

Going MULTI – How to Easily Achieve High Multiplexing in Real-Time PCR

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Overview

- Introduction
- Multiplex real-time PCR – The Challenges
- Multiplex real-time PCR – The Solutions
- Application Data
- Recommendations
- Summary

The Advantages of real-time multiplex PCR

- Increased reliability of quantification –
  Coamplification of internal controls / reference genes
- Conservation of precious samples – *More data / sample*
- Increased throughput – *More targets per run*
- Reduced reagent costs – *More results / € $ ¥*

The Challenges of multiplex real-time PCR

- "**Optimization** of each individual reaction before combining it into a multiplex reaction is **necessary**."
  (Brisson *et al.* (2004), in "A-Z of quantitative PCR",ed. S. Bustin, 619-642)

- "The **development** of an efficient multiplex PCR usually requires …**multiple attempts** to optimize reaction conditions."
  (Markoulatos *et al.* (2002), Multiplex Polymerase Chain Reaction: A Practical approach; J. Clin. Lab. Anal., **16**, 47-51)

- "Successful multiplexing is **never trivial**."
  (Bustin (2000), Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction; J. Mol. Lab. Endocrinol., **25**, 169-193)

Multiplex real-time PCR is difficult !

General Problems in real-time PCR

- Poor PCR specificity
- Lack of PCR sensitivity
- Assay design

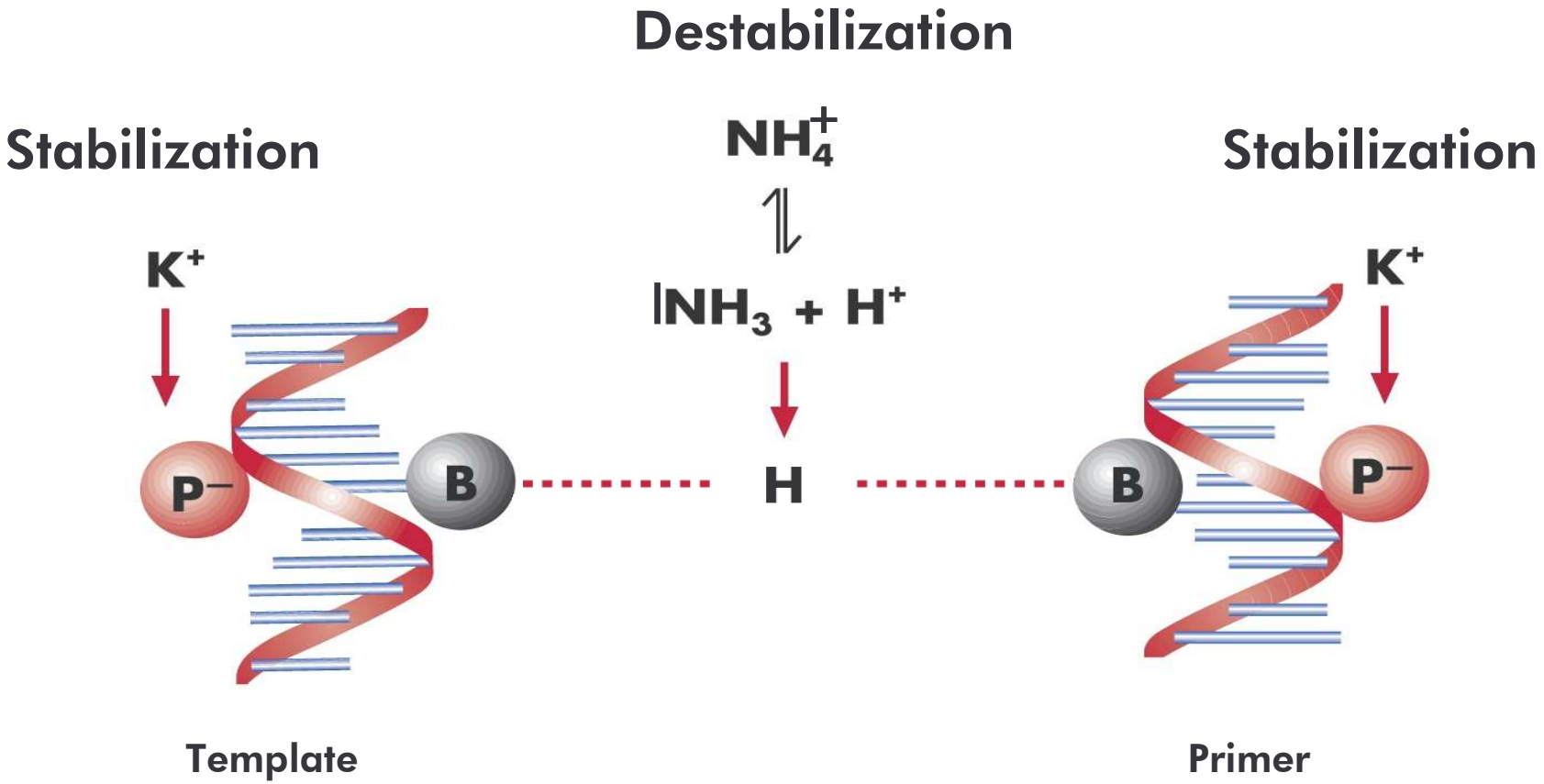
Factors Influencing PCR Specificity

- Amount of starting template
- Primer design
- Cations contained in reaction buffer
- Initial generation of artifacts by *Taq* DNA polymerase

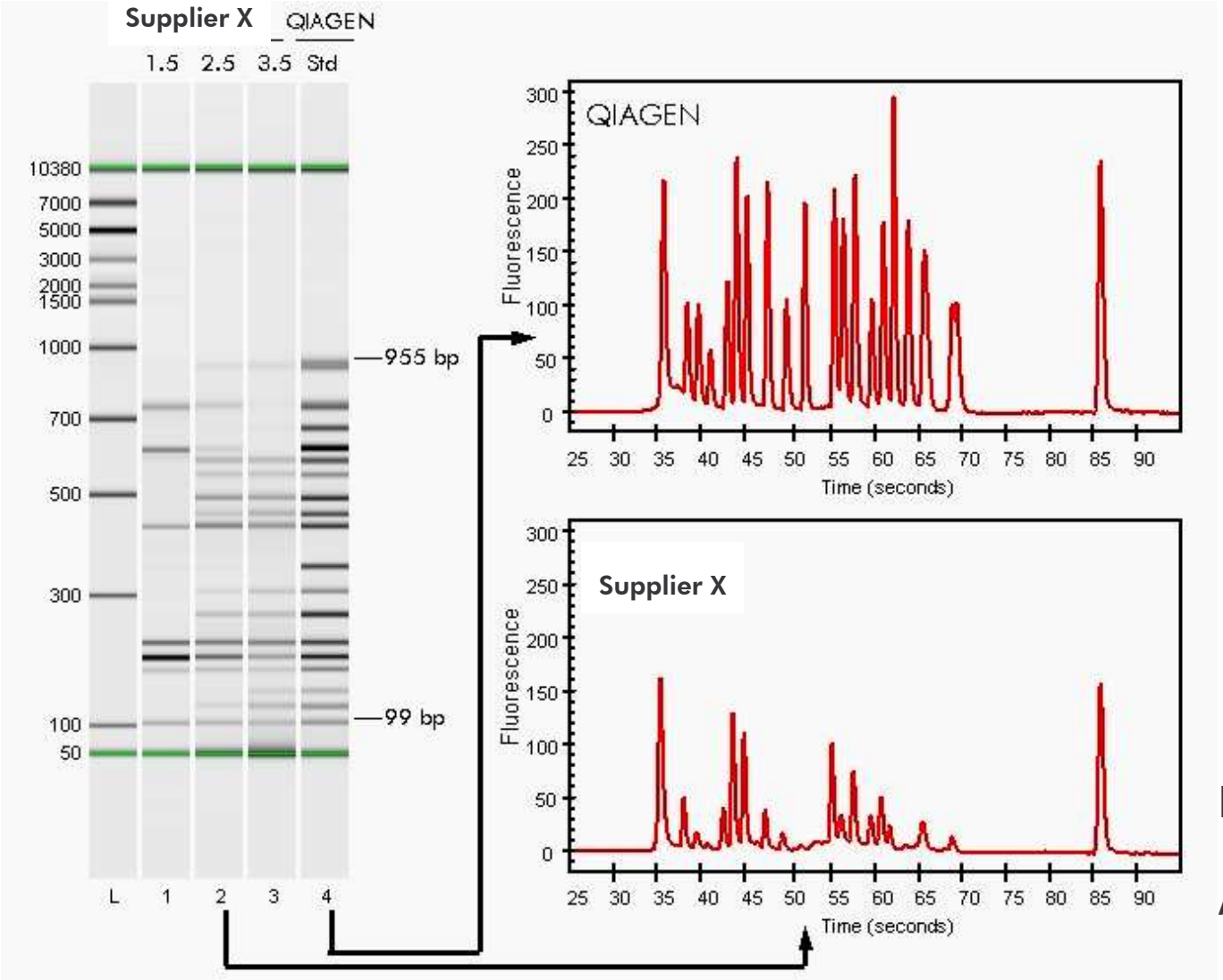
Causes for Poor PCR Specificity

- Relatively high primer concentration
  - Required for efficient primer annealing during short annealing step
- Low template amount or low abundance of target
  - Excess primer molecules
- Complementary primer/probe sequences
  - Primer dimer formation
- Amplicon length
  - Short fragments (e.g. primer dimers) compete efficiently for rxn components

Key Technologies to Improve Annealing Specificity: Unique PCR Buffer Composition



Effect of Buffer System on 19-plex PCR



Profiles from

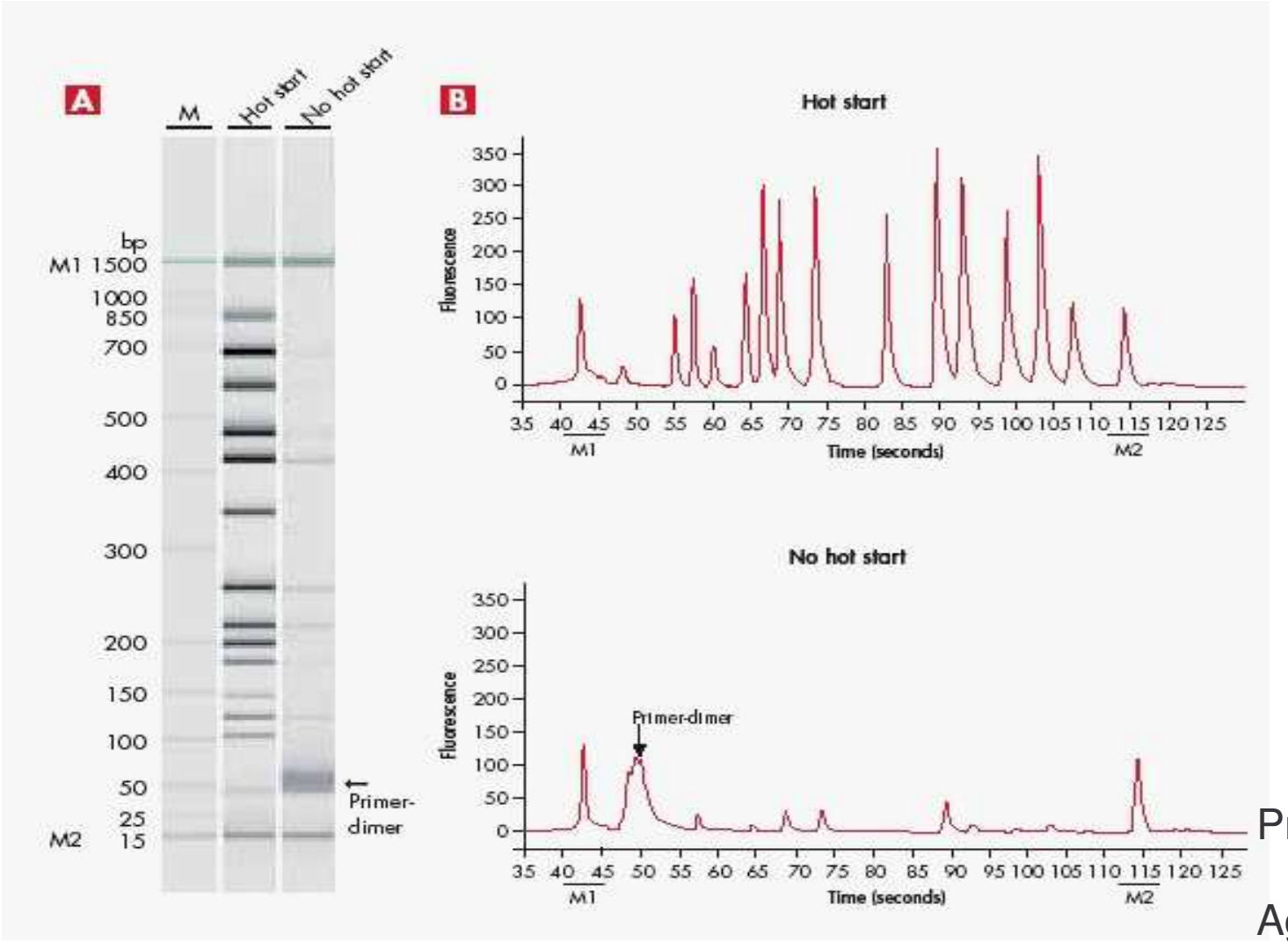
Agilent Bioanalyzer 2100

Key Technologies to Improve Specificity: The Enzyme

- Specialized Reaction Buffer Chemistry

- HotStarTaq DNA Polymerase
  - Unique chemical modification of recombinant *Taq* DNA
  - Polymerase becomes active by initial heat incubation step
  - Robust reactivation independent from PCR environment (pH, salts)

Effect of Hot Start on Multiplex PCR



Profiles from

Agilent Bioanalyzer 2100

Specific Problems in real-time multiplex PCR

- Potential mutual interference of various primers and probes
  - Increased risk of primer dimer formation
- Different hybridization properties of primers and probes
  - Not all primers and probes are created equal
- Smaller PCR products (and primer dimers!) amplified more efficiently
  - Poor product yields or missing PCR product
- Preferential or exclusive amplification of the most abundant target
  - Non-reliable results, deviation from single-plex data

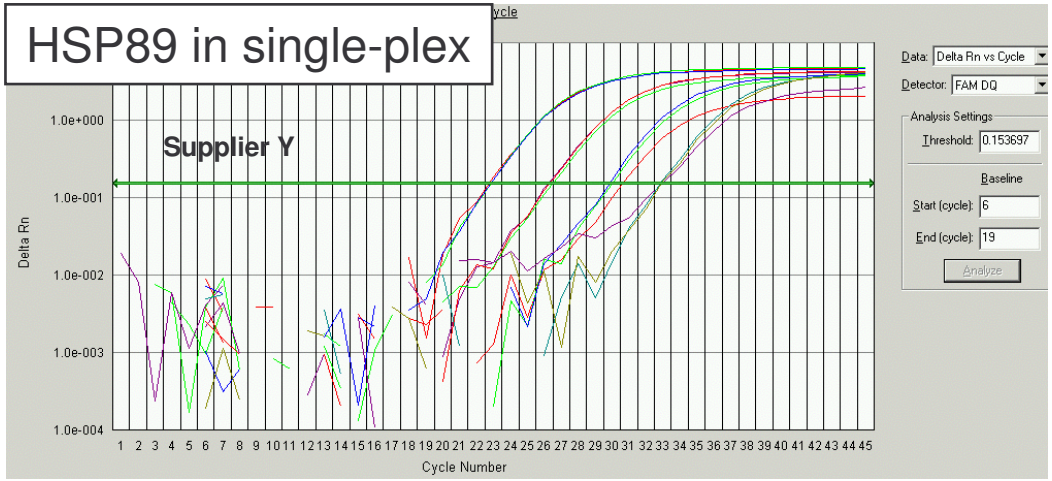
The Less Abundant Target: Inhibition in Later PCR Cycles

- Utilization of substrates
- Thermal inactivation / limiting concentration of polymerase
- Increased pyrophosphate concentration
- Destruction of product because of Taq 5'-3' exo activity
- **Binding of Taq to short dsDNA w/o sequence specificity**



Common Problems in Real-time Multiplex PCR

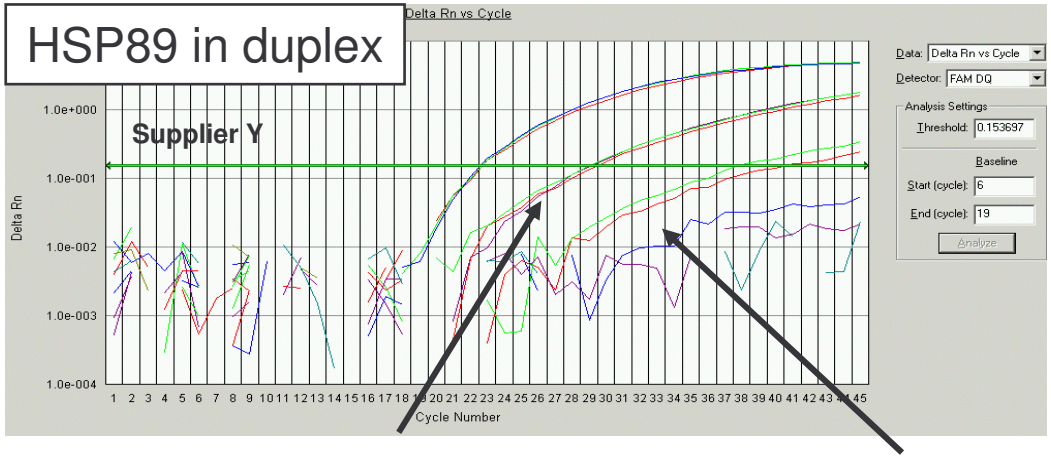
HSP89 in single-plex



Detection of HSP89 in various cDNA amounts:

10 ng, 1 ng, 100 pg, 10 pg

HSP89 in duplex



Detection of HSP89 in various cDNA amounts:

10 ng, 1 ng, 100 pg, 10 pg

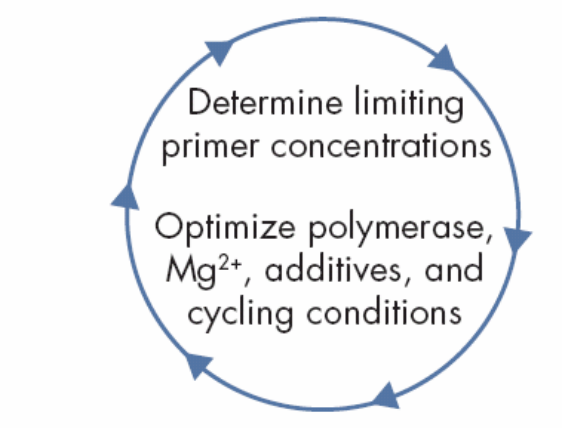
plus 10^6 copies reference gene (GAPD)

Shifted (higher) C_T values

Low HSP89 transcript levels not detected

Optimal multiplex PCR: The Individual Approach

- Increase Taq concentration for specific primer-probe systems
  - Expensive; increased risk of non-specific products
- Optimize Mg^{2+} concentration
  - Increased risk of non-specific primer-probe annealing
- Optimize primer-probe sequences and concentrations (e.g. limited primer conc.)
  - Cumbersome, time-consuming
- Optimize cycling conditions
  - Not universally applicable



Wheel of Fortune or Vicious Circle?

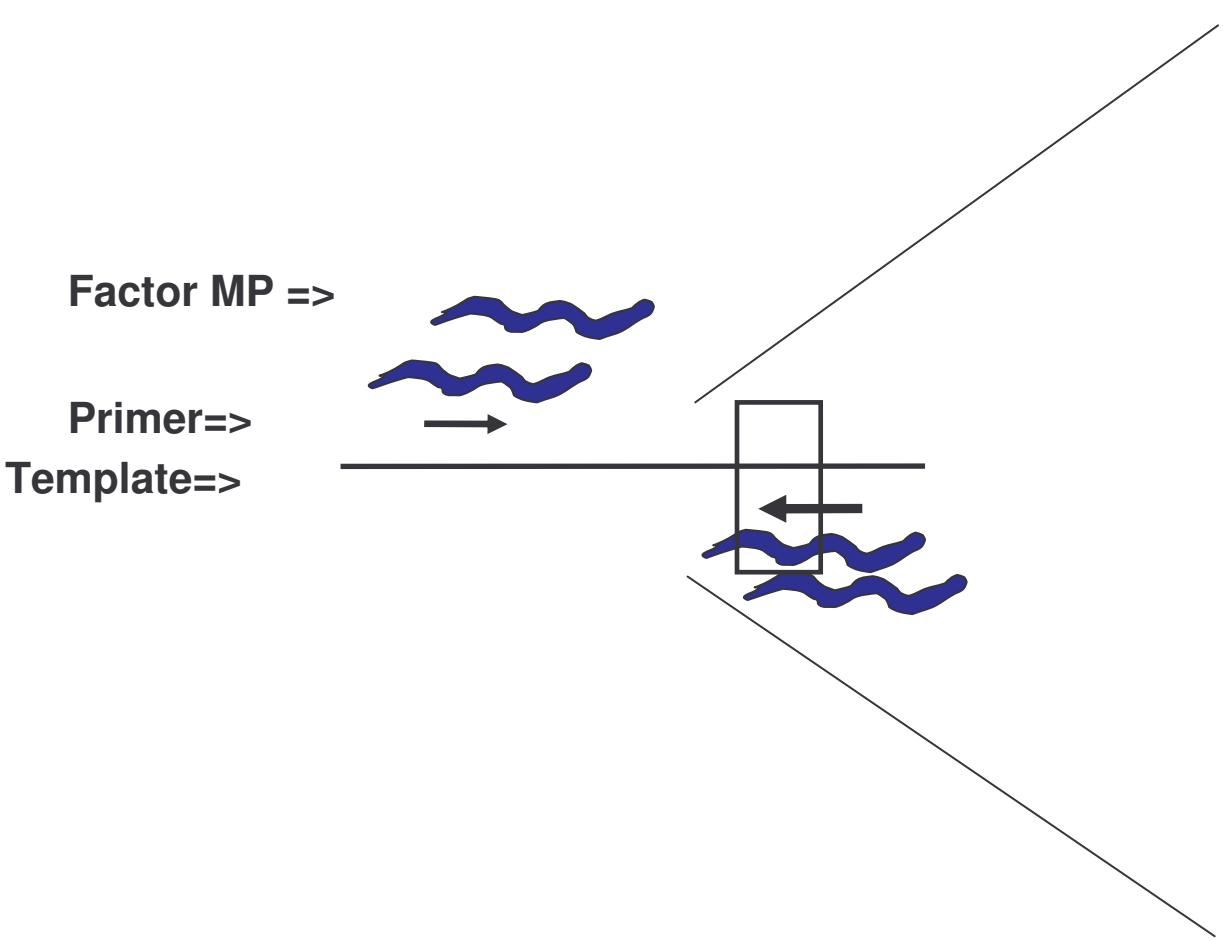
Optimal multiplex PCR: The Generic Approach

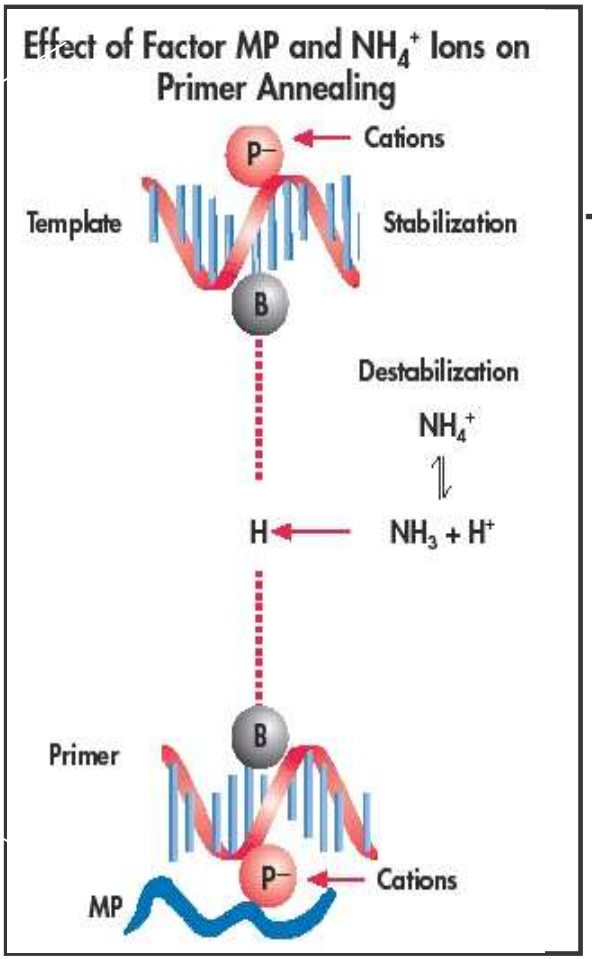
- Optimized buffer chemistry
- Optimized enzyme
- Macromolecular crowding

Macromolecular Crowding

- Steric exclusion of rxn volume by inert macromolecules
- Increase of effective concentration of reactants
- Improved hybridization of primer/probes and template
- More efficient binding between Taq and primer-template-DNA
- Broadening of suitable reaction conditions

Factor MP balances the Primer Annealing Efficiency



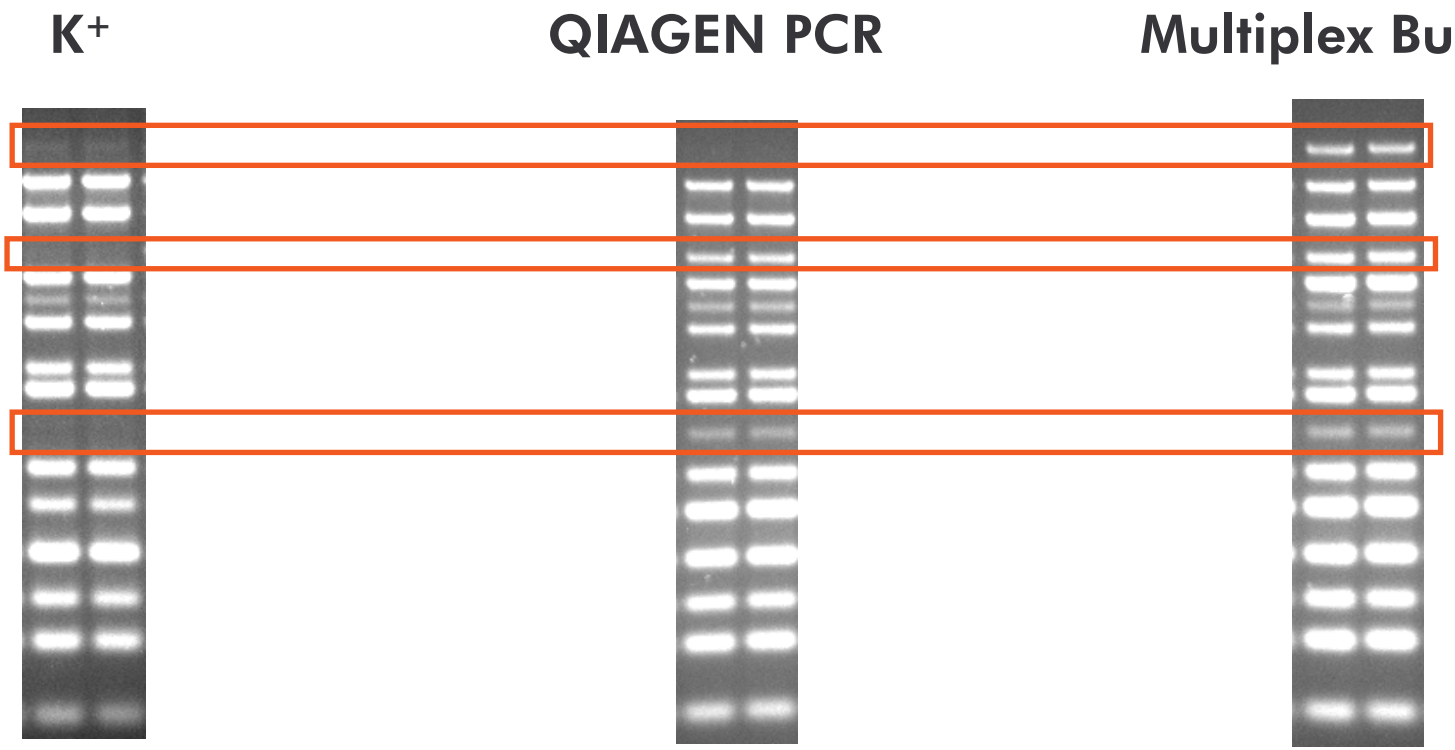


Template

Primer

Effect of Factor MP

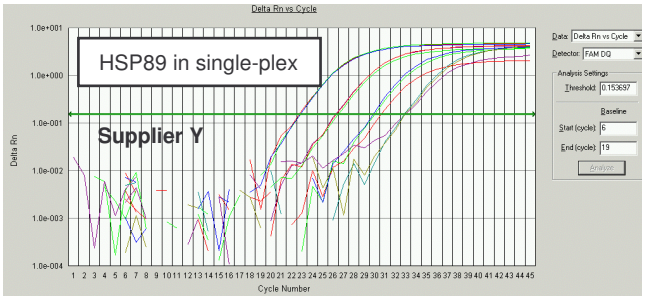
Comparison of different Reaction Chemistries

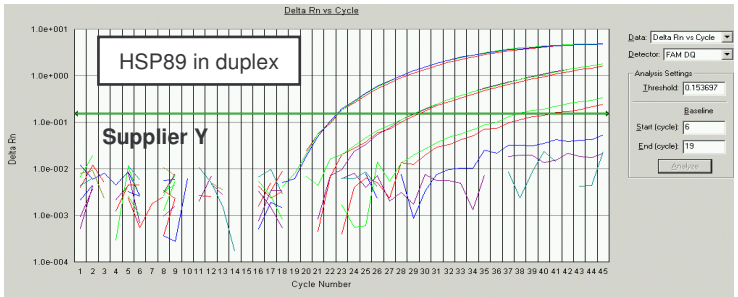


16-plex [bp]
955
845
756
662
610
564
523
446
414
363
310
269
222
181
150
99

Effect of Novel Multiplex Real-time PCR Chemistry

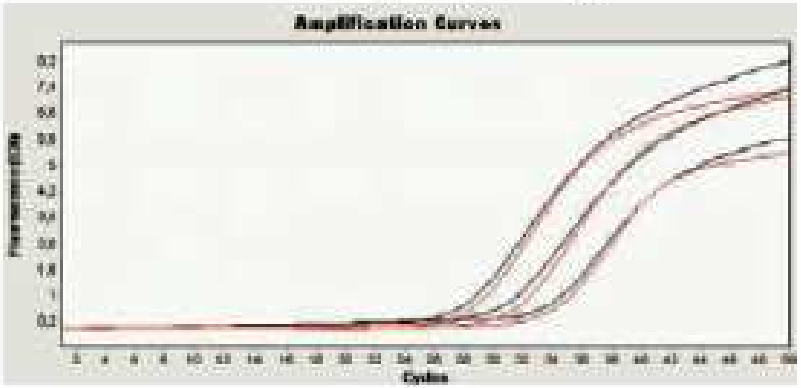
	QIAGEN		Supplier Y	
cDNA	Single	Duplex	Single	Duplex
10 ng	22.17	21.23	22.76	22.60
1 ng	25.55	24.71	26.30	29.18
100 pg	28.71	28.53	30.14	39.20
10 pg	32.06	34.49	32.89	45.00



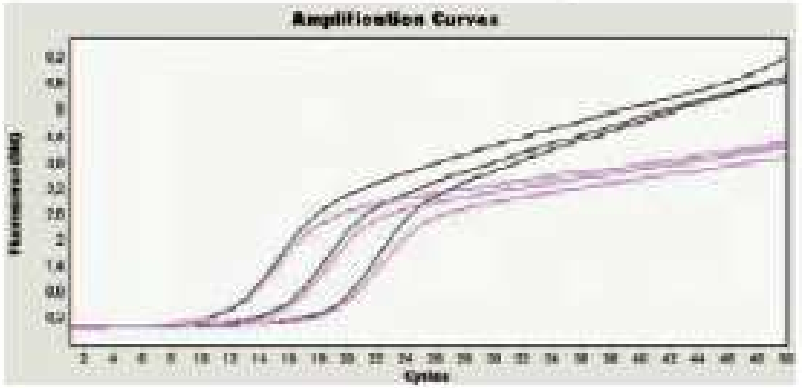


Equivalent C_T Values in Single and 4plex PCRs (I)

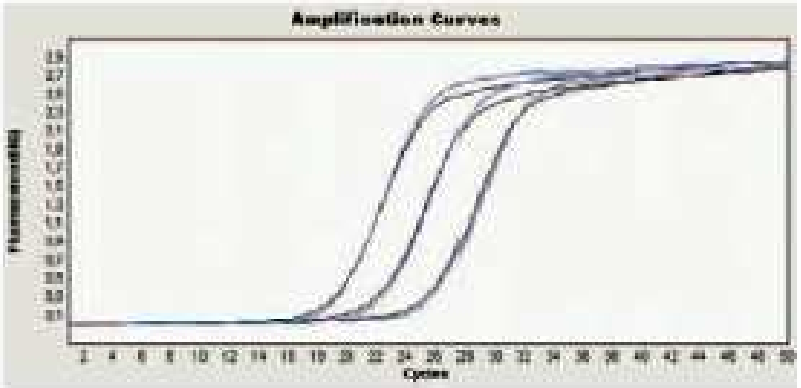
FAM channel (t(8;14) transcript)



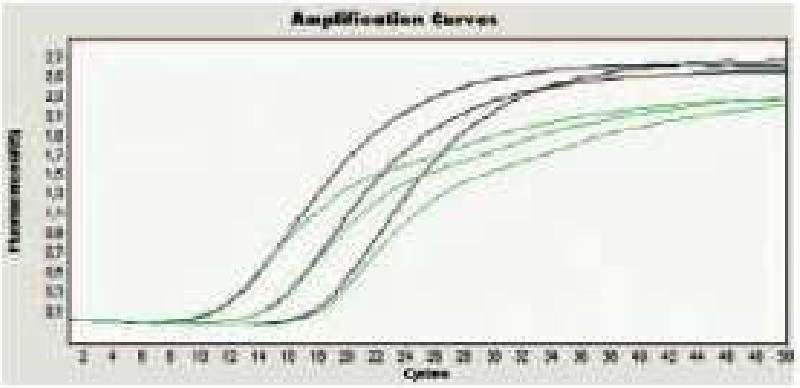
HEX channel (18S rRNA)



Texas Red channel (HSP89)

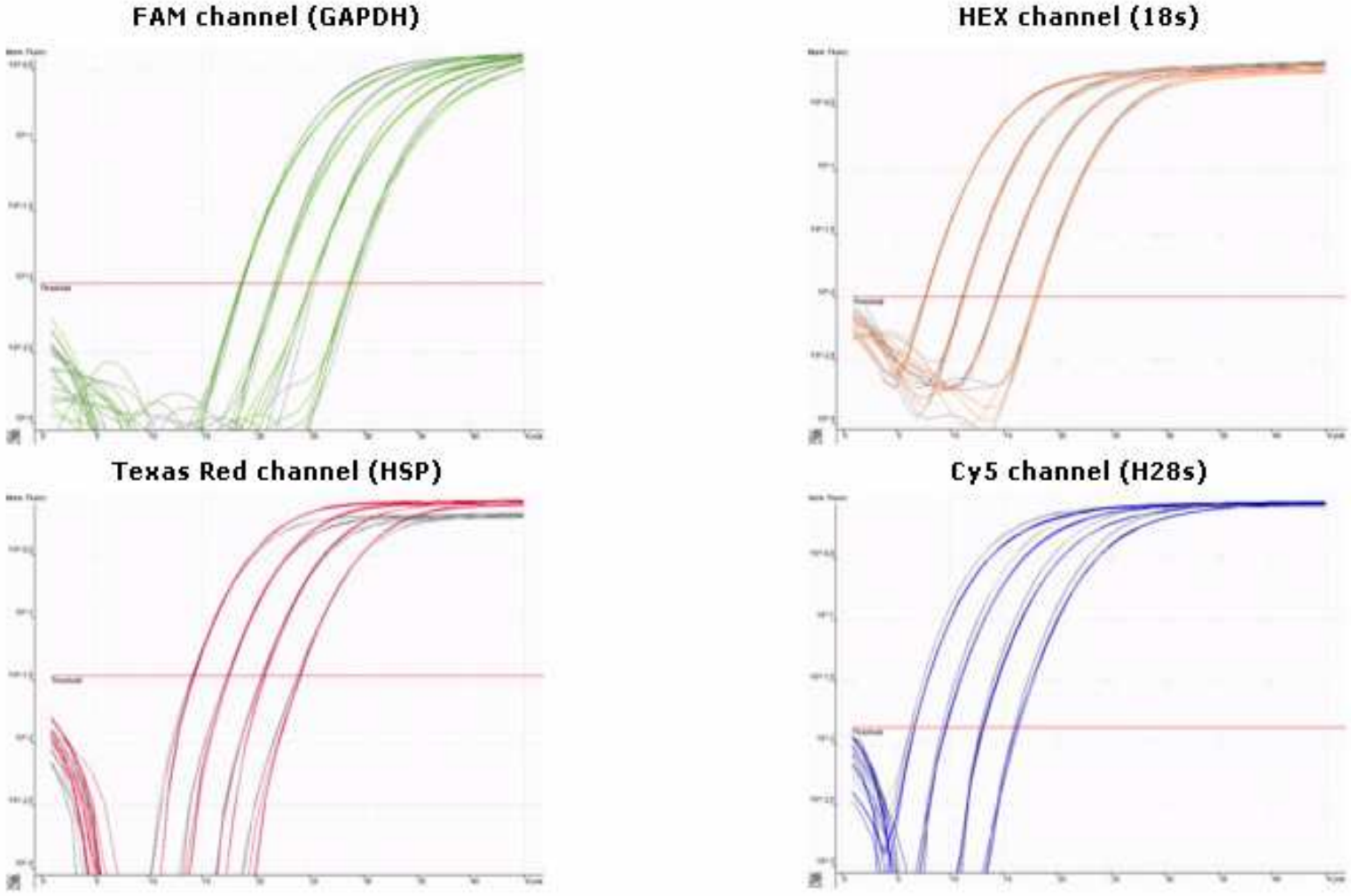


Alexa Fluor 660 channel (28S rRNA)



LightCycler II

Equivalent C_T Values in Single and 4plex PCRs (II)



Rotorgene 3000

Strongly Varying Target Abundance in Triplex PCR (I)

- The Goal
  Show identity of results obtained in single-plex (i.e. one primer-probe combination only/reaction) and in triplex (all 3 targets coamplified in the same reaction)

- The Challenge
  The 3 targets vary strongly in their abundance (differences up to 5 logs)

- The Targets and Templates

t14;18	20 copies	gDNA
GAPD	10^6 copies	plasmid DNA
NFKB	10^3-10^5 copies	cDNA

Strongly Varying Target Abundance in Triplex PCR (II)

	Detection of		
	t(8;14) (20 copies)	GAPDH (10^6 copies)	NFκB (copies in parentheses)
Triplex PCR	34.31	20.37	21.92 (10^5)
Single PCRs	34.07	20.54	21.83 (10^5)
Triplex PCR	34.61	20.62	25.03 (10^4)
Single PCRs	34.00	20.46	25.19 (10^4)
Triplex PCR	35.17	19.94	28.38 (10^3)
Single PCRs	34.43	20.50	28.65 (10^3)

Experiment performed on ABI PRISM 7900

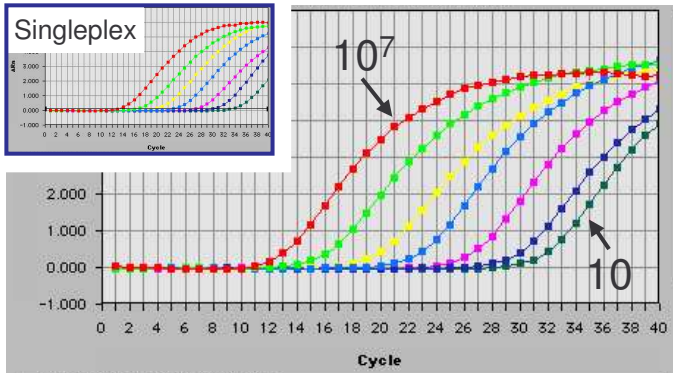
Examples (I)
Reliable Duplex PCR without Optimization

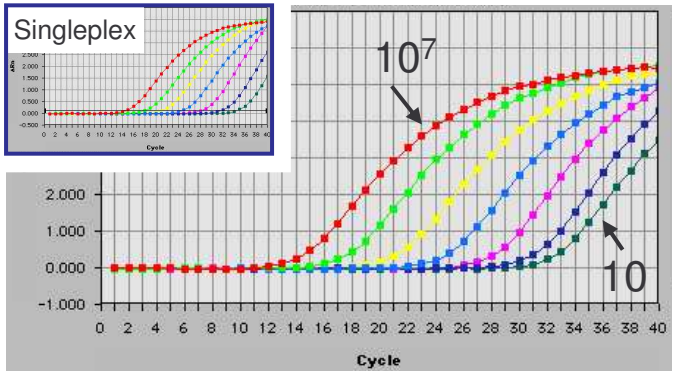
Duplex-Assay 1

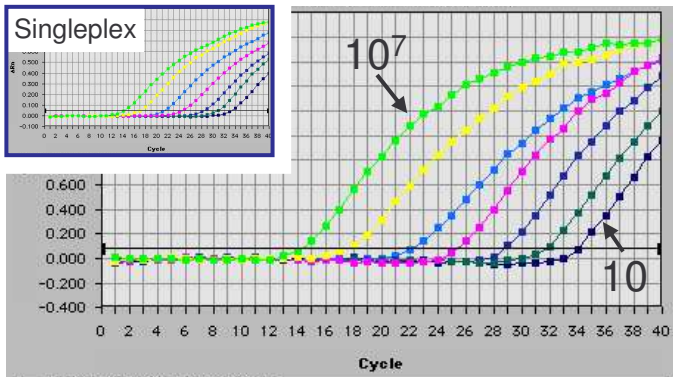
Duplex-Assay 2

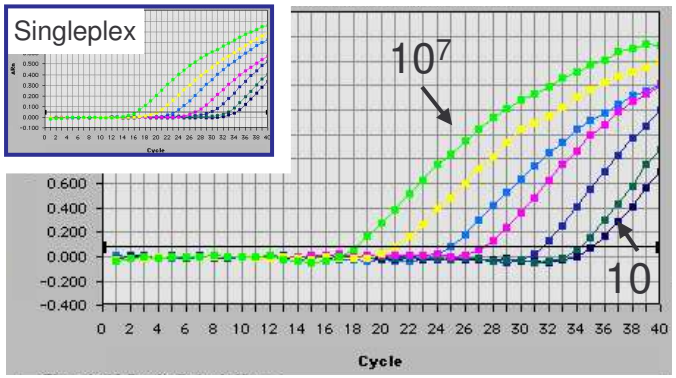
FAM

VIC





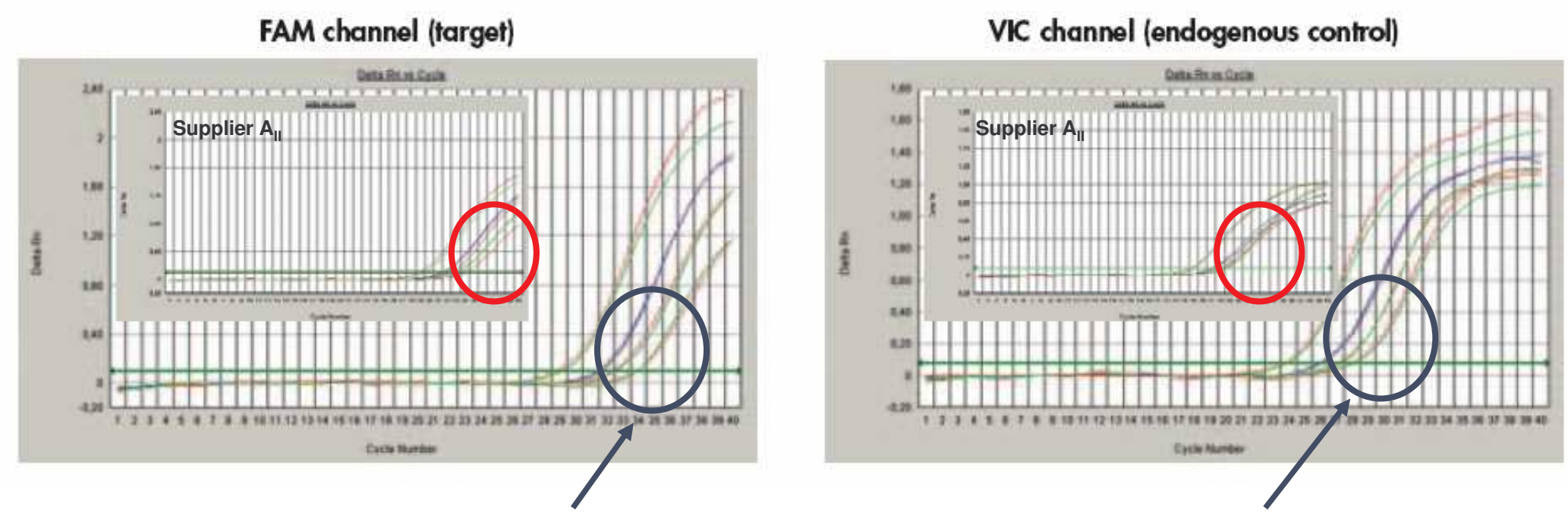




ABI 7700, 10 fold dilutions of a DNA standard

Data kindly provided by Dr. John Coleman, Wyeth Research

Examples (II)
Resolution of Small Differences in Copy Number

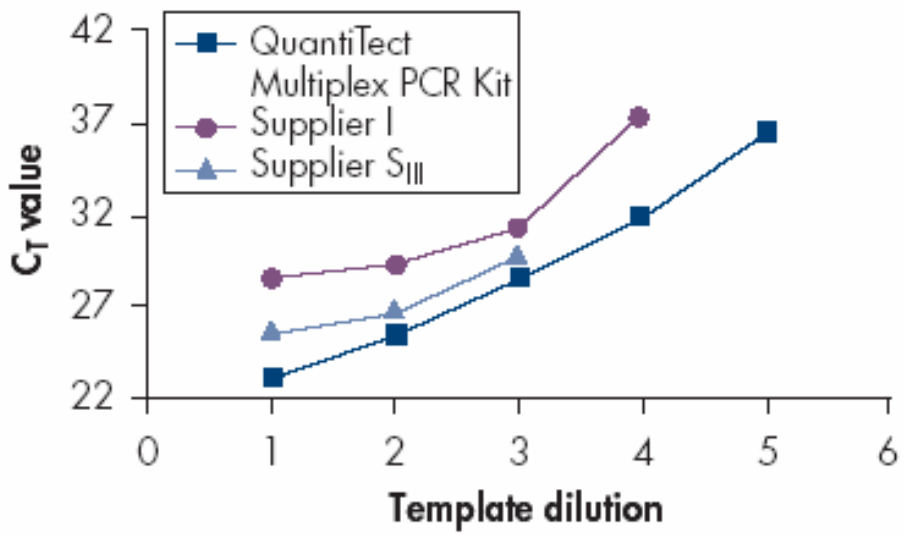


Resolution of 2-fold differences using QT Multiplex PCR Kit

Data kindly provided by Dr. Louise R. Simard, Centre de recherche de l'Hôpital Sainte-Justine, Montréal, QC, Canada.

Examples (III)
Sensitive ASF Virus Detection in Multiplex Real-Time PCR

A



B

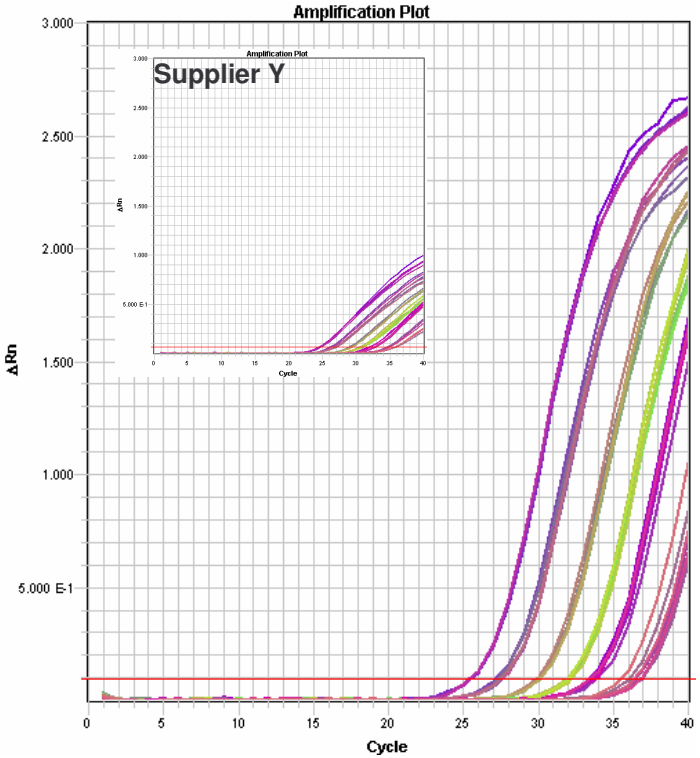
Product	Template dilution C_T value*				
	1	2	3	4	5
QuantiTect Multiplex PCR Kit	23.07	25.42	28.57	31.86	36.42
Supplier I	28.66	29.26	31.13	37.37	N.D.
Supplier S_{II}	25.48	26.61	29.56	N.D.	N.D.

* Data for coamplified internal control not shown

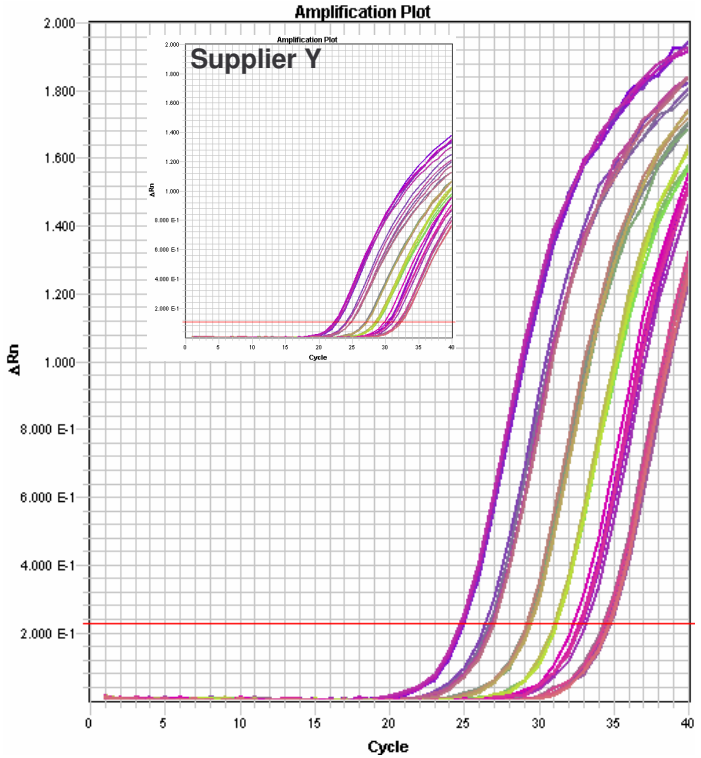
Data kindly provided by Dr. Bernd Hoffmann, Friedrich-Loeffler-Institut, Greifswald, Germany

“The results we obtained with your reagents were impressive”, Dr. Virginia M. Litwin, BMS

Gene of Interest (FAM)



Endogenous Control (VIC)



ABI 7900, 4 fold dilutions starting at 135 copies

Data kindly provided by Dr. Virginia Litwin, Bristol-Myers Squibb

Multiplex Real-Time PCR Essentials

- Test functionality of primers and probes in single-plex first
  - Ideally check integrity and quantity of primers and probes
- PCR products as short as possible (60-150 bp)
- Set baseline and threshold for *each* reporter dye to obtain most accurate quantitation
  - Make sure to activate the detector for each reporter dye used
  - Make sure that your instrument is calibrated for each reporter dye used
- Perform appropriate controls
  - Run single-plex and multiplex and compare data (ideally in identical run)
- Choose suitable reporter dye and quencher combinations

Recommendations

ABI PRISM 7500

Table 8. Suitable Combinations of Reporter Dyes for Multiplex Analysis Using the Applied Biosystems 7500

Type of assay	Filter A*	Filter B*	Filter C*	Filter D†	Filter E*
Duplex	6-FAM	HEX JOE VIC Yakima Yellow‡		ROX (passive reference)	
Duplex	6-FAM		Bodipy TMR§ NED	ROX (passive reference)	
Triplex¶	6-FAM	HEX JOE VIC	Bodipy TMR§ NED	ROX (passive reference)	
4plex¶	6-FAM	HEX JOE VIC	Bodipy TMR§ NED	ROX (passive reference)	Cy5

* Use filter A, filter B, and filters C and E to detect the least abundant target, the second least abundant target, and the 2 most abundant targets, respectively. Reporter dyes marked in bold are available from Operon Biotechnologies.

† Filter D is for detecting ROX passive reference dye, a component of the 2x QuantiTect Multiplex PCR Master Mix.

‡ QuantiTect Endogenous Control Assays from QIAGEN use QuantiProbes carrying a Yakima Yellow fluorophore, which is compatible with VIC calibration. Choose VIC from the list of calibrated dyes to set up detection of Yakima Yellow dye.

§ Before using the Bodipy TMR dye, a pure-dye calibration of the real-time cycler using this dye must be performed. See the manufacturer's manual for details. Supplementary protocols for pure dye calibration of major real-time cyclers are available from QIAGEN Technical Services (see back cover).

¶ Each probe in the triplex or 4plex assay must contain a nonfluorescent quencher.

Rotorgene 3000

Table 6. Suitable Combinations of Reporter Dyes for Multiplex Analysis Using the Rotor-Gene 3000 System*

Type of assay	Channel 1 (470/510†)	Channel 2 (530/555†)	Channel 3 (585/610†)	Channel 4 (625/665†)
Duplex	6-FAM	HEX JOE VIC Yakima Yellow‡		
Duplex	6-FAM		Texas Red ROX	
Duplex	6-FAM			Cy5
Triplex	6-FAM	HEX JOE VIC	Texas Red ROX	
Triplex	6-FAM	HEX JOE VIC		Cy5
Triplex	6-FAM		ROX§ Cy3.5§	Cy5
4plex	6-FAM	HEX JOE VIC	ROX§	Cy5

* Reporter dyes marked in bold are available from Operon Biotechnologies.

† The numbers indicate the wavelengths of the excitation light source and the detection filter.

‡ QuantiTect Endogenous Control Assays from QIAGEN use QuantiProbes carrying a Yakima Yellow fluorophore, which can be detected by channel 2 (the JOE channel).

§ We do not recommend using channel 3 to detect Texas Red dye if channel 4 is also being used. This is because Texas Red dye is partly detected by channel 4.

LC 2.0

Table 8. Suitable Combinations of Reporter Dyes for Multiplex Analysis Using the LightCycler 2.0 System*

Type of assay†	Detection channel 1 (530 nm filter)	Detection channel 2 (560 nm filter)	Detection channel 3 (610 nm filter)
Duplex	6-FAM	HEX JOE VIC Yakima Yellow‡	
Duplex	6-FAM		Texas Red ROX
Triplex	6-FAM	HEX JOE VIC	Texas Red ROX

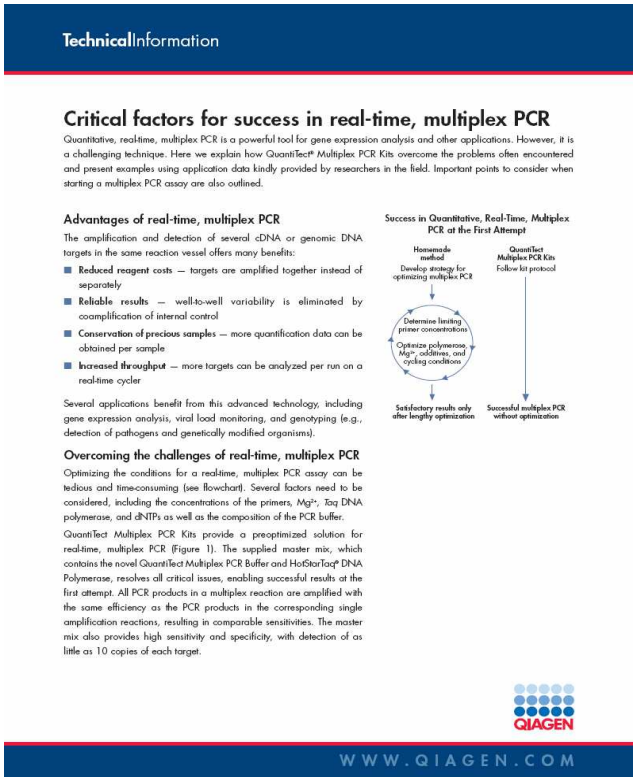
* Reporter dyes marked in bold are available from Operon Biotechnologies.

† A supplementary protocol on performing 4plex assays using the LightCycler 2.0 system is available at www.qiagen.com/goto/qmpcr.

‡ QuantiTect Endogenous Control Assays from QIAGEN use QuantiProbes carrying a Yakima Yellow fluorophore, which can be detected using the 560 nm filter.

…and many more!

Further Information



TechnicalInformation

Critical factors for success in real-time, multiplex PCR

Quantitative, real-time, multiplex PCR is a powerful tool for gene expression analysis and other applications. However, it is a challenging technique. Here we explain how QuantiTect® Multiplex PCR Kits overcome the problems often encountered and present examples using application data kindly provided by researchers in the field. Important points to consider when starting a multiplex PCR assay are also outlined.

Advantages of real-time, multiplex PCR

The amplification and detection of several cDNA or genomic DNA targets in the same reaction vessel offers many benefits:

- **Reduced reagent costs** — targets are amplified together instead of separately
- **Reliable results** — well-to-well variability is eliminated by coamplification of internal control
- **Conservation of precious samples** — more quantification data can be obtained per sample
- **Increased throughput** — more targets can be analyzed per run on a real-time cycler

Several applications benefit from this advanced technology, including gene expression analysis, viral load monitoring, and genotyping (e.g., detection of pathogens and genetically modified organisms).

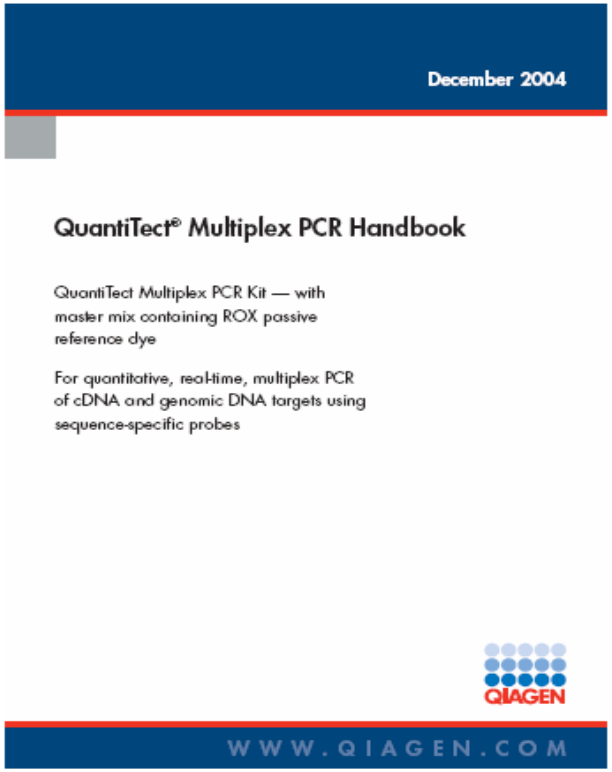
Success in Quantitative, Real-Time, Multiplex PCR at the First Attempt

Overcoming the challenges of real-time, multiplex PCR

Optimizing the conditions for a real-time, multiplex PCR assay can be tedious and time-consuming (see flowchart). Several factors need to be considered, including the concentrations of the primers, Mg^{2+}, Taq DNA polymerase, and dNTPs as well as the composition of the PCR buffer.

QuantiTect Multiplex PCR Kits provide a preoptimized solution for real-time, multiplex PCR (Figure 1). The supplied master mix, which contains the novel QuantiTect Multiplex PCR Buffer and HotStarTaq® DNA Polymerase, resolves all critical issues, enabling successful results at the first attempt. All PCR products in a multiplex reaction are amplified with the same efficiency as the PCR products in the corresponding single amplification reactions, resulting in comparable sensitivities. The master mix also provides high sensitivity and specificity, with detection of as little as 10 copies of each target.

WWW.QIAGEN.COM



December 2004

QuantiTect® Multiplex PCR Handbook

QuantiTect Multiplex PCR Kit — with master mix containing ROX passive reference dye

For quantitative, real-time, multiplex PCR of cDNA and genomic DNA targets using sequence-specific probes

WWW.QIAGEN.COM

http://www1.qiagen.com/products/pcr/quantitativepcr

Novel Multiplex Real-Time PCR Chemistry

- Multiplex PCR buffer with Synthetic Factor MP
  - Increases local primer concentration at template cDNA or DNA
  - Improves hybridization efficiency of potentially suboptimal primers and probes
- Default cycling parameters & protocols for optimal performance
- No tedious optimization required
  → “Plug & Play” master mix reagent
- High sensitivity and reproducibility
- Works with various dual-labeled probes on all cyclers

Multiplex real-time PCR WAS difficult !

Thank You For Your Attention !

