Novel Amplification & Detection Chemistries

Dirk Löffert, Director R&D Modification/Amplification

dirk.loeffert@qiagen.com

March 5th, 2004

Presentation Overview: Amplification

- Importance of Reaction Chemistry on Real-time PCR Specificity
  - Technology principle
- PCR Specificity & Quantitation Accuracy
  - Effect of specificity on assay efficiency
  - Effect of efficiency on quantitation accuracy using $\Delta\Delta C_T$ method
- Enabling Quantitative Multiplex Real-time PCR

Presentation Overview: Detection

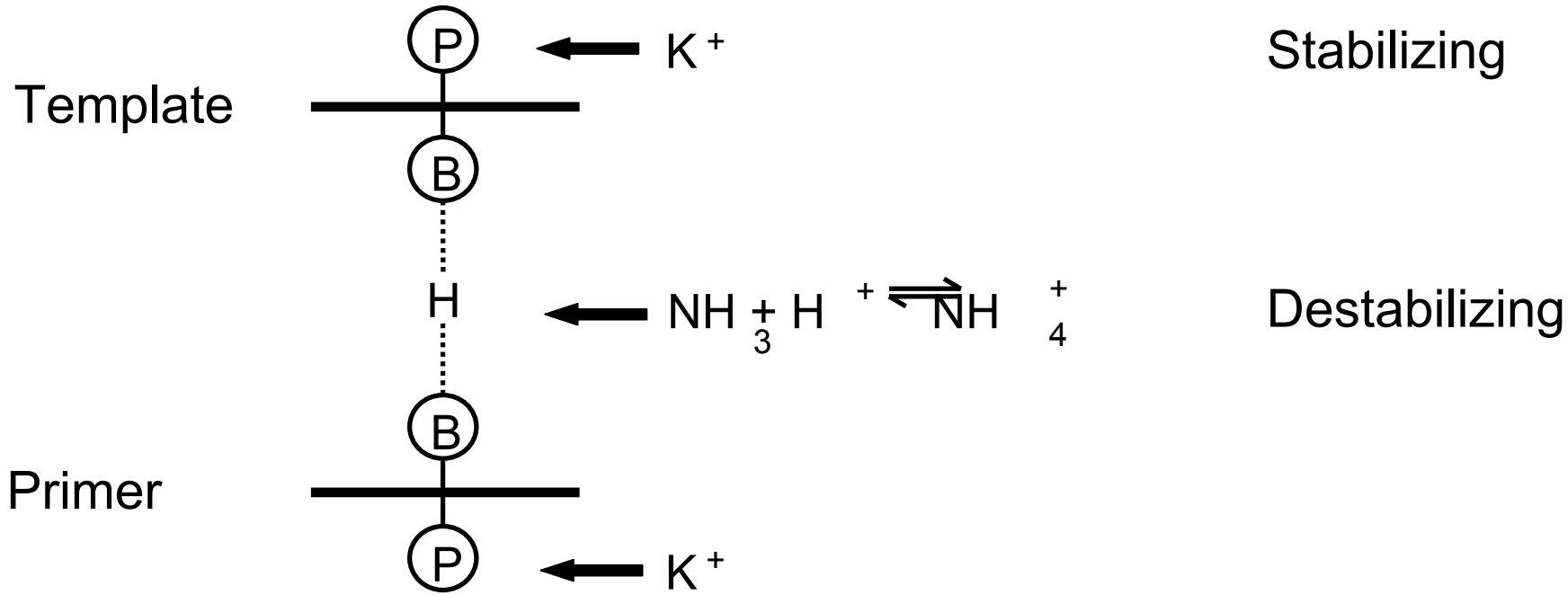
- Functionally Validated QuantiTect Gene Expression Assays
  - Bioinformatics
  - Functional Validation
  - Verification
  - Assay Manufacturing
  - Assay Database
- Novel Primer/Probe Technology
  - Working Principle
  - Prerequisites for Assay Design at Difficult Sequences; e.g. Splice Sites
    - Effect of Minor Grove Binder & Superbases
- Simple Assay Design with WEB-based Free Design Software
  - QuantiTect Custom Assays

Key Technologies - Amplification

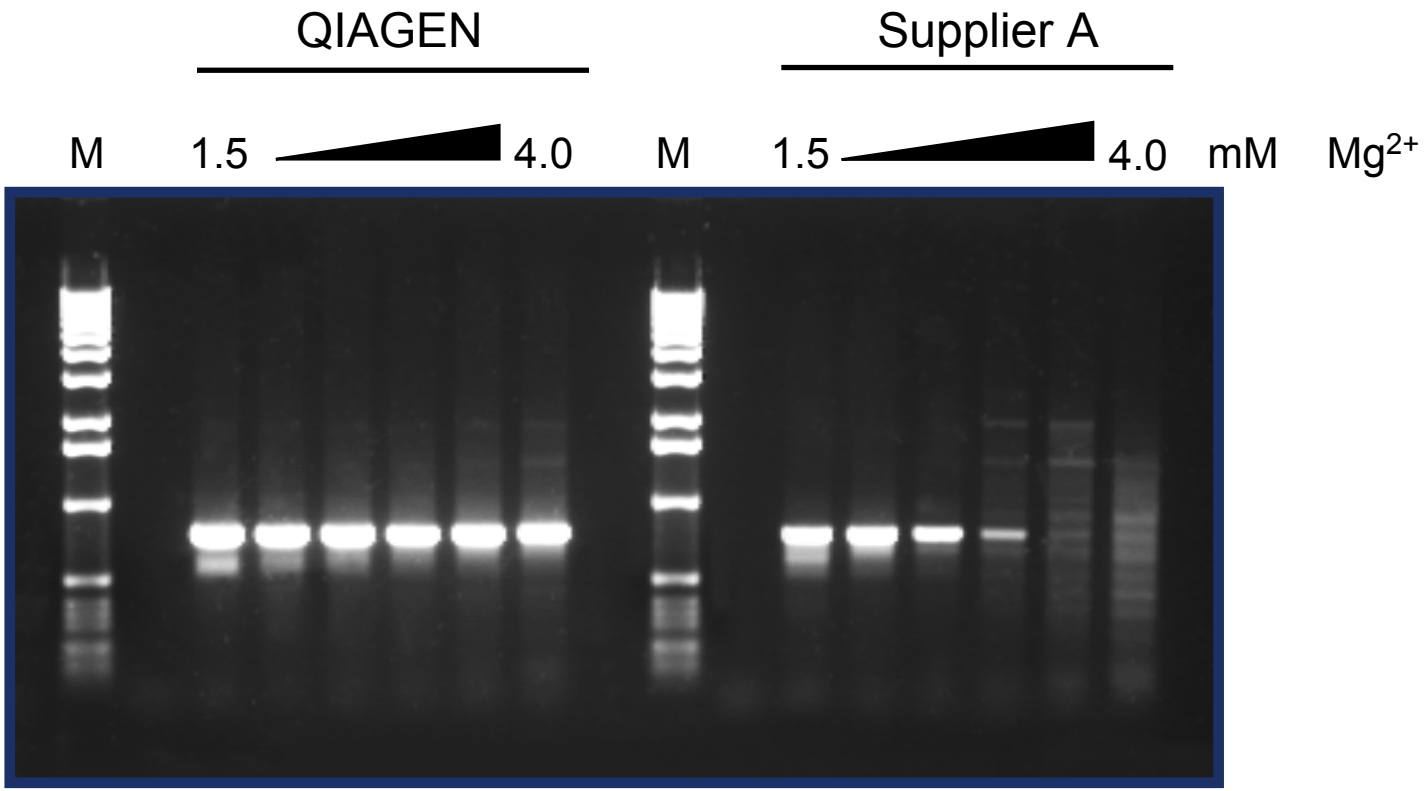
- Novel Reaction Buffering Chemistries for PCR
  - Higher PCR specificity due to:
    - Increased primer annealing specificity in each PCR cycle
    - Less dependent on other parameters such as temperature & Mg^{2+} concentration

- HotStarTaq DNA Polymerase
  - Unique chemical modification of recombinant *Taq* DNA Polymerase
  - Becomes active by initial heat incubation step
  - Robust reactivation independent from PCR environment (e.g. sample pH)

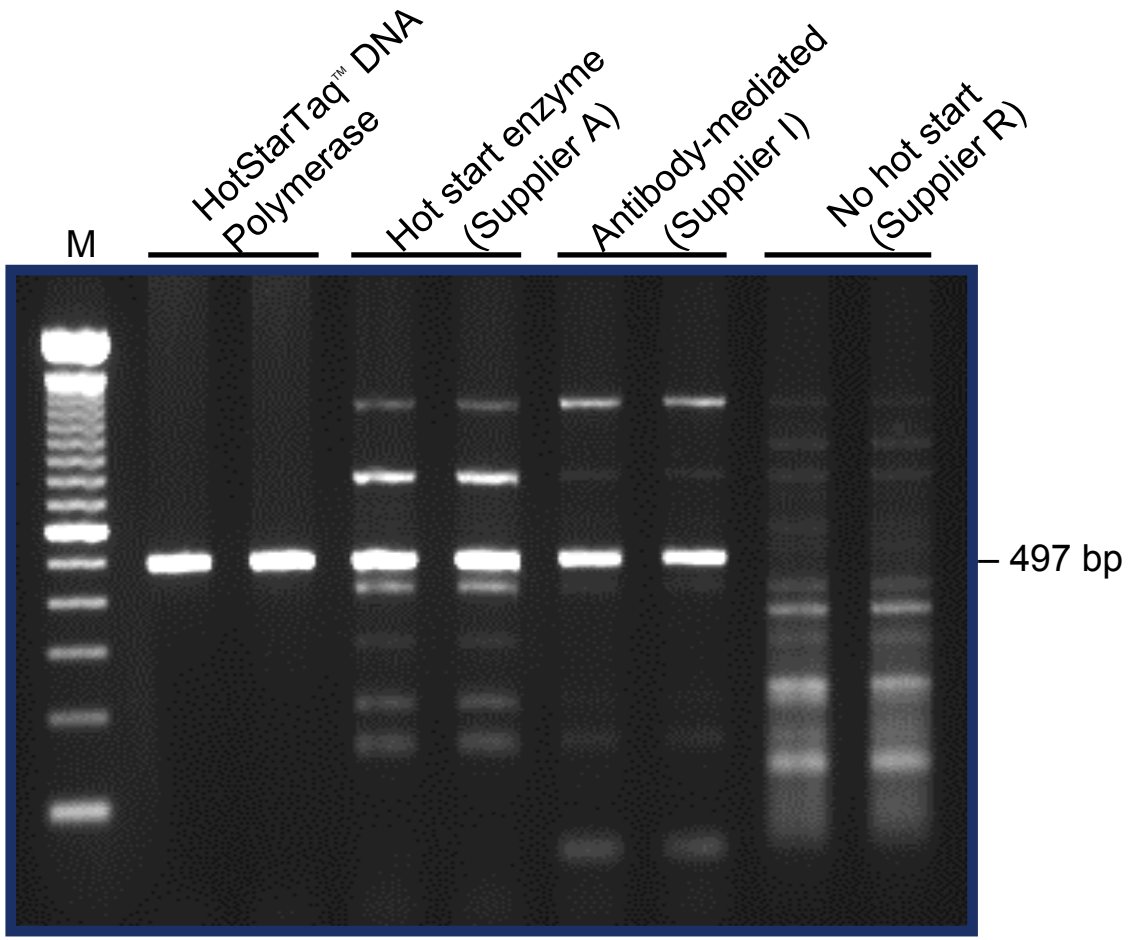
Effects of Cations in PCR Buffer



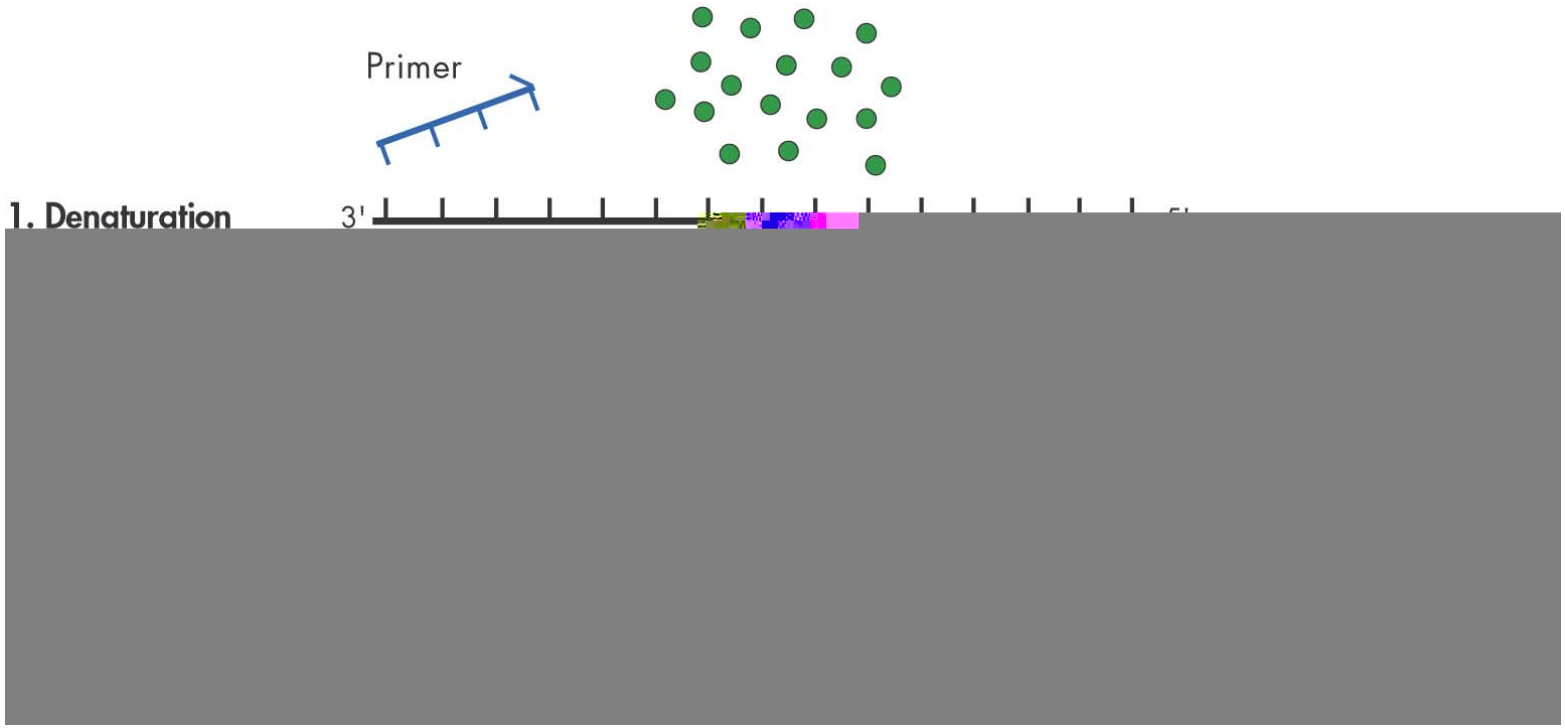
NH_4^+ Buffers Destabilize Non-Specifically Bound Primers



Combined Effect of Hot Start Methods & PCR Buffers

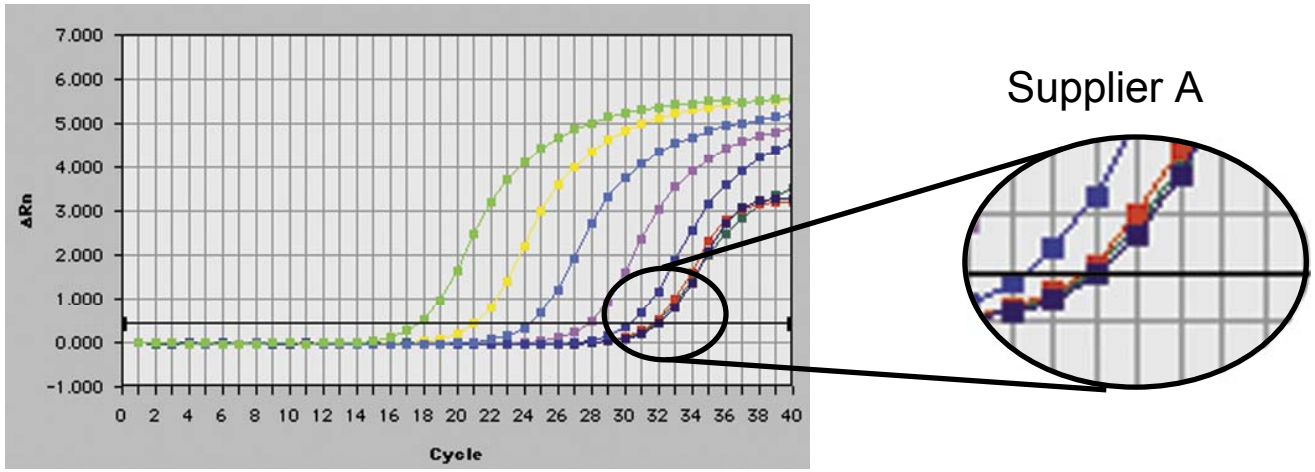


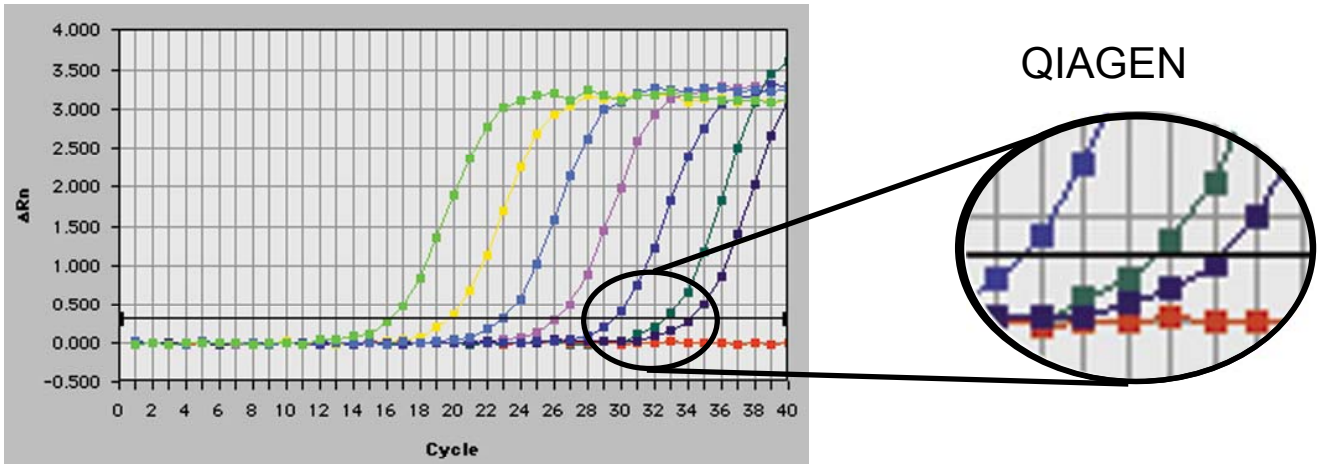
SYBR Green Detection



→ Detection of specific & non-specific PCR products

Effect of Specificity on SYBR Green Real-Time PCR





Copies

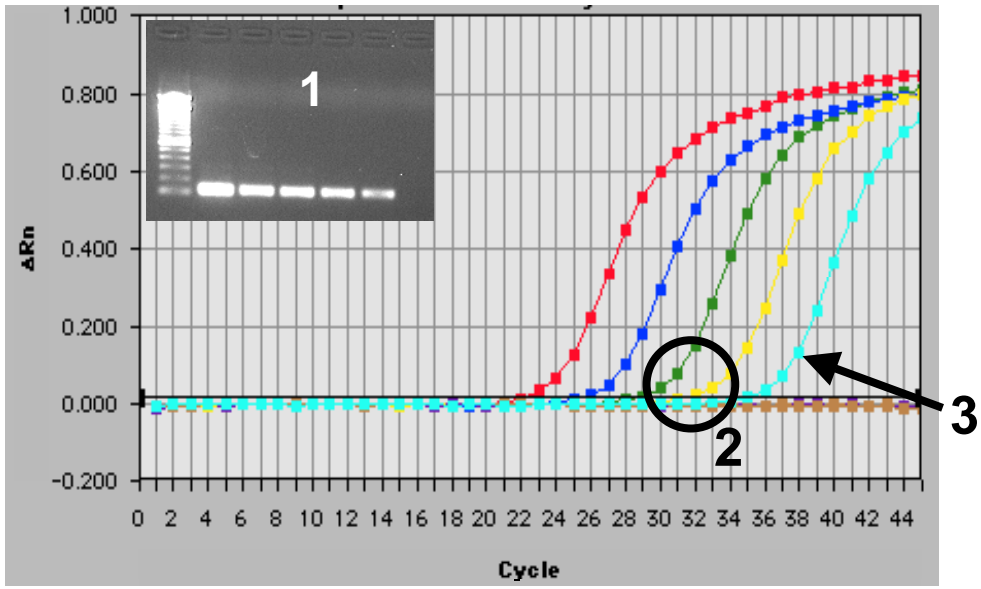
- 10^2
- 10
- 5
- 0

PCR Specificity & Quantitation Accuracy

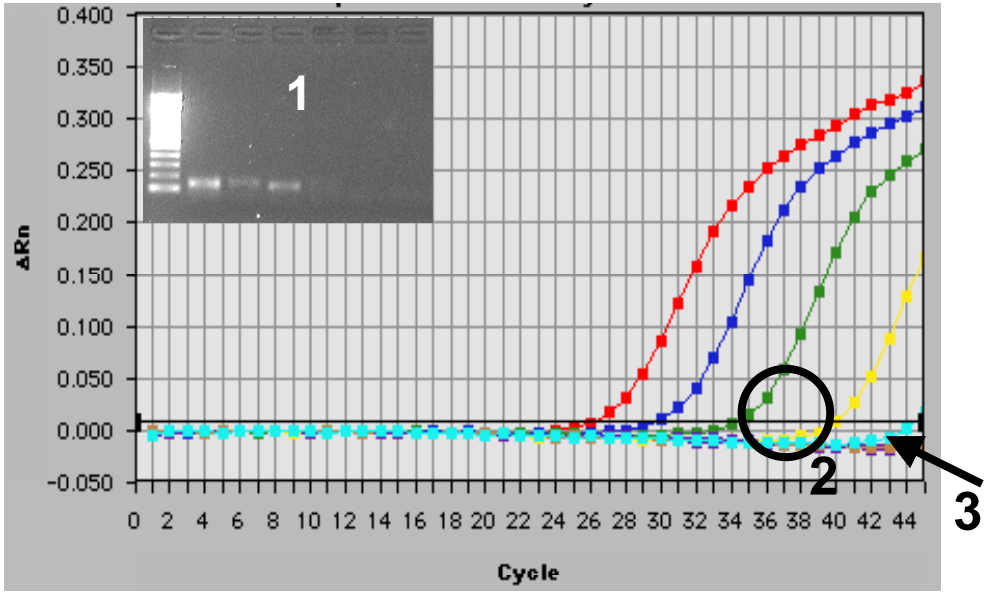
- Effect of PCR Specificity on:
  - Uniformity of C_T distances for template dilutions
  - Real-time PCR assay sensitivity
  - Real-time PCR efficiency
    - Effect on quantitation accuracy using $\Delta\Delta C_T$ method

Improved PCR Efficiency & Specificity Using Probes

QIAGEN



Supplier A



1: Specific PCR Product

2: Uniform C_T distance (ca 3.3) for 10fold dilution

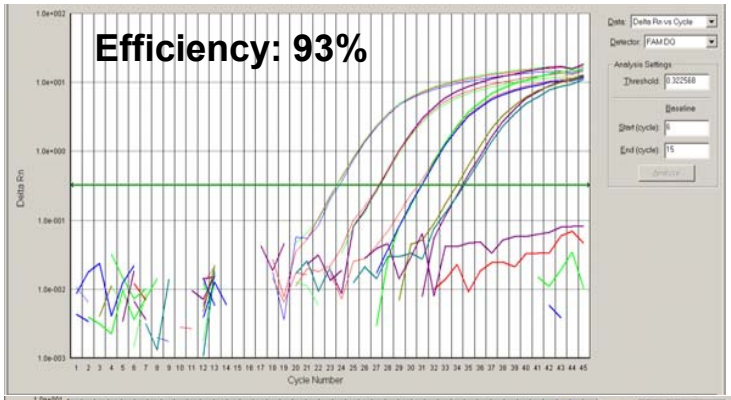
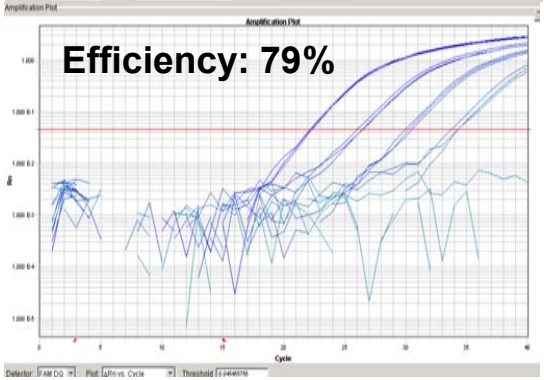
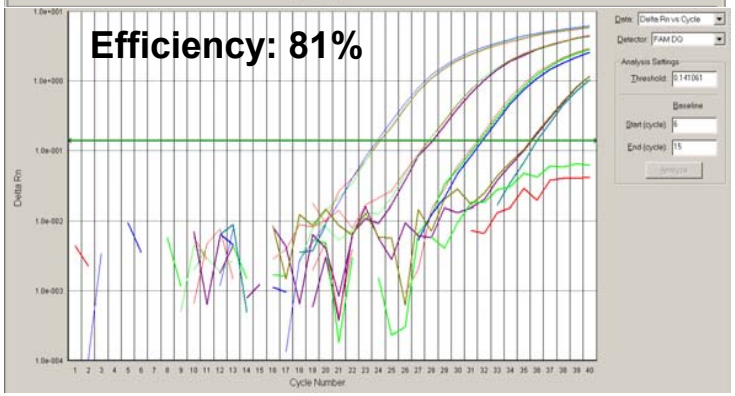
3: All dilutions amplified

1: Non-specific PCR product / low yield

2: Non-uniform C_T distance for 10fold dilution

3: Lowest template amount not detected

Specificity Can Improve PCR Efficiency of Assays

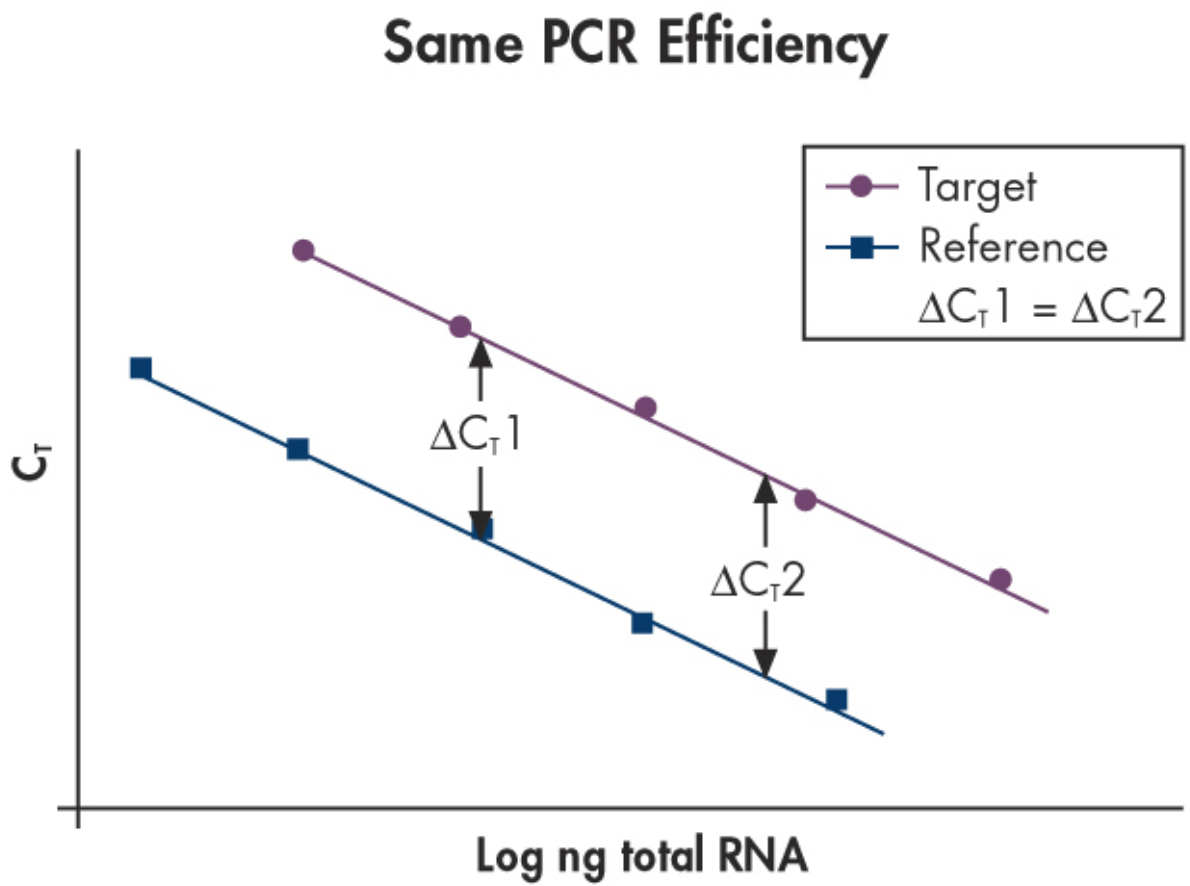
	Gene A	Gene B
QIAGEN		
Supplier A		
	ABI SDS 7900	**ABI SDS 7000**

Example for Analysis Using $\Delta\Delta C_T$ Method

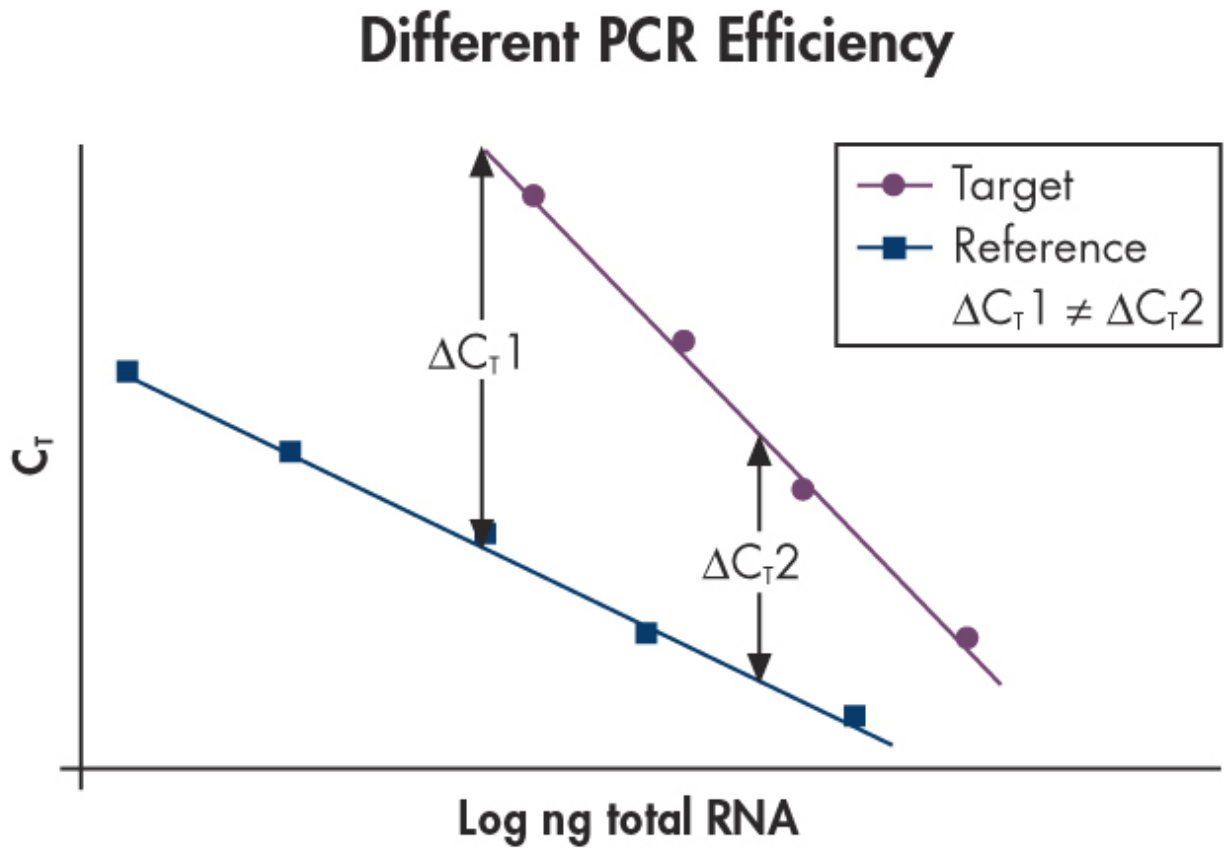
- Does not employ use of standard curves
- Compares ΔC_T of unknown sample (e.g treated cells or diseased tissue)
  - *Gene of interest* (e.g. TNF) and *endogenous reference gene* (e.g. HPRT)
- with ΔC_T of calibrator sample (non-treated control sample)

Sample	Average C_T TNF	Average C_T HPRT	ΔC_T TNF – HPRT	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_T$ calibrator	Expression of TNF compared to calibrator ($2^{-\Delta\Delta C_T}$)
Untreated (calibrator sample)	36.5	22.8	13.7	0	1
PMA	31.0	23.1	7.9	–5.8	55.7

Pre-Requisite: Same Real-time PCR Assay Efficiency



Difference in PCR Efficiencies Affect Accuracy of $\Delta\Delta C_T$ Method



Impact of Efficiency on Quantitation ($\Delta\Delta C_T$)

- PCR efficiency: $E = 10^{(-1/S)} - 1$
  - Calculated from slope (S) of standard curve
- Error in (%) = $[(2^n/(1+E)^n \times 100)] - 100$
- Example:
  - Difference of 10% (0.9 versus 1.0) results in 261% error at C_T 25

    → Estimated expression level about 3.6fold lower than calculated
- Due to High PCR Specificity, QuantiTect Kits:
  - Achieve High PCR efficiency
  - Contribute to accuracy of quantitation

A Challenge: Multiplex Quantitative Real-time PCR

- Multiplex Quantitative Real-time PCR Offers
  - Higher reliability through in-tube quantitation control
  - Saves sample material
  - Reduces time for reaction setup
  - Reduces reagent costs

- Common Problems with Multiplex Quantitative Real-time PCR
  - Usually tedious optimization required, e.g. primer limitation because of:
    - Preferred amplification of the more abundant transcript

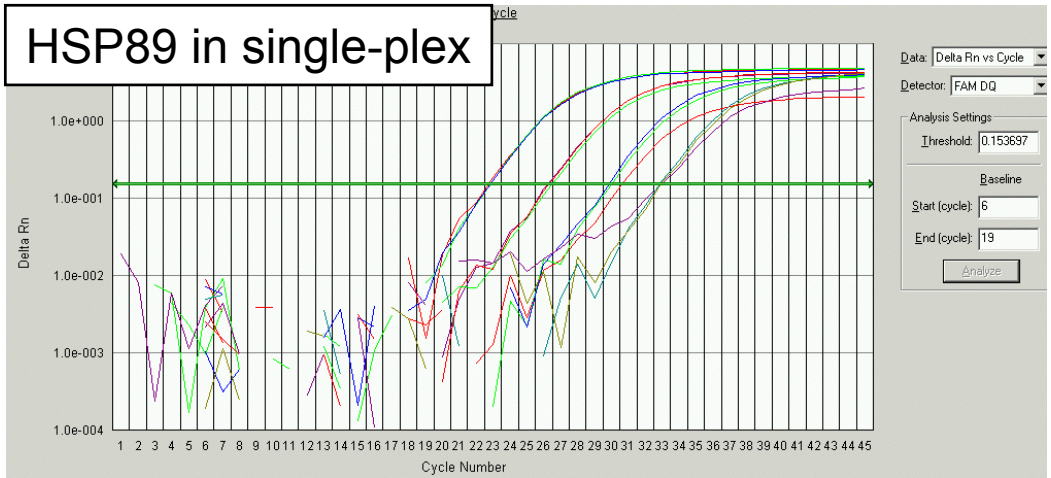
      → Reduced sensitivity

      → Smaller linear range

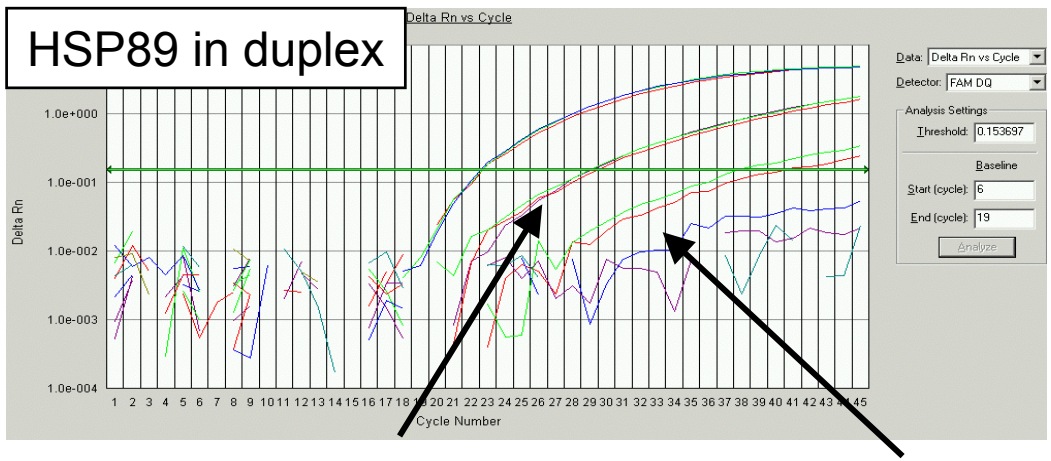
      → C_T of „single-plex“ and duplex reactions vary

      → Inaccurate quantitation

Common Problems in Multiplex PCR



- Detection of HSP89 in various cDNA amounts:
  - 10 ng, 1 ng, 100 pg, 10 pg



- Detection of HSP89 in various cDNA amounts:
  - 10 ng, 1 ng, 100 pg, 10 pg
  - plus 10^6 copies reference gene

Shifted (higher) C_T values

Low HSP89 transcript levels not detected

Effect of Novel Multiplex Real-time PCR Chemistry

	QIAGEN Novel Multiplex Reagent		**Supplier A**	
cDNA	Single	Duplex	Single	Duplex
10ng	22,17	21,23	22,76	22,60
1ng	25,55	24,71	26,30	29,18
100pg	28,71	28,53	30,14	39,20
10pg	32,06	34,49	32,89	45,00

Novel Multiplex Real-time PCR Chemistry

- Especially optimized reaction chemistry (adaption of HotStarTaq & multiplex PCR buffer) solves multiplex real-time PCR problems
  - Default cycling parameters & protocols for various cyclers
  - No tedious optimization required such as primer limitation
    - “Plug & Play” master mix reagent
  - Works with different dual-labeled probes such as TaqMan probes and QuantiProbes on most cyclers

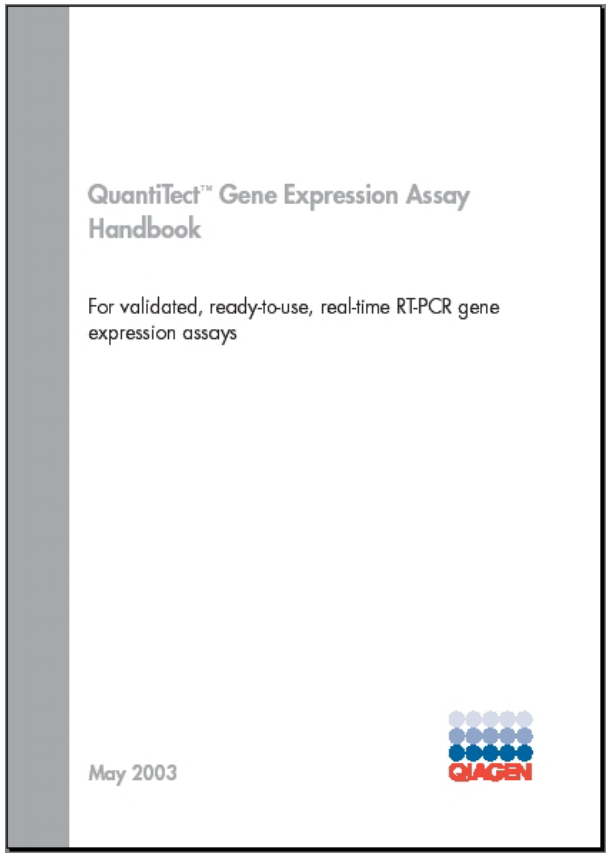
→ Will be launched in second half of 2004

QuantiTect SYBR Green & Probe Kits

- High PCR specificity through hot start and cations in buffer
  → High assay sensitivity & linearity
  → Accurate quantitation
- Kit formats for 2step- & 1step RT-PCR available
- Optimized for use with any real-time cycler
- Optimized for use of any sequence-specific probe type (QuantiTect Probe kits)
- New QuantiTect Multiplex PCR Kit will be launched second half of 2004

QuantiTect Gene Expression Assays

- QuantiTect Gene Expression Assays
  - Cost- & time saving
  - Functionally validated ready-to-use assays
  - Validated with QuantiTect Probe Kits
  - 10x Primer/Probe mix in single tube
  - For RNA detection only
  - For all real-time cyclers
  - Online search tool


QuantiTect™ Gene Expression Assay Handbook

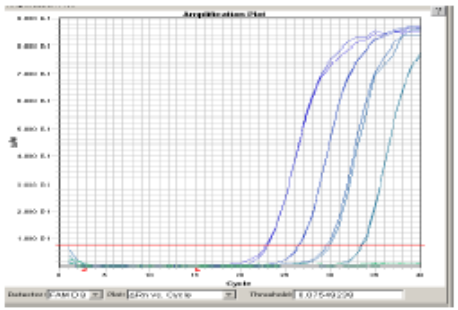
For validated, ready-to-use, real-time RT-PCR gene expression assays

May 2003

QuantiTect Gene Expression Assay Validation

Step 1:
Bioinformatics
e.g. RefSeq Database
RNA specific design
Homology search
etc.

Step 2: Functional Validation

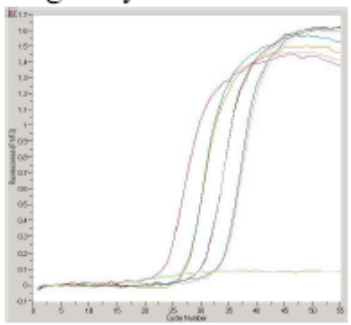


Validation of Mm_Runx1 Gene Expression Assay on ABI Prism 7900 with log dilutions of mouse liver cDNA (blue), genomic DNA (green) and non template controls.

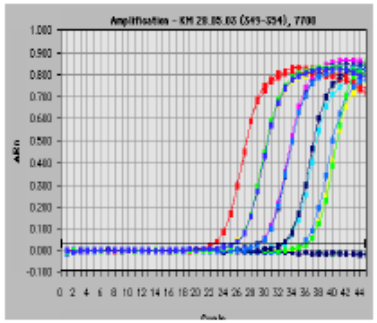
Specification	Passed
High Efficiency	√
Detection of 10 copies	√
No Detection of genomic DNA	√
Linearity (C_T - template amount)	√
One-Step RT-PCR functionality	√

Step 3: Verification

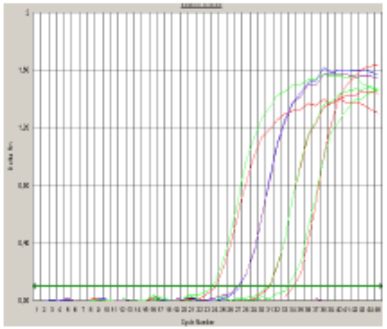
Light Cycler



ABI Prism 7700



ABI Prism 7000



Step 4:
Manufacturing
Quality Control
Analytical QC
Functional QC in
real-time PCR assay

QuantiTect Gene Expression Assay Search Tool

Search for QuantiTect Gene Expression Assays

You may combine up to 5 single words in each field, separated by blank space.

More search help

		Example	
Gene name:		*phosphoglycerate kinase*	Help
Gene symbol:		*PGK1*	Help
RefSeq ID:		*NM_000291*	Help
Species:	Human		
Keywords:	interleukin	*anemia housekeeping transferase*	Help

Search

View all assays | Design your own assays

QuantiTect Gene Expression Assay Search Tool

Search for QuantiTect Gene Expression Assays

Your search for:

Keyword: interleukin
AND **Species:** Human

produced **6** results. Showing **1** - **6**:

Add to cart	Assay name	Dye label	Gene name	Gene symbol	Species	Locus ID at NCBI	Cat. No.	Price
☐	Hs_IL12A_Assay (100)	FAM	interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	IL12A	Human	3592	241047	$140.00
☐	Hs_IL12B_Assay (100)	FAM	interleukin 12B	IL12B	Human	3593	241049	$140.00
☐	Hs_IL1RN_Assay (100)	FAM	interleukin 1 receptor antagonist	IL1RN	Human	3557	241055	$140.00
☐	Hs_IL4_Assay (100)	FAM	interleukin 4	IL4	Human	3565	241039	$140.00
☐	Hs_IL6_Assay (100)	FAM	interleukin 6 (interferon, beta 2)	IL6	Human	3569	241041	$140.00
☐	Hs_IL8_Assay (100)	FAM	interleukin 8	IL8	Human	3576	241043	$140.00

Add Checked Items to Cart

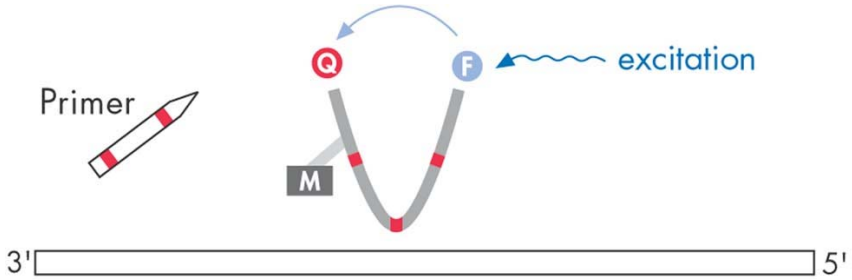
Back

Search again | View all assays | Design your own assays

QuantiProbe Technology

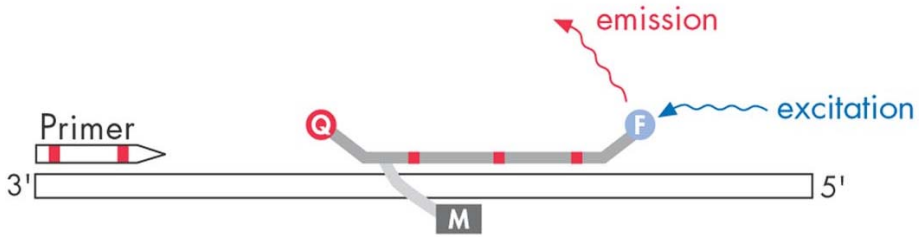
Denaturation

No signal



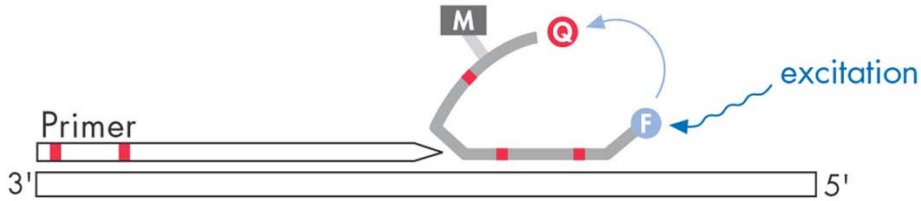
Annealing

Signal

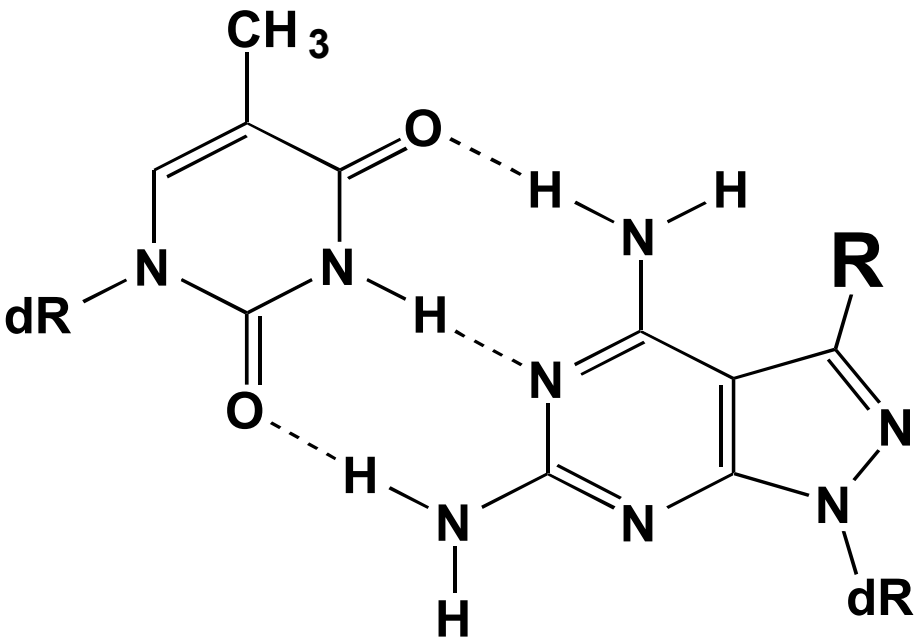


Extension

No signal



Super A Base Paired with T



Super A & T

- 3 Hydrogen Bonds instead of 2
- 2.5°C higher T_m
- Improves Hybridization
- More flexibility to place primers and/ore probes at pre-selected sequences

Courtesy of Epoch Biosciences

QuantiProbe Technology

- MGB and Superbases stabilize primer and probe binding
  - Allows to design shorter probes as well as primers
  - Offers more assay design flexibility when placing primer and/or probes at specific sites, e.g.
    - Splice sites
    - Select small sequence portion with minor homology to other gene family members
- Optimized for use with any real-time cycler with QuantiTect Probe Kits

QuantiTect Custom Assays

- QuantiTect Custom Assays
  - Simple & fast design
    - Express or custom mode
    - Design across splice sites for RNA specific detection
    - Easily exclude undesired sequences from design
    - Target probe at desired location
    - Superbases & MGB enable design for “difficult” sequences
  - Free WEB-based software
  - Ready-to-use 20x probe solution and 20x primer mix
  - Optimized with QuantiTect Probe Kits for any real-time cycler

QuantiTect Custom Assays – Sequence Input Format

Design your own QuantiTect Custom Assays

Step 1: Input nucleotide sequences

Design Mode: Express Custom

Design Method: Gene Expression

Input Type: Target Sequence(s) (in FastA format) Help

GI Number(s) Search GenBank for a GI Number

```
> Mm_Il12b
          1 agaaggaaca gtgggtgtcc aggcacatca
gaccaggcag ctcgcagcaa agcaaggtaa
         61 gttctctcct cttccctgtc gctaactccc
tgcatctaga ggctgtccag attcagactc
        121 caggggacag gctacccctg aaccaggcag
cgtgggagtg ggatgtgtcc tcagaagcta
        181 accatctcct ggtttgccat cgttttgctg
gtgtctccac tcatggccat gtgggagctg
        241 gagaaagacg tttatgttgt agaggtggac
tggactcccg atgcccctgg agaaacagtg
        301 aacctcacct gtgacacgcc tgaagaagat
gacatcacct ggacctcaga ccagagacat
        361 ggagtcatag gctctggaaa gaccctgacc
```

Continue >>

Sequence - FastA Format

Design your own QuantiTect Custom Assays

Step 1: Input nucleotide sequences

Design Mode: Express Custom

Design Method: Gene Expression

Input Type: Target Sequence(s) (in FastA format) Help

GI Number(s) Search GenBank for a GI Number

6680396

Continue >>

GenBank Identifier - GI Number

QuantiTect Custom Assay Software
Data Input Sequence FastA & Splice Site

Design your own QuantiTect Custom Assays

Step 1: Input nucleotide sequences

Design Mode: (•) Express ( ) Custom

Design Method: Splice Site

Input Type: (•) Target Sequence(s) (in FastA format) Help

( ) GI Number(s) Search GenBank for a GI Number

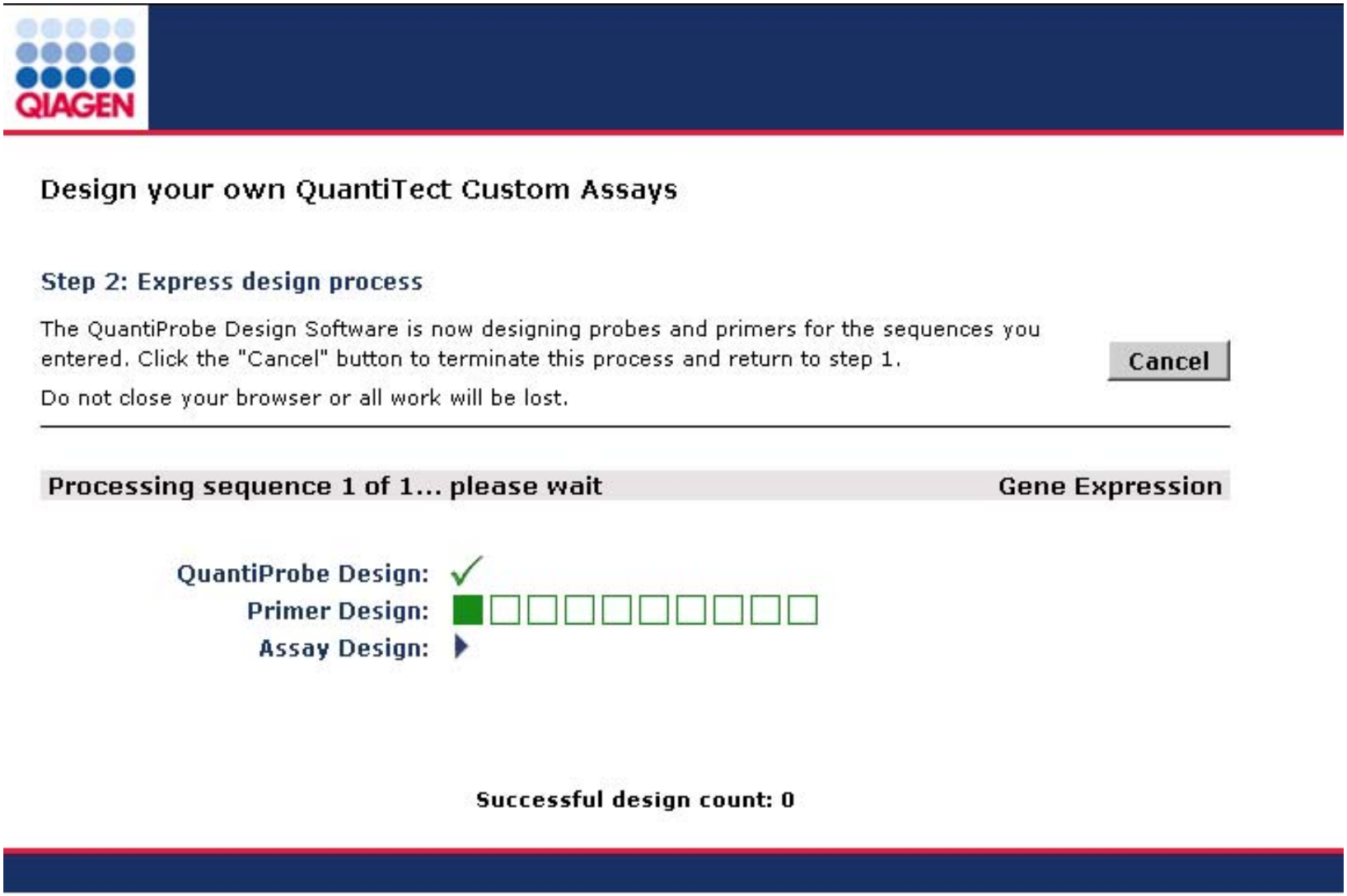
```
>Hs_BRCA1/NM_007294

GGCAGTTTGTAGGTCGCGAGGGAAGCGCTGAGGATCAGGAAGGGGG
CACTGAGTGTCCGTGGGGGAATCCTCGTGATAGGAACTGGAATATG
CCTTGAGGGGGACACTATGTCTTTAAAAACGTCGGCTGGTCATGAG
GTCAGGAGTTCCAGACCAGCCTGACCAACGTGGTGAAACTCCGTCT
CTACTAAAAATACAAAAATTAGCCGGGCGTGGTGCCGCTCCAGCTA
CTCAGGAGGCTGAGGCAGGAGAATCGCTAGAACCCGGGAGGCGGAG
GTTGCAGTGAGCCGAGATCGCGCCATTGCACTCCAGCCTGGGCGAC
AGAGCGAGACTGTCTCAAAACAAAACAAAACAAAACAAAACAAAAA
ACACCGG[]
GTTCATTGGAACAGAAAGAAATGGATTTATCTGCTCTTCGCGTTGA
AGAAGTACAAAATGTCATTAATGCTATGCAGAAAATCTTAGAGTGT
CCCATCTG[]
```

Continue >>

[ ] - indicating splice site

QuantiTect Custom Assays - Design



QuantiTect Custom Assay Software
Result: One Optimal QuantiProbe / Primer Set

Design your own QuantiTect Custom Assays

Step 2: Express design process

Processing sequence 1 of 1... please wait

QuantiProbe Design: ✓
Primer Design:
Assay Design:

Successful design count: 0

Design your own QuantiTect Custom Assays

Final step: Review assays / Add to cart

Check the details of the assays listed on this page.

After checking the details of the assays:

- Click here to export the details of the assays as a CSV file.
- Click "Add to Cart" to add all assays listed to the shopping cart.

Delete	Assay Name	Method/Mode	Price
☐	Hs_BRCA1/NM_007294	Splice Site/Express	
	Edit Assay Name		

Oligo	T_m	GC%	Length	5' Pos	3' Pos	Sequence
Forward Primer	60.9	38	21	594	614	GATATAACCAAAAGGAGCCTA
Reverse Primer	65.9	48	21	700	680	GCATACTCCAAACCTGTGTCA
Probe 1	65.85	38	16	624	639	ACGAGAT*T*T*A*GT*CAAC

Scale	Reactions (Help)	Price
◉ Small	500	
○ Medium	1,500	
○ Large	5,000	

Delete Number of assays: 1 Total:

Design Another Assay Add To Cart >>

Hybridisation Display of Assay Location

Hybridization Display

```
  1 GGCAGTTTGT AGGTCGCGAG GGAAGCGCTG AGGATCAGGA AGGGGGCACT GAGTGTCCGT
    CCGTCAAACA TCCAGCGCTC CCTTCGCGAC TCCTAGTCCT TCCCCCGTGA CTCACAGGCA

 61 GGGGGAATCC TCGTGATAGG AACTGGAATA TGCCTTGAGG GGGACACTAT GTCTTTAAAA
    CCCCCTTAGG AGCACTATCC TTGACCTTAT ACGGAACTCC CCCTGTGATA CAGAAATTTT

121 ACGTCGGCTG GTCATGAGGT CAGGAGTTCC AGACCAGCCT GACCAACGTG GTGAAACTCC
    TGCAGCCGAC CAGTACTCCA GTCCTCAAGG TCTGGTCGGA CTGGTTGCAC CACTTTGAGG

181 GTCTCTACTA AAAATACAAA AATTAGCCGG GCGTGGTGCC GCTCCAGCTA CTCAGGAGGC
    CAGAGATGAT TTTTATGTTT TTAATCGGCC CGCACCACGG CGAGGTCGAT GAGTCCTCCG

241 TGAGGCAGGA GAATCGCTAG AACCCGGGAG GCGGAGGTTG CAGTGAGCCG AGATCGCGCC
    ACTCCGTCCT CTTAGCGATC TTGGGCCCTC CGCCTCCAAC GTCACTCGGC TCTAGCGCGG

301 ATTGCACTCC AGCCTGGGCG ACAGAGCGAG ACTGTCTCAA AACAAAACAA AACAAAACAA
    TAACGTGAGG TCGGACCCGC TGTCTCGCTC TGACAGAGTT TTGTTTTGTT TTGTTTTGTT

361 AACAAAAAAC ACCGGGTTCA TTGGAACAGA AAGAAATGGA TTTATCTGCT CTTCGCGTTG
    TTGTTTTTTG TGGCCCAAGT AACCTTGTCT TTCTTTACCT AAATAGACGA GAAGCGCAAC

421 AAGAAGTACA AAATGTCATT AATGCTATGC AGAAAATCTT AGAGTGTCCC ATCTGTCTGG
    TTCTTCATGT TTTACAGTAA TTACGATACG TCTTTTAGAA TCTCACAGGG TAGACAGACC

481 AGTTGATCAA GGAACCTGTC TCCACAAAGT GTGACCACAT ATTTTGCAAA TTTTGCATGC
    TCAACTAGTT CCTTGGACAG AGGTGTTTCA CACTGGTGTA TAAAACGTTT AAAACGTACG

                                                            GATATAA
541 TGAAACTTCT CAACCAGAAG AAAGGGCCTT CACAGTGTCC TTTATGTAAG AATGATATAA
    ACTTTGAAGA GTTGGTCTTC TTTCCCGGAA GTGTCACAGG AAATACATTC TTACTATATT

    CCAAAAGGAG CCTA           ACGAGAT TTAGTCAAC
601 CCAAAAGGAG CCTACAAGAA AGTACGAGAT TTAGTCAACT TGTTGAAGAG CTATTGAAAA
    GGTTTTCCTC GGATGTTCTT TCATGCTCTA AATCAGTTGA ACAACTTCTC GATAACTTTT

661 TCATTTGTGC TTTTCAGCTT GACACAGGTT TGGAGTATGC AAACAGCTAT AATTTTGCAA
    AGTAAACACG AAAAGTCGAA CTGTGTCCAA ACCTCATACG TTTGTCGATA TTAAAACGTT
                        A CTGTGTCCAA ACCTCATACG

721 AAAAGGAAAA TAACTCTCCT GAACATCTAA AAGATGAAGT TTCTATCATC CAAAGTATGG
    TTTTCCTTTT ATTGAGAGGA CTTGTAGATT TTCTACTTCA AAGATAGTAG GTTTCATACC
```

Thank You For Your Attention !