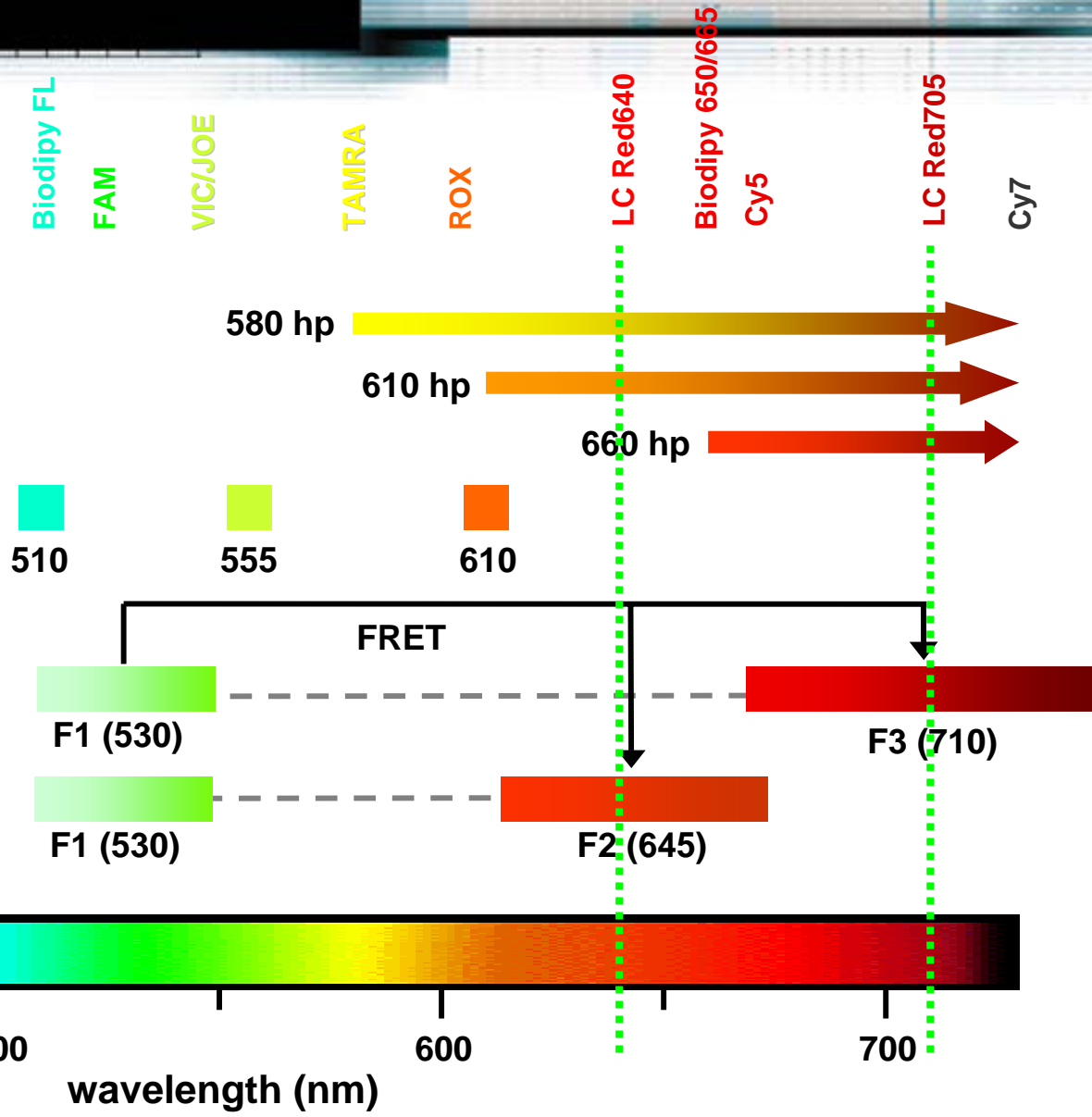


Transfer of the HSV Real-Time Assay to the Rotor-Gene™ Instrument



Rotor-Gene™

LightCycler®



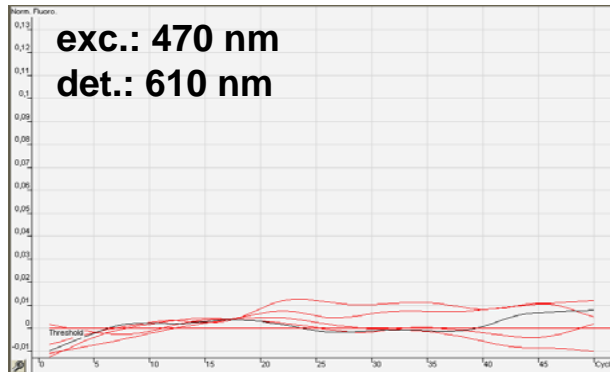
Rotor-Gene™ Channel Setup

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

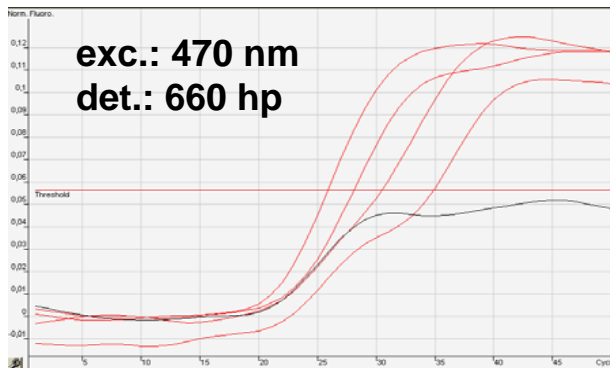
excitation	detection	fluorophore
470 nm	610 nm	LC Red640
470 nm	610 hp	LC Red640/705
470 nm	660 hp	LC Red705
625 nm	660 hp	LC Red705

➔ No unique channel for LC Red640 available !

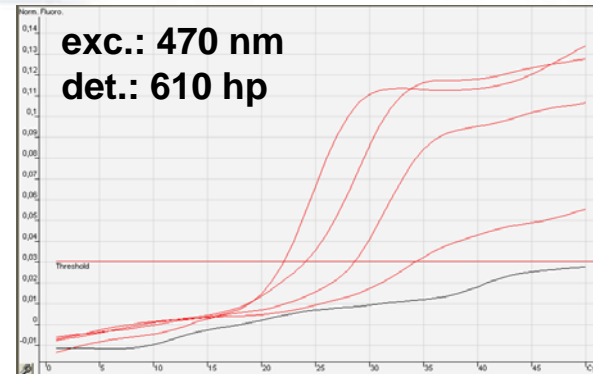
No Discrimination between LC Red640 and LC Red705



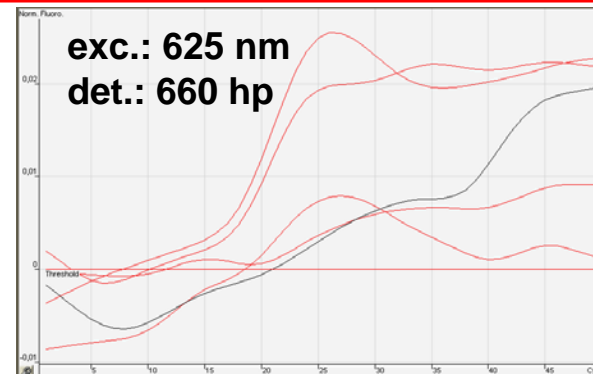
no detectable signal - emission max. of LC Red640 is higher



detection of IC (LC Red705) only



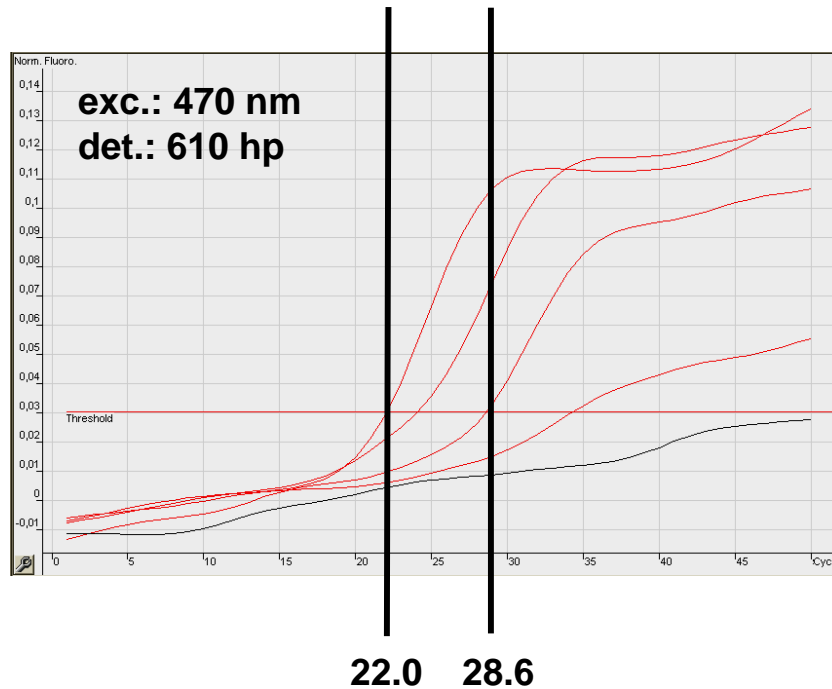
610 hp detects all emissions of 610 nm and higher - no discrimination between LC Re640 and LC Red705



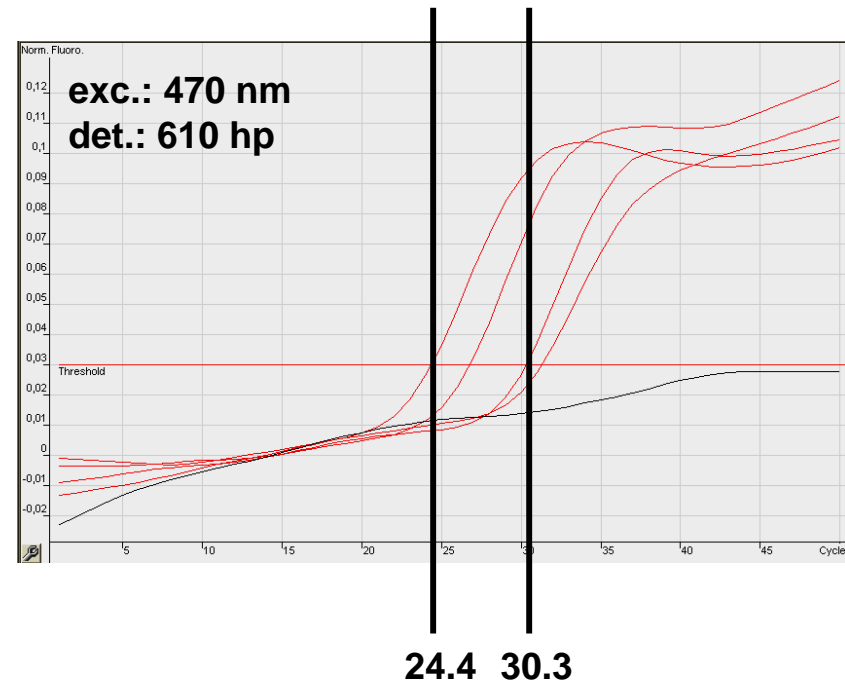
absorption max. of LC Red705 is around 680 nm - it can, thus, not efficiently be excited

Both PCR Reactions are Detected in One Channel

PCR setup **including IC**



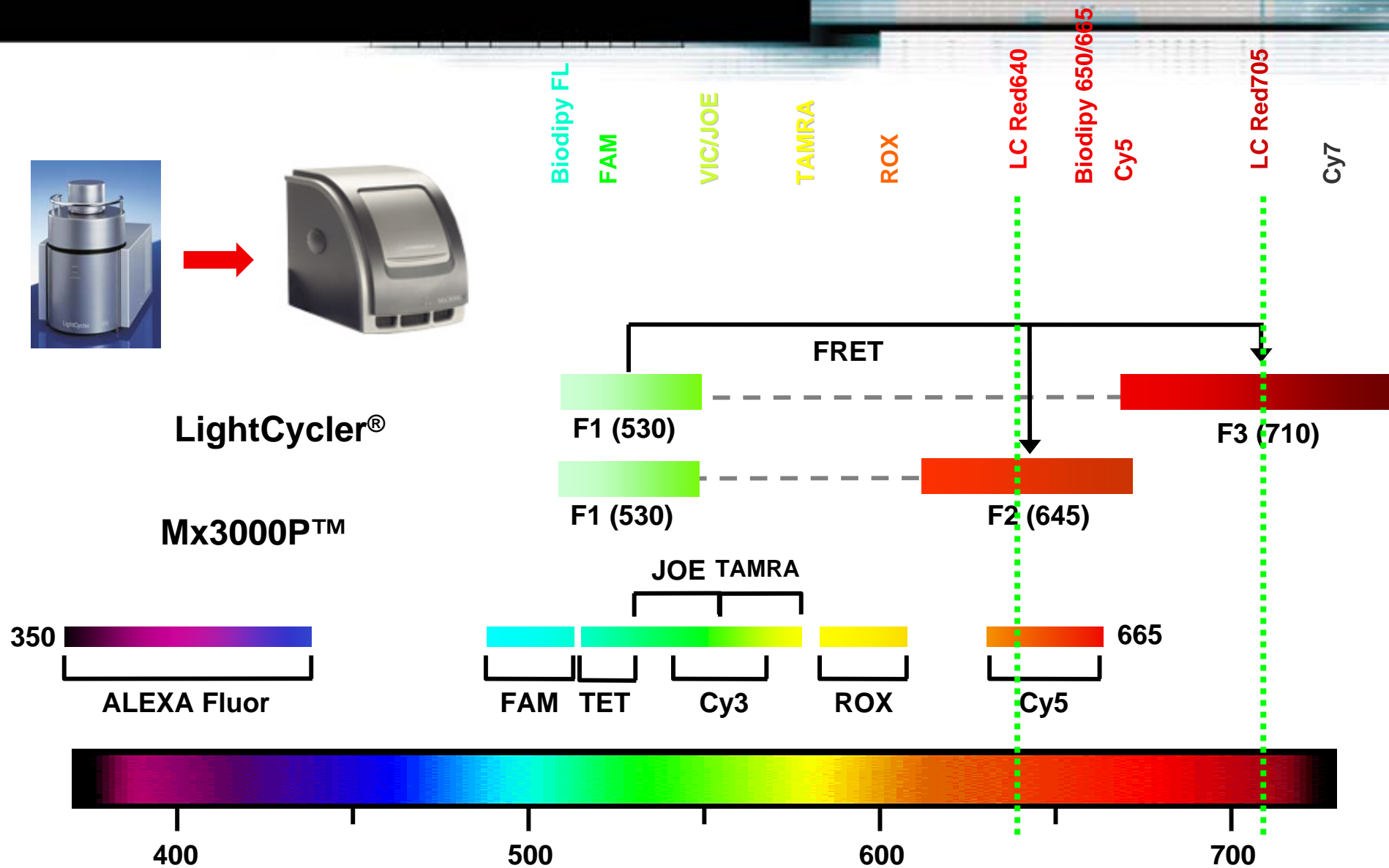
PCR setup **excluding IC**



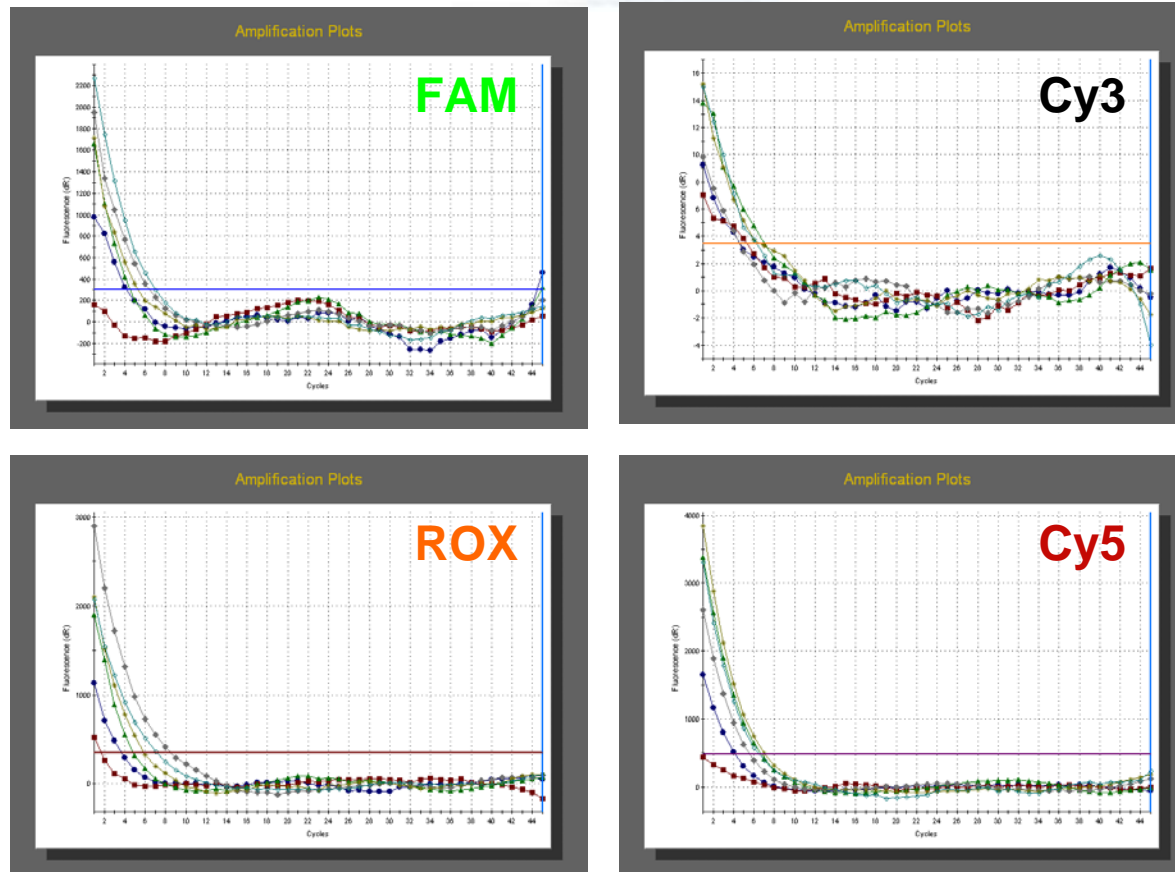
increase of Ct values due to overall increased detected fluorescence

➔ a LC assay cannot easily be transferred to the Rotor-Gene™ ←
(alternative: quenched FRET, other fluorescent dyes)

Transfer of the HSV Real-Time Assay to the Mx3000P™ Instrument

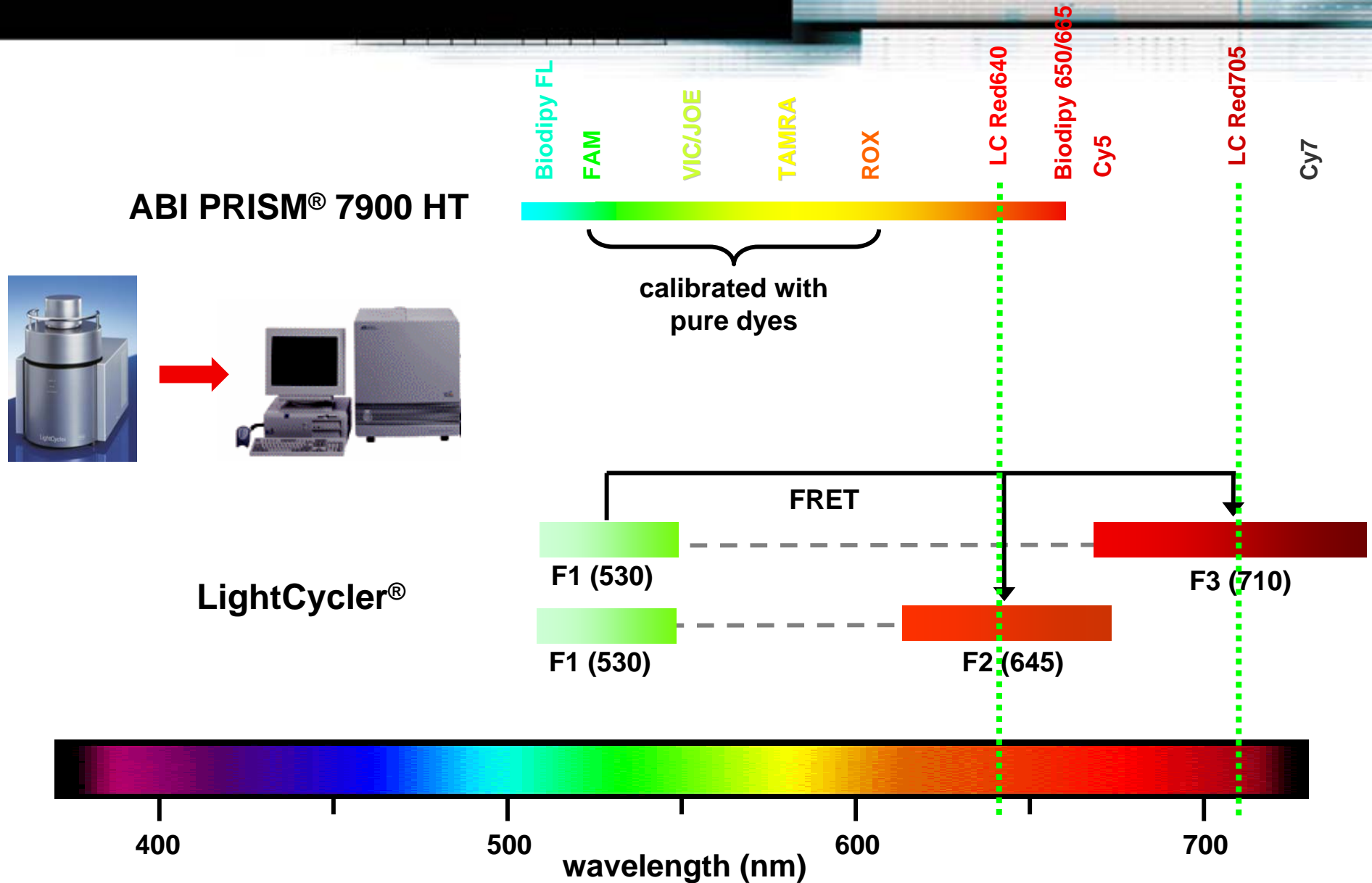


Transfer of the HSV Real-Time Assay to the Mx3000P™ Instrument

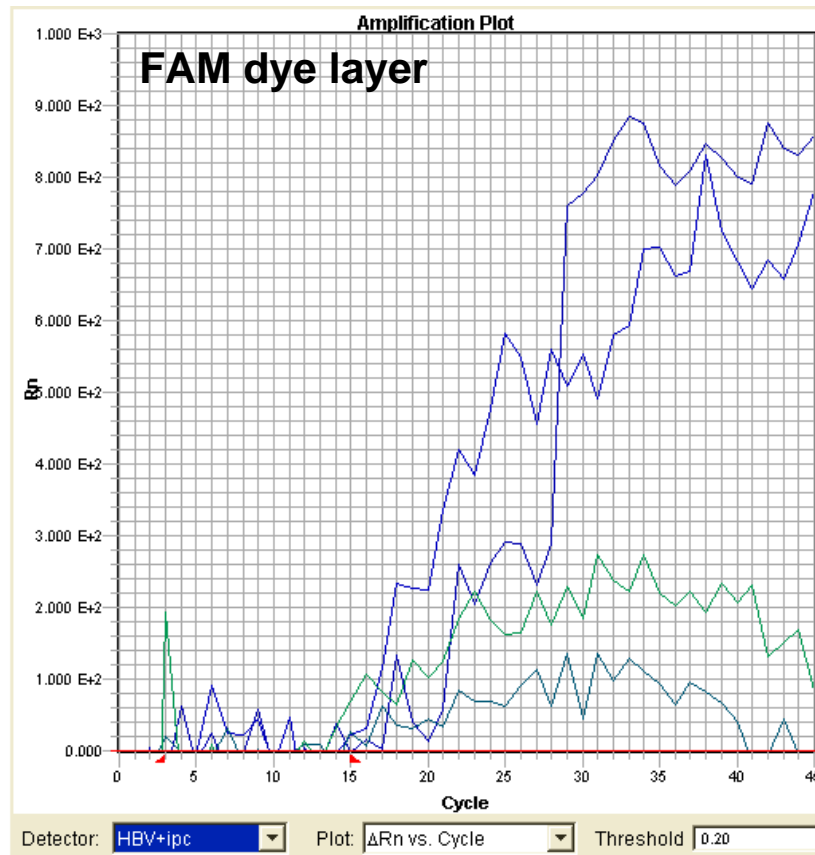


→ amplification of neither HSV standards nor the internal control can be detected ←

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



Transfer of the HSV Real-Time Assay to the ABI PRISM[®] 7900 Instrument

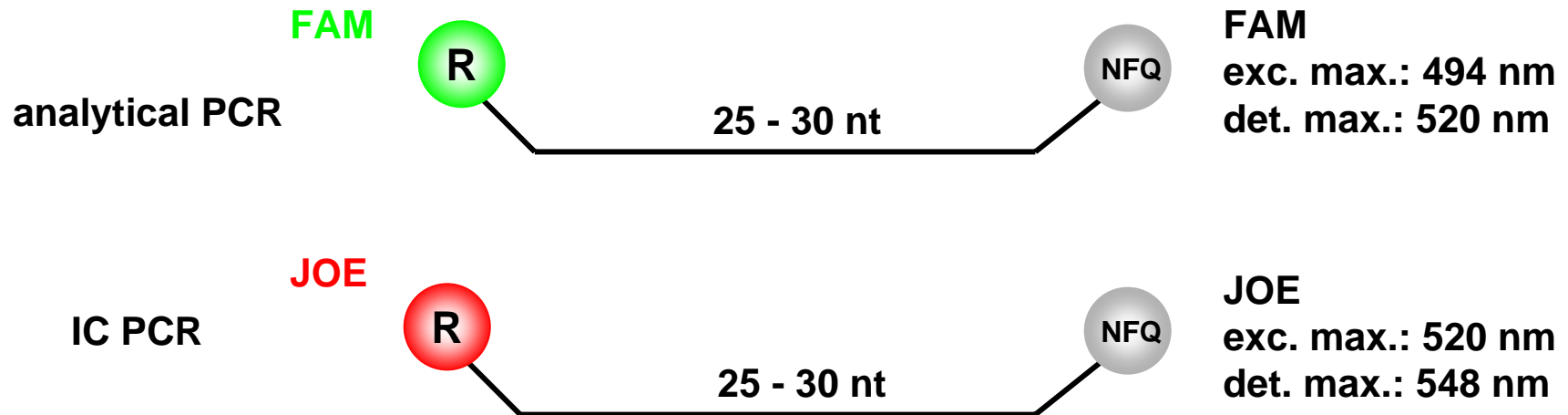


Detection of fluorescence signals due to FAM-labeled oligo probe 1

**HSV quantitation standard series
(10^1 - 10^4 copies/ μ l)**

Alternative Detection Format for Rotor-Gene™, ABI PRISM®, Mx3000P™ Instruments

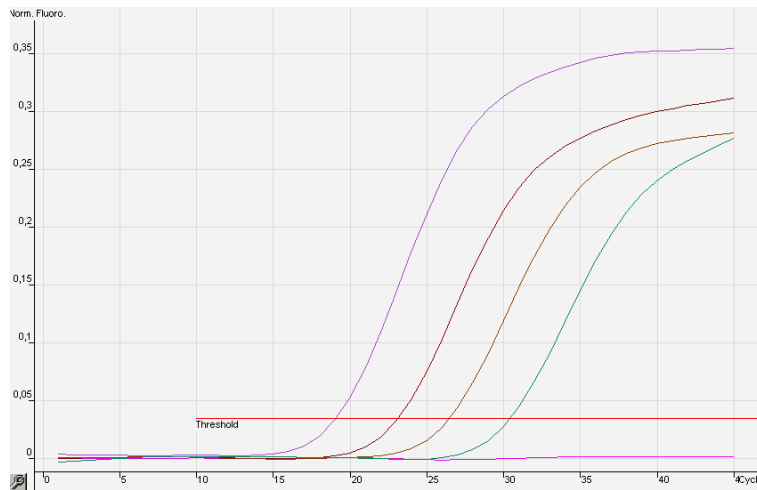
Use of two dual labeled probes: TaqMan probes



R: reporter fluorophore
NFQ: non-fluorescent quencher

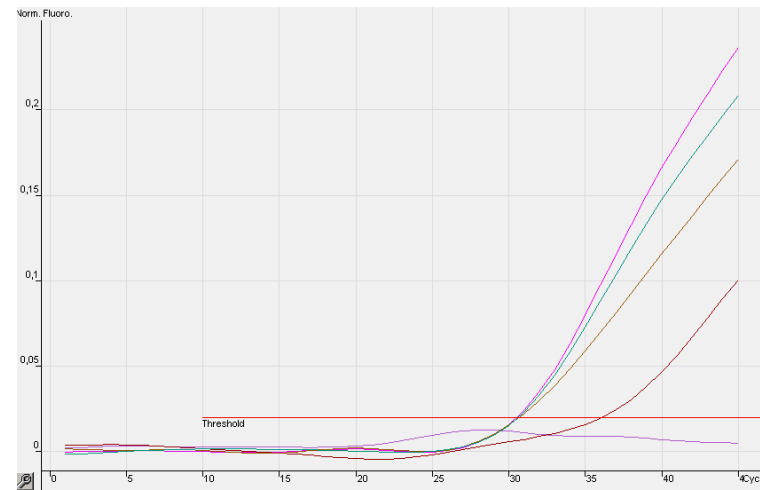
Example of a Rotor-Gene™ Assay Using Dual-Labeled Probes

**analytical PCR in
FAM channel**



**Malaria quantitation standard
series of defined concentrations**

**IC PCR in JOE
channel**

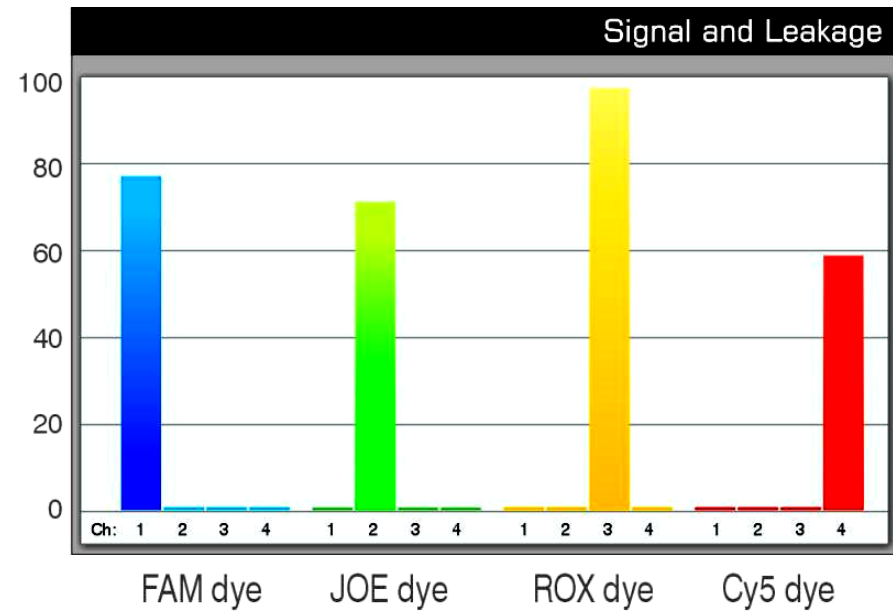


**Internal control PCR
(competition effect)**

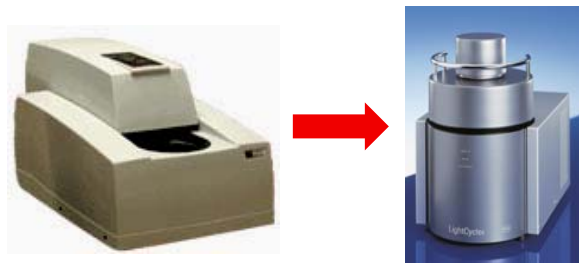
➔ good fluorescence signal separation in two channels !!!

No Color Compensation Required on the Rotor-Gene™

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

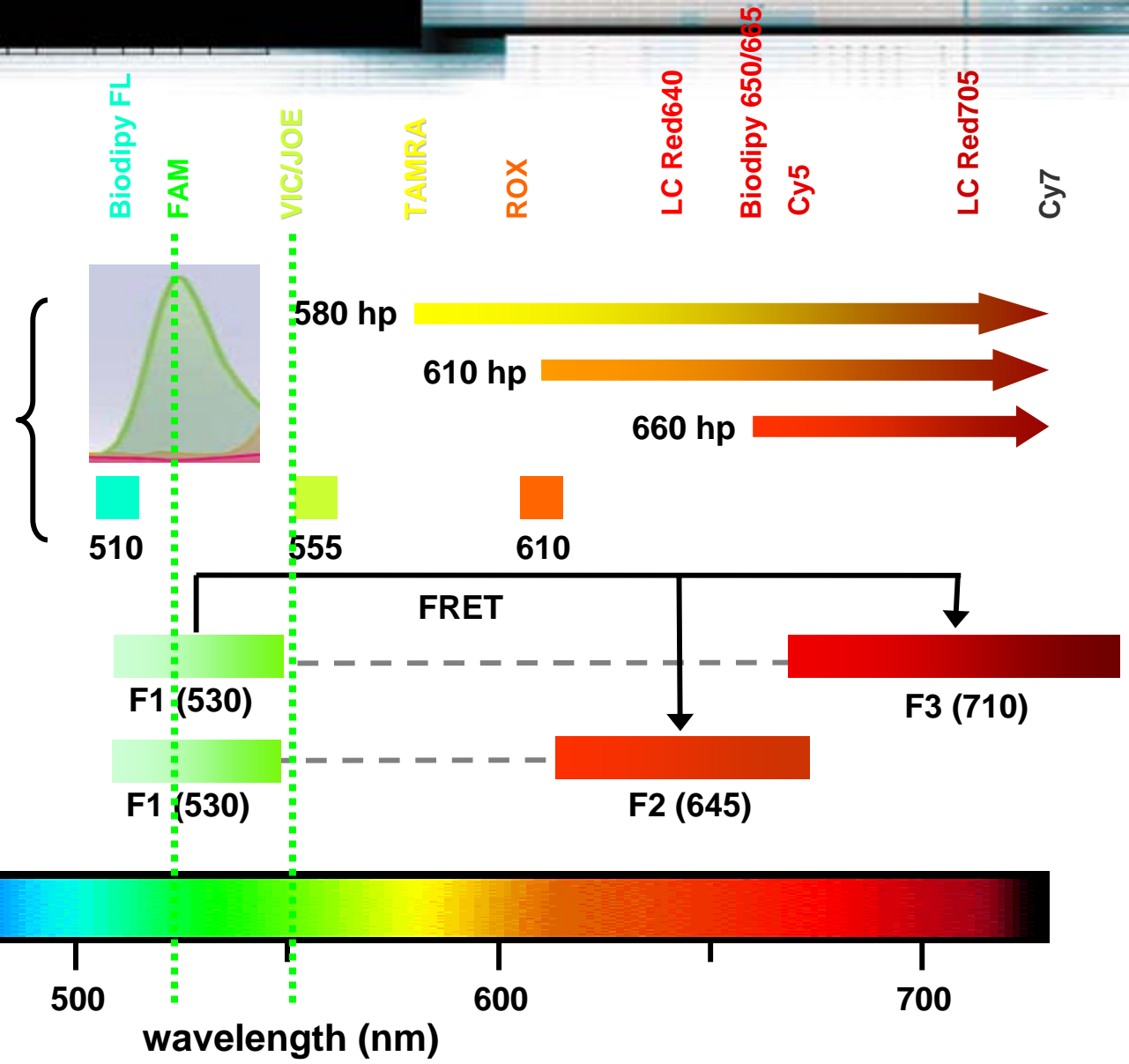


Transfer of the Malaria Rotor-Gene™ Assay on the LightCycler®



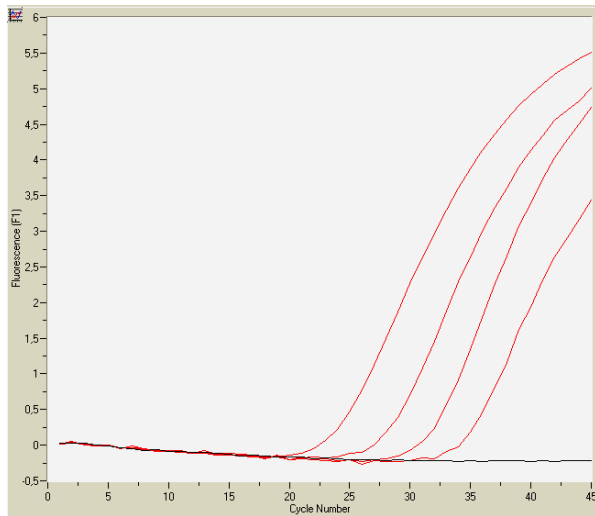
Rotor-Gene™

LightCycler®



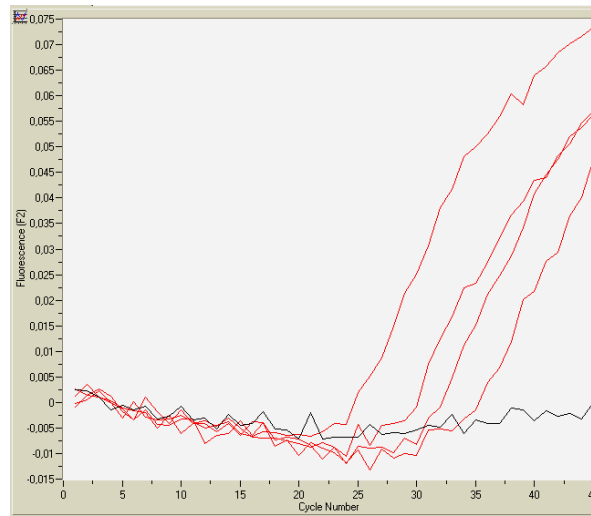
Transfer of the Malaria Rotor-Gene™ Assay on the LightCycler®

F1



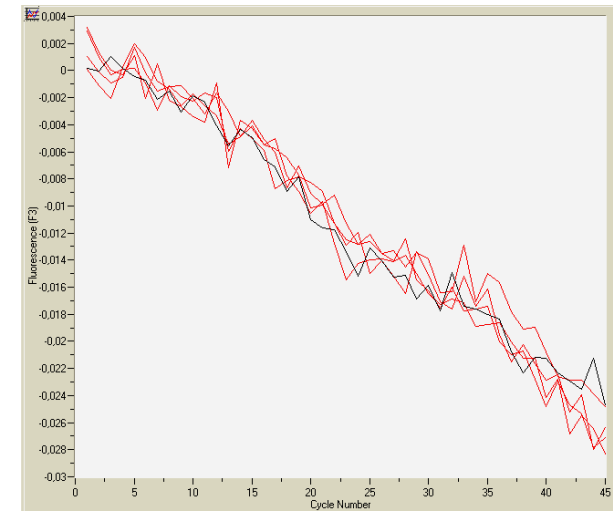
**analytical Malaria PCR
detected in FAM channel**

F2



**despite color compensation
the F1 signal strikes through
into the F2 channel**

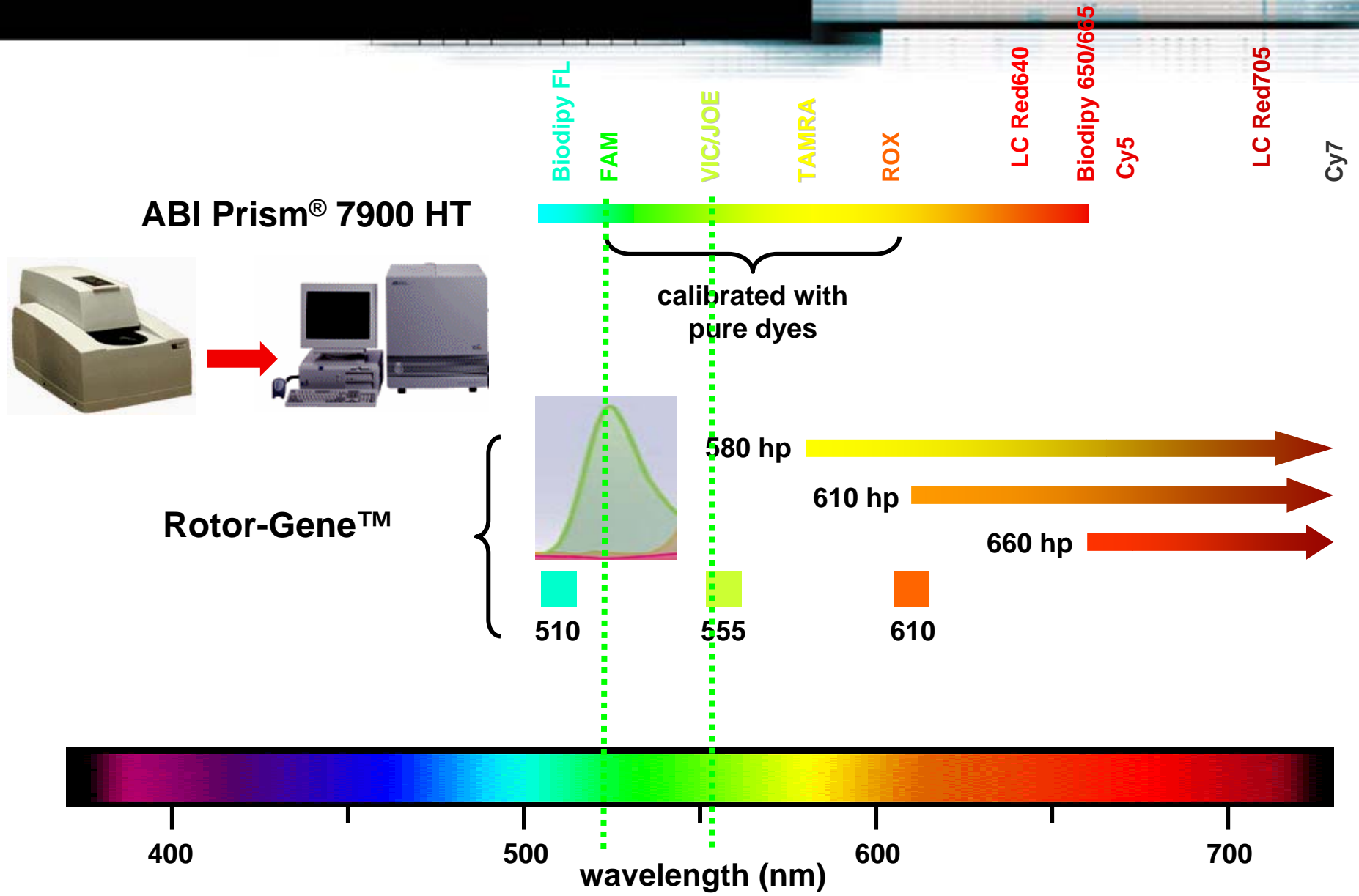
F3



**no fluorescences can be
measured in the F3 channel**

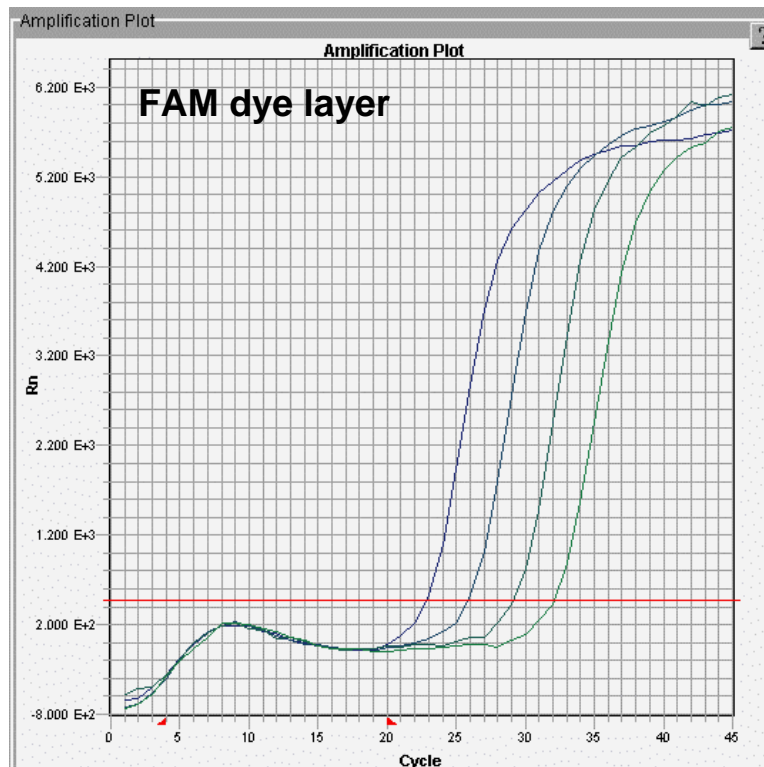
➔ no IC detection as JOE cannot be excited by the LightCycler®

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

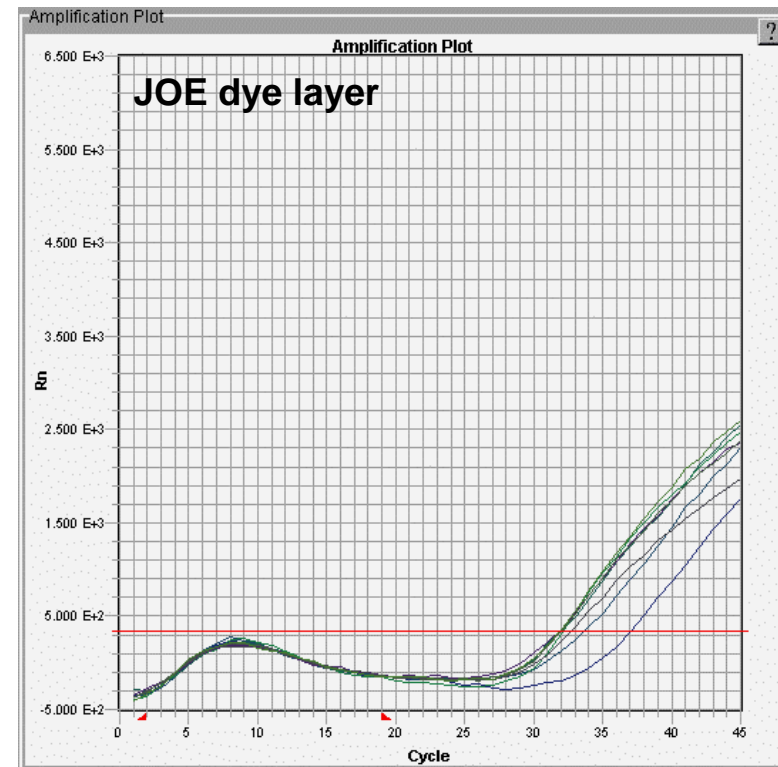


Transfer of the Malaria Rotor-Gene™ Assay on the ABI PRISM® 7900HT

analytical PCR



IC PCR



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

Transfer of the HBV ABI PRISM[®] Real-Time Assay to the Mx3000P[™] Instrument



Biodipy FL

FAM

VIC/JOE

TAMRA

ROX

LC Red640

Biodipy 650/665

Cy5

LC Red705

Cy7

ABI PRISM[®] 7000

~530 ~550 ~570 ~610

Mx3000P[™]

JOE TAMRA

350

ALEXA Fluor

FAM

TET

Cy3

ROX

Cy5

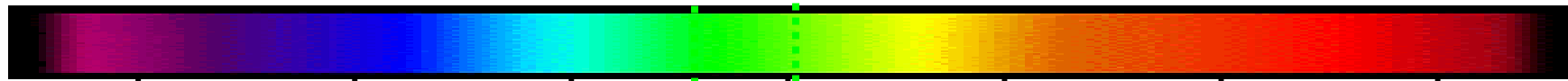
665

400

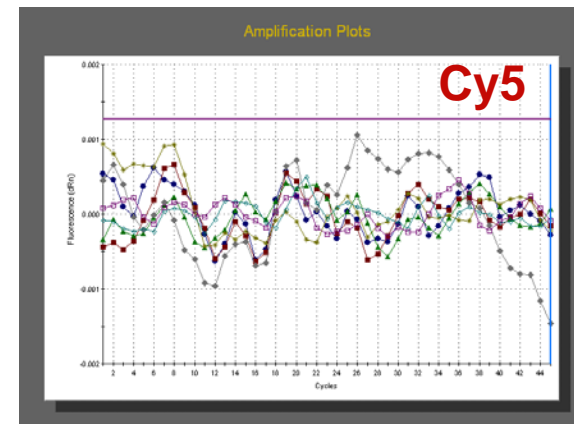
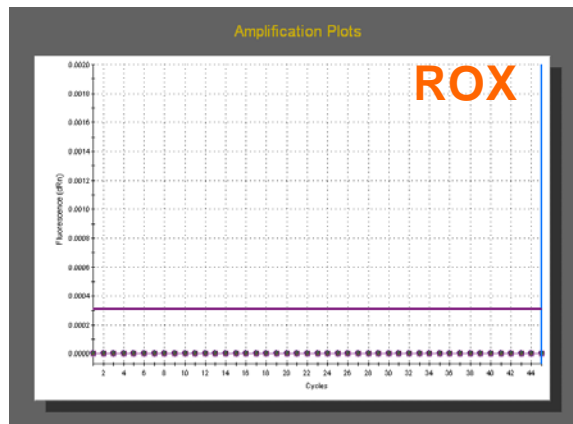
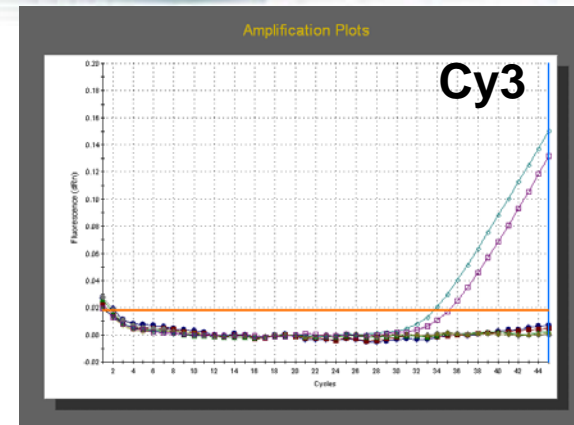
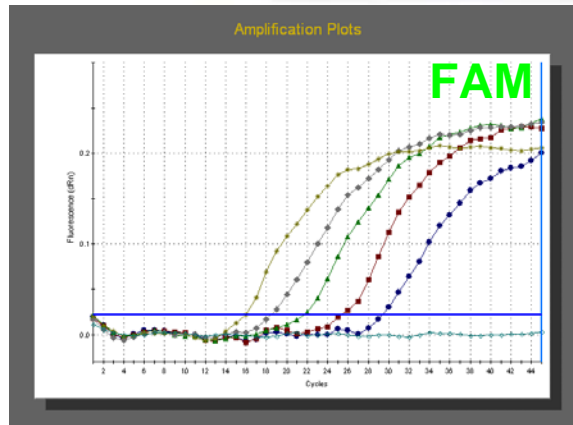
500

600

700



Transfer of the HBV ABI PRISM® Real-Time Assay to the Mx3000P™ Instrument



➔ HBV quantitation standards can easily be detected (FAM filter), whereas the IC signals (Cy3) are rather weak

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 μ l**
 - RG/TM: allow reaction volumes of up to 100 μ l**
- ➔ increased total volume allows a larger volume of sample material**
- ✓ **Passive reference dyes**
- ✓ **Temperature profile**
- ✓ **... well, try and see !**