Department of Molecular Biology

Iraqi Center for Cancer and Medical Genetics Research

Real Time PCR Training

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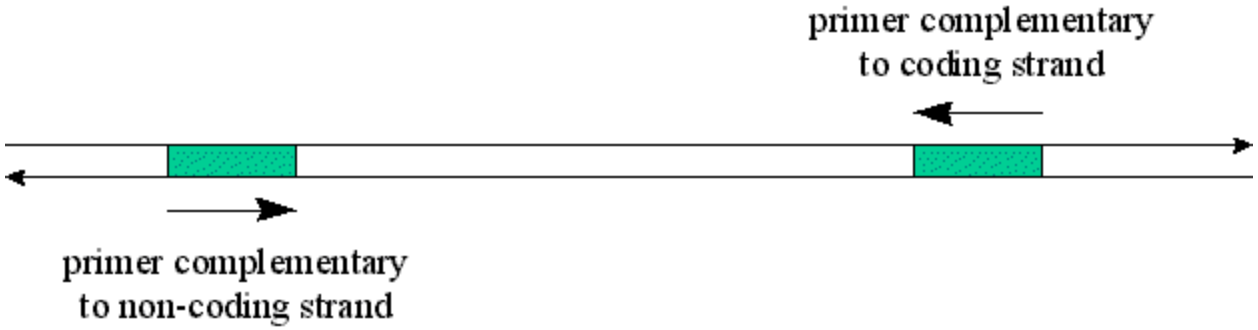
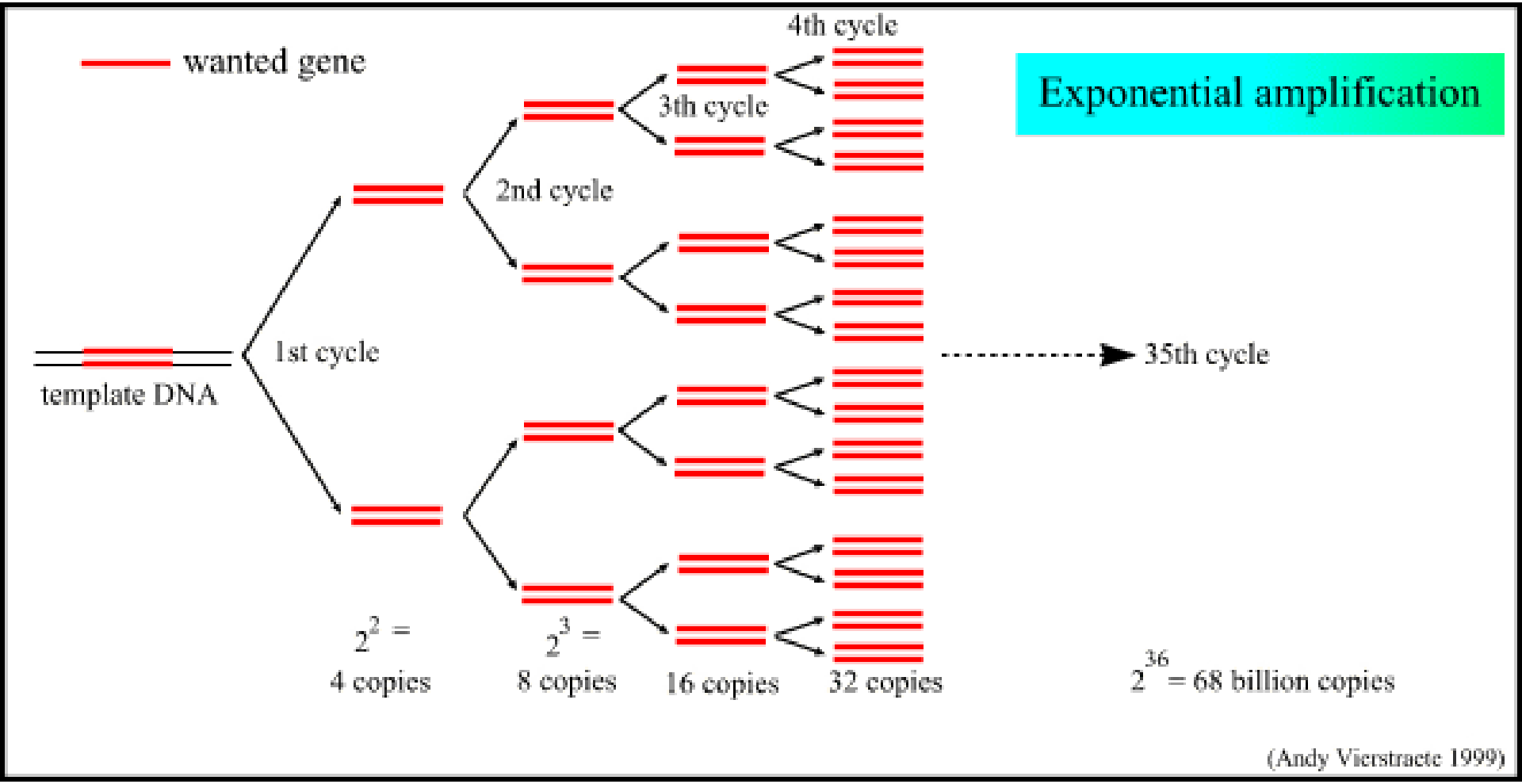
PCR - Polymerase Chain Reaction

- PCR is an *in vitro* technique for the amplification of a region of DNA which lies between two regions of known sequence.
- PCR amplification is achieved by using oligonucleotide primers.
  - These are typically short, single stranded oligonucleotides which are complementary to the outer regions of known sequence.



- The oligonucleotides serve as *primers* for DNA polymerase and the denatured strands of the large DNA fragment serves as the *template*.
  - This results in the synthesis of new DNA strands which are complementary to the parent template strands.
  - These new strands have defined 5' ends (the 5' ends of the oligonucleotide primers), whereas the 3' ends are potentially ambiguous in length.

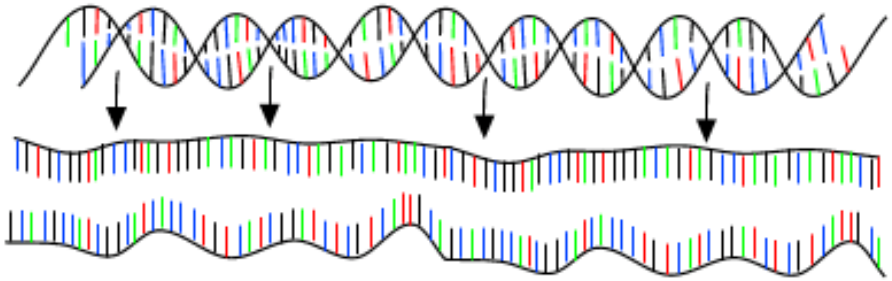


Exponential amplification

(Andy Vierstraete 1999)

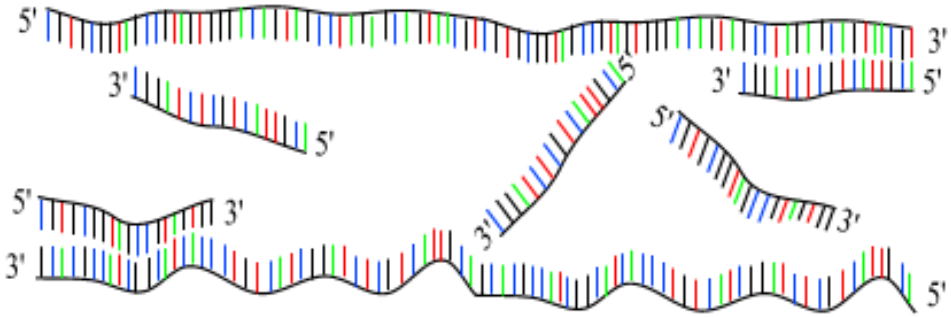
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

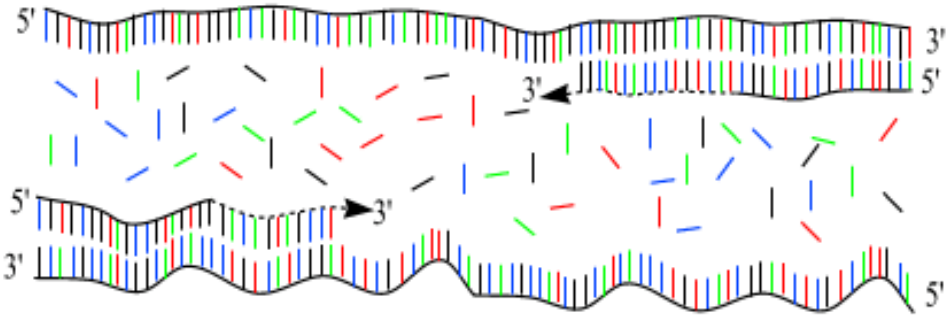
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



Step 3 : extension

2 minutes 72 °C

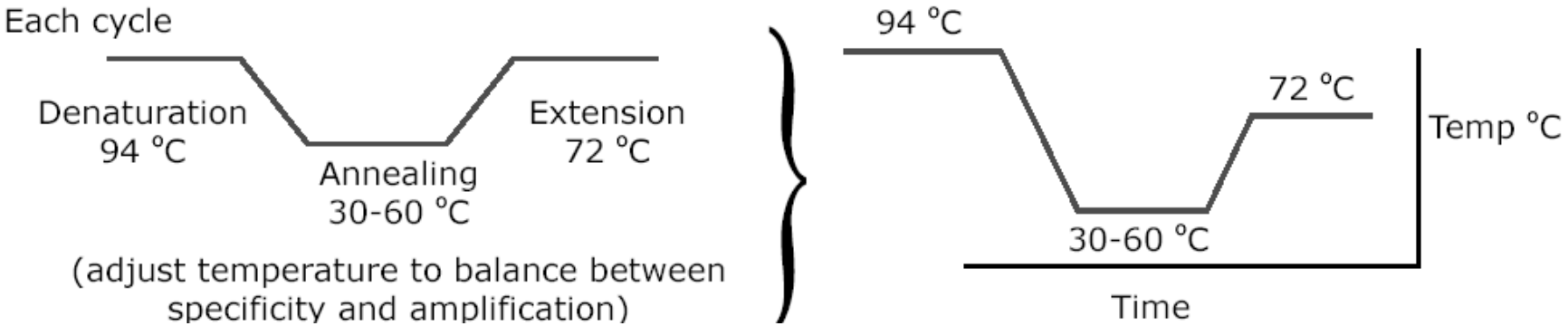
only dNTP's

(Andy Vierstraete 1999)

PCR reaction contains

- Target DNA (example: environmental DNA)
- 2 primers (20-30 nts long)
- Thermostable DNA polymerase
- Nucleotides (dNTPs)
- Mg^{2+} (cofactor for DNA polymerase)

Mix is subjected to temperature cycling



- http://ocw.mit.edu/NR/rdonlyres/Civil-and-Environmental-Engineering/1-89Fall-2004/321BF8FF-75BE-4377-8D74-8EEE753A328C/0/11_02_04.pdf

Primer selection

- **Primer is an oligonucleotide sequence – will target a specific sequence of opposite base pairing (A-T, G-C only) of single-stranded nucleic acids**

- For example, there is a
  - ¼ chance (4-1) of finding an A, G, C or T in any given DNA sequence; there is a
  - 1/16 chance (4-2) of finding any dinucleotide sequence (eg. AG); a
  - 1/256 chance of finding a given 4-base sequence.
- Thus, a **sixteen base sequence will statistically be present only once in every (=4 294 967 296, or 4 billion)**: this is about the size of the human or maize genome, and 1000x greater than the genome size of *E. coli*.

Primer Specificity

- Universal – amplifies ALL bacterial DNA for instance
- Group Specific – amplify all denitrifiers for instance
- Specific – amplify just a given sequence

Forward and reverse primers

- If you know the sequence targeted for amplification, you know the size which the primers should be anealing across
- If you don’t know the sequence… What do you get?

DNA Polymerase

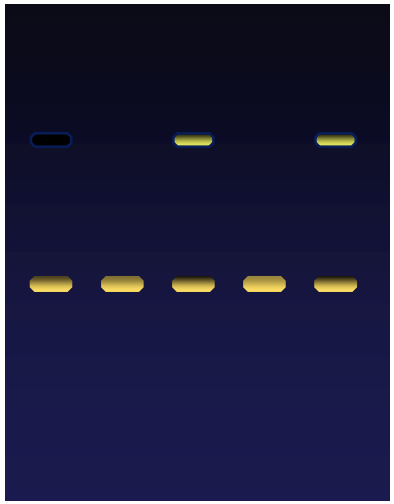
- DNA Polymerase is the enzyme responsible for copying the sequence starting at the primer from the single DNA strand
- Commonly use Taq, an enzyme from the hyperthermophilic organisms *Thermus aquaticus*, isolated first at a thermal spring in Yellowstone National Park
- This enzyme is heat-tolerant → useful both because it is thermally tolerant (survives the melting T of DNA denaturation) which also means the process is more specific, higher temps result in less mismatch – more specific replication

Published papers with ‘PCR’

- 1989 - 219
- 1990 – 496
- 1991 – 711
- 1992 – 906
- 1993 –1030
- 1994 – 857 (>4000)
- 1995 – 823
- 1996 – 796
- 1997 – 732
- 1998,10 - >73,000
- 1999,4 - >81,000
- 2000,10 – 121,305
- 2001,2 – 125,563
- 2002,3 – 149,572
- 2003,2 – 170,841
- 2004,2,23-195,193
- 2004,2,26-195,265
- 2006,3,22 - 255,788
- 2006/4/18 – 257,737
- 2007/3/9 – 283,607
- 2007/4/11 - 286,486

- 1. simple
- 2. powerful
  - A. sensitive – sensitivity
  - B. specific – specificity
  - C. reliable – reliability; fidelity
- 3. fast

What is Wrong with Agarose Gels?



* Poor precision
* Low sensitivity
* Short dynamic range < 2 logs
* Low resolution
* Non-automated
* Size-based discrimination only
* Results are not expressed as numbers
* Ethidium bromide staining is not very quantitative
* Same end-point results with different initial amount of template
* Different end-point results with the same initial amount of template

ABI: Real-Time PCR vs Traditional PCR

(www)

Real-Time PCR

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection

TheScientist tuesday
MAGAZINE OF THE LIFE SCIENCES EVERY DAY, ONLINE

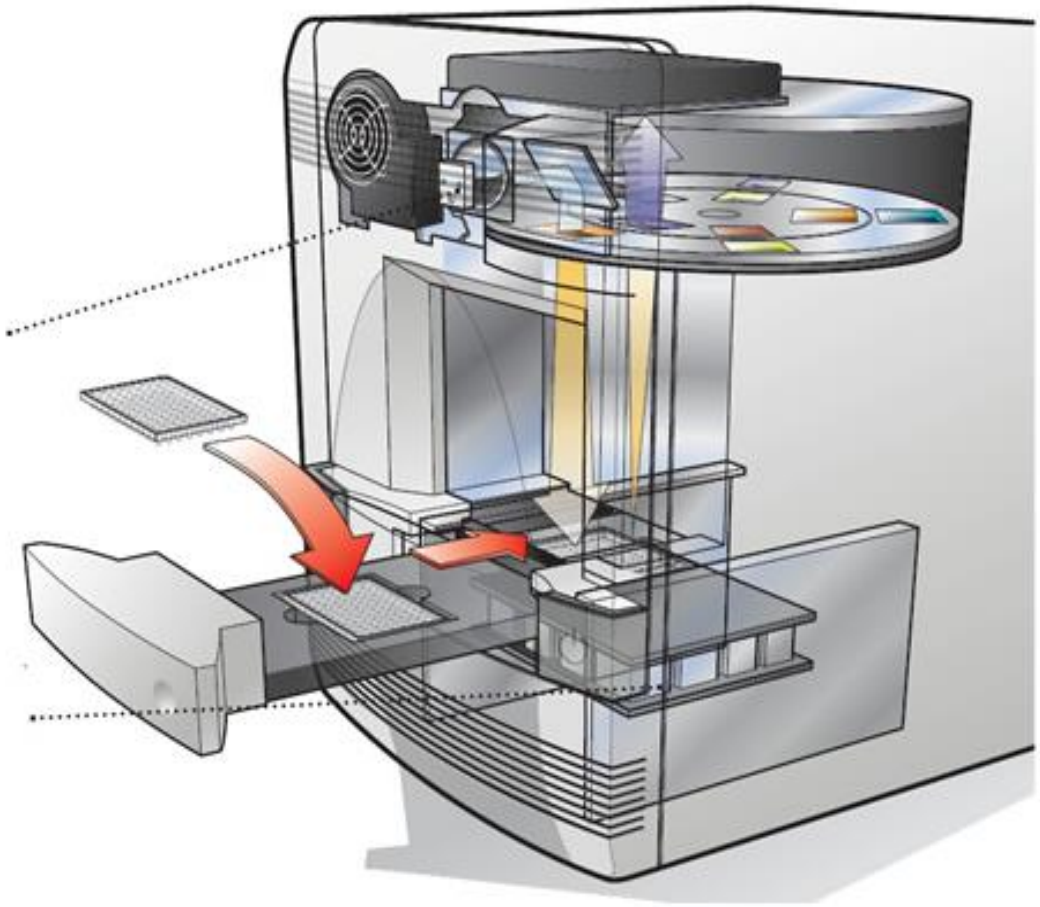
How it Works: Real Time PCR

Brendan Maher

The instrumentation is basic: a thermal cycler for amplification, a light source for excitation of fluorescent probes (see chemistries below), a camera for recording, and a computer to control the instrument and record data. Increasingly sophisticated instruments, such as those capable of multiplex experiments, are becoming affordable in academic labs.

The light source in the Applied Biosystems 7500 (represented here) is a simple halogen lamp shone through one of five different excitation filters over the entire sample. A CCD camera positioned above the sample records fluorescence from behind one of five emission fi lters. Some makes and models use a scanning head that moves over the plate, exciting and reading fluorescence in the wells individually.

Many qPCR instruments including the ABI 7500 use a Peltier element for heating and cooling. Peltier coolers use electron flow between semiconductor couples to heat or cool one side of a plate depending on the direction of current. Other systems use liquid or air fl ow or mechanical transition between blocks of different temperatures to cycle the samples.

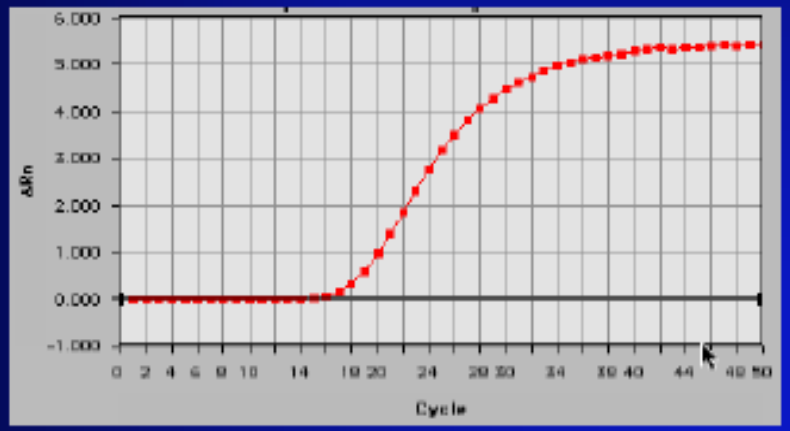


(www)

Real-time PCR is kinetic

- Detection of "amplification-associated fluorescence" at each cycle during PCR
- No gel-based analysis at the end of the PCR reaction
- Computer based analysis of the cycle-fluorescence time course

Increasing fluorescence



Linear plot

} Log-view augments this part

PCR cycle

Nigel Walker, NIEHS (www)

Real-time PCR advantages

* not influenced by non-specific amplification

* amplification can be monitored real-time

* no post-PCR processing of products
(high throughput, low contamination risk)

* ultra-rapid cycling (as fast as 25 minutes)

* wider dynamic range of up to 10^{10}-fold

* requirement of 1000-fold less RNA than conventional assays
(minimum 6 picogram = one diploid genome equivalent)

* detection is capable down to a two-fold change

* confirmation of specific amplification by melting curve analysis

* most specific, sensitive and reproducible

* not much more expensive than conventional PCR
(except equipment cost)

Real-Time PCR Principles

Three general methods for the quantitative assays:

1. Hydrolysis probes

(TaqMan, Beacons)

2. Hybridization or FRET probes

(Light Cycler)

3. DNA-binding (intercalating) agents

(SYBR Green, Eva Green, LC Green)

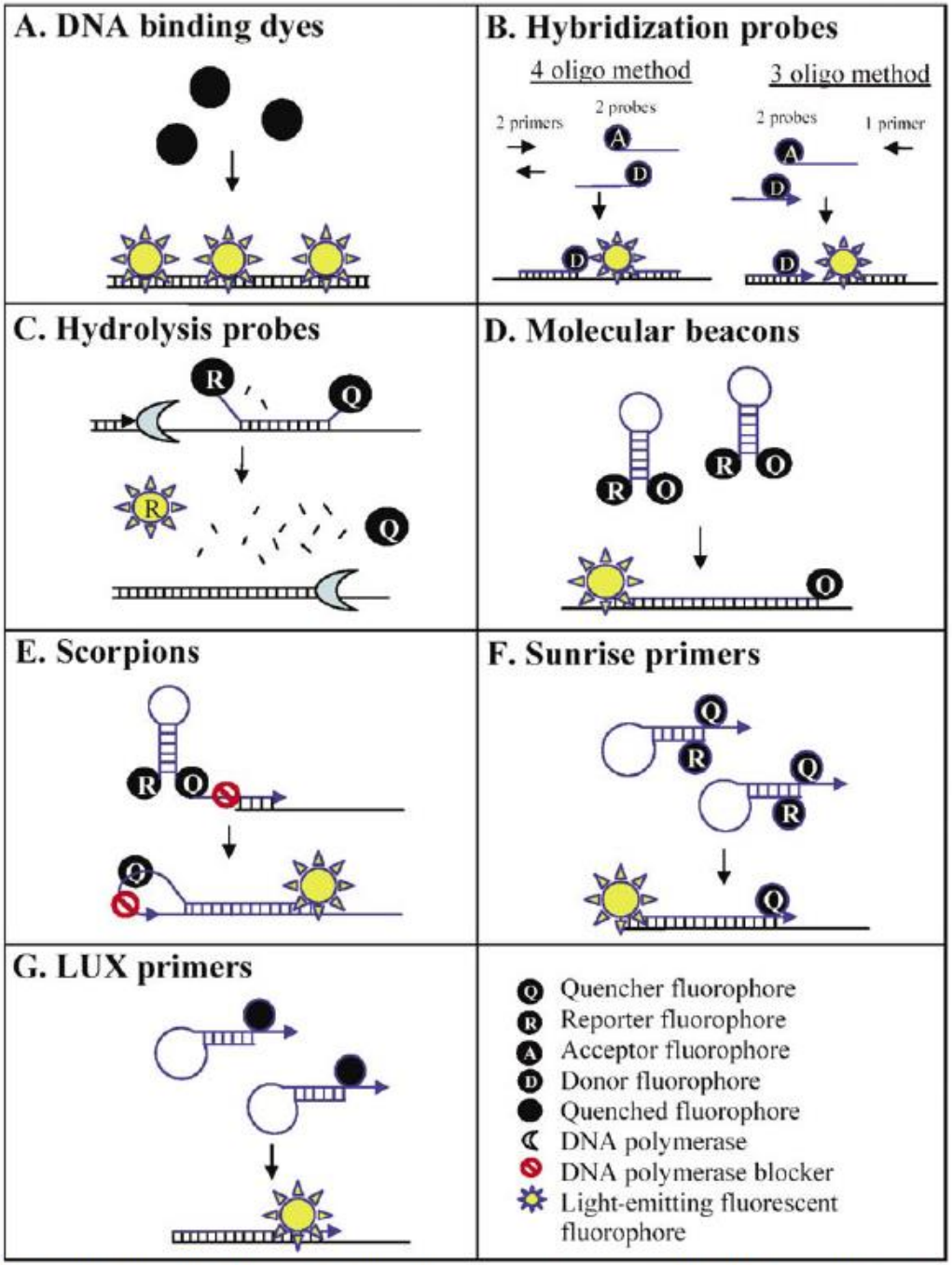


Figure 3. Real-time PCR detection chemistries. Probe sequences are shown in blue while target DNA sequences are shown in black. Primers are indicated by horizontal arrowheads. Not all unlabeled PCR primers are shown. Oligo, oligonucleotide.

Real-time PCR for mRNA quantitation

Marisa L. Wong and Juan F. Medrano

BioTechniques 39:75-85 (July 2005)

(www)

TheScientist tuesday
MAGAZINE OF THE LIFE SCIENCES EVERY DAY, ONLINE

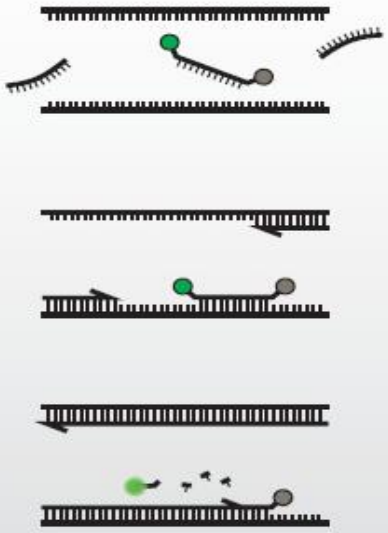
This month:
TheScientist
RE-ENGINEERING HUMANS

Volume 20 | Issue 12 | Page 68

How it Works: Real Time PCR

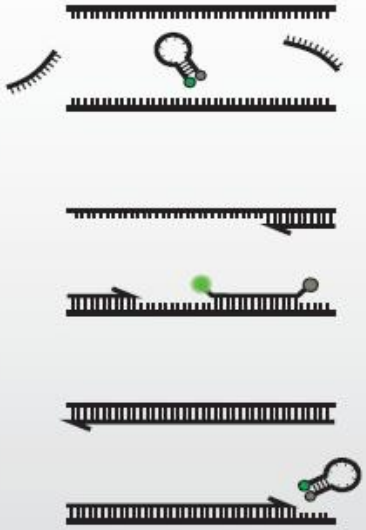
Brendan Maher

TaqMan requires a sequence-specific probe that connects fluorophore and quencher.



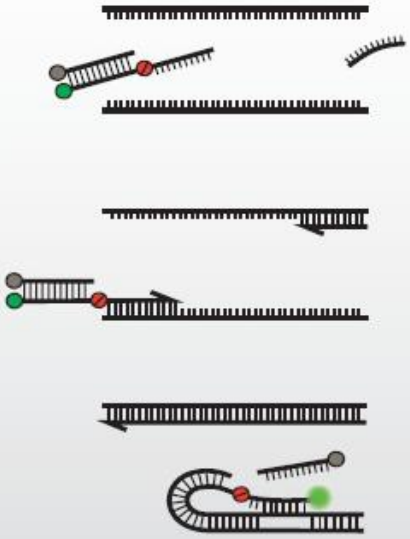
* Pros: specificity, different colors can be used in multiplex assays
* Cons: some background noise due to irreversibility of the reaction

Molecular Beacons uses sequence specific probes that take on a hairpin structure.



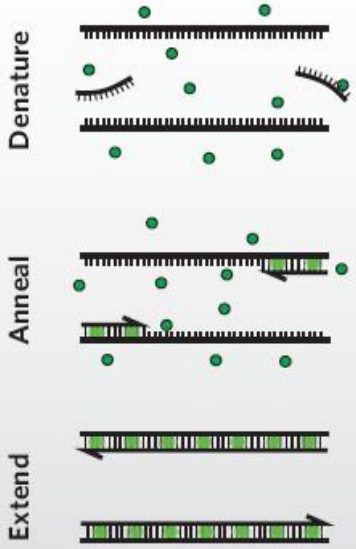
* Pros: greater specificity, reversible fluorescence means lower background
* Cons: some non-specific interactions of the hairpins can lead to false positives

Scorpions chemistry combines probe and primer. A polyermase blocker prevents unwanted replication.



* Pros: high specificity, faster cycling
* Cons: probe/primer design is involved and pricey

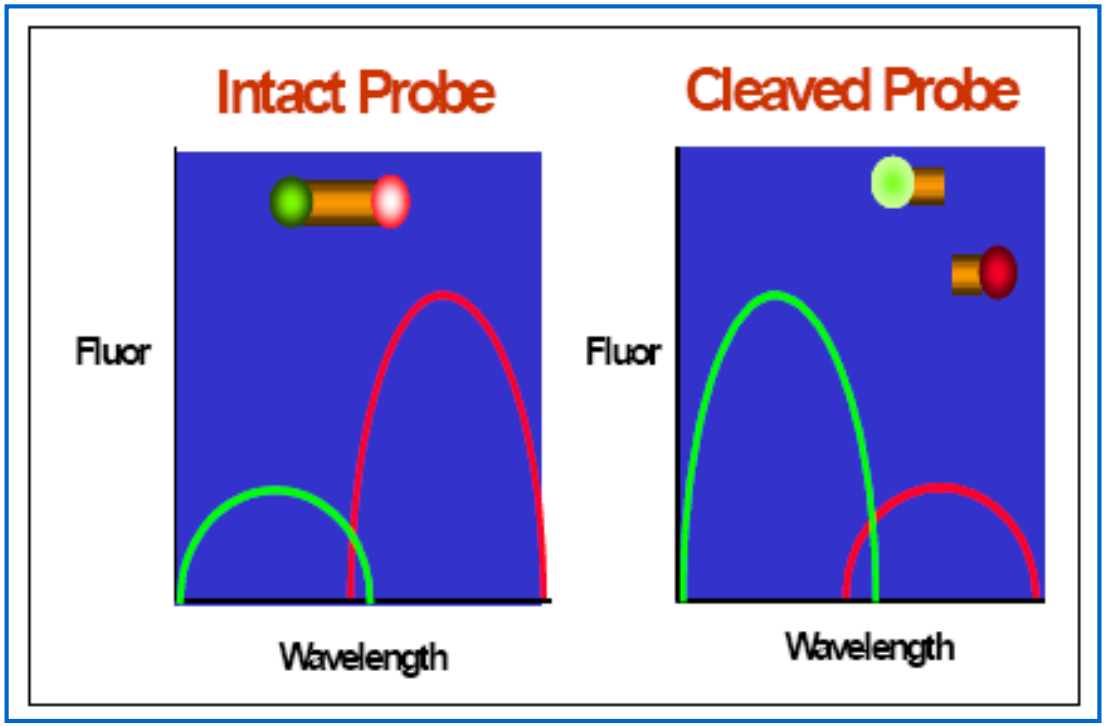
SYBR Green I fluoresces only when bound to dsDNA.



* Pros: relatively cheap, doesn't require probe design
* Cons: nonspecificity can lead to false positives, not attuned for complex protocols

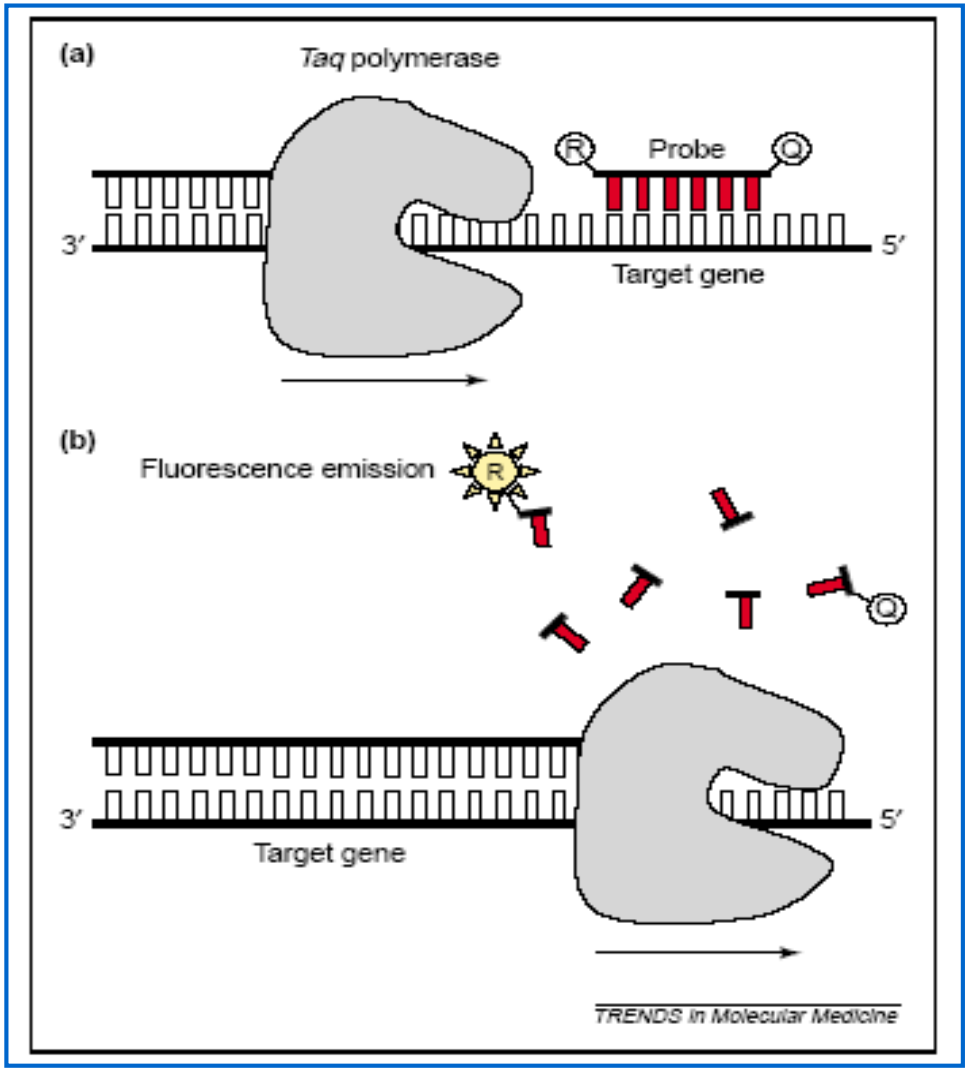
(www)

FRET = Förster/fluorescence resonance energy transfer

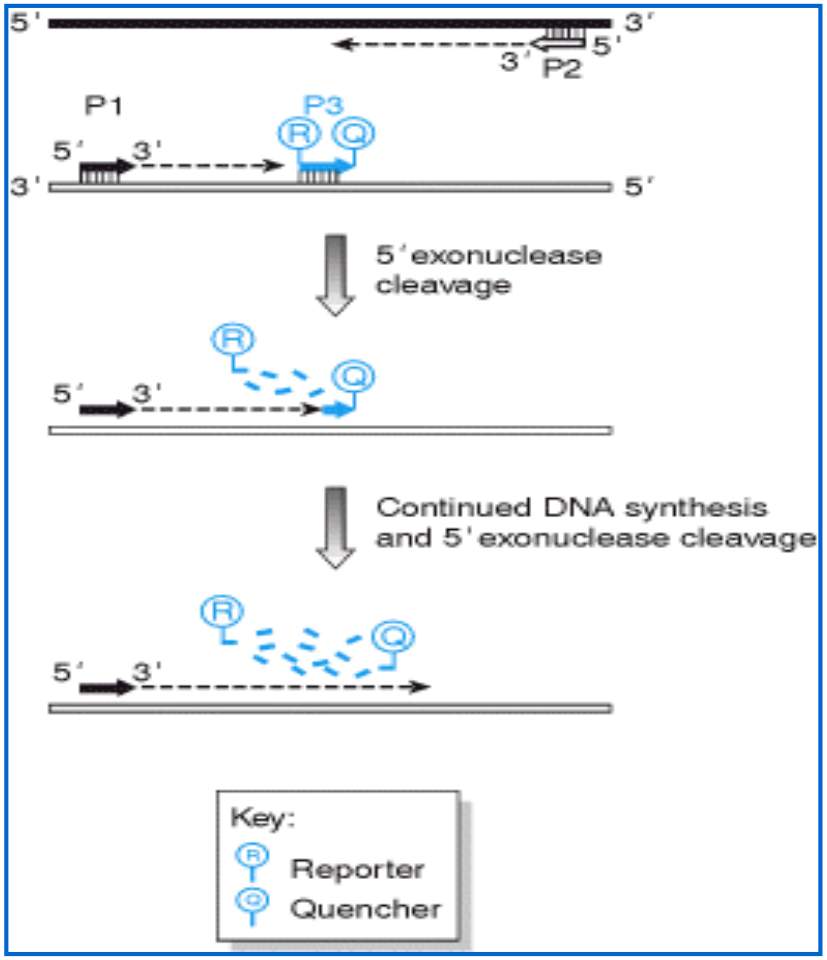


ABI: Real-Time PCR vs Traditional PCR (www)

DNA Polymerase 5' Exonuclease Activity



Mocellin et al. Trends Mol Med 2003 (www)



TaqMan 5' Exonuclease Assay

In addition to two conventional PCR primers, P1 and P2, which are specific for the target sequence, a third primer, P3, is designed to bind specifically to a site on the target sequence downstream of the P1 binding site. P3 is labelled with two fluorophores, a reporter dye (R) is attached at the 5 end, and a quencher dye (D), which has a different emission wavelength to the reporter dye, is attached at its 3 end. Because its 3' end is blocked, primer P3 cannot by itself prime any new DNA synthesis. During the PCR reaction, *Taq* DNA polymerase synthesizes a new DNA strand primed by P1 and as the enzyme approaches P3, its 5' exonuclease activity degrades the P3 primer from its 5' end. The end result is that the nascent DNA strand extends beyond the P3 binding site and the reporter and quencher dyes are no longer bound to the same molecule. As the reporter dye moves away from the quencher, the resulting increase in reporter emission intensity is easily detected.

Human Molecular Genetics 2. NCBI Books (www)

Dye and Quencher Choice

When designing a fluorescent probe, it is necessary to ensure that the fluorophore and quencher pair is compatible, given the type of detection chemistry. In addition, when designing multiplexed reactions the fluorophores and quenchers chosen for the different targets should minimize the spectral overlap between them, to avoid possible crosstalk issues (Table 1).

Filter Set	Ex Wavelength	Em Wavelength
Alexa 350	350	440
FAM/SYBR Green	492	516
TET	517	538
HEX/JOE/VIC	535	555
CY3	545	568
TAMRA	556	580
ROX/Texas Red	585	610
CY5	635	665
FR 640	492	635
FR ROX	492	610
FR CY5	492	665

Table 1

Parameters of the Mx3000P system filter sets. FR 640, FR ROX and FR Cy5 are available only as a custom set.

Most common choices are FAM and VIC/HEX/JOE as reporters and TAMRA or NFQ as a quencher

(www)

Table 1. Combinations of Reporter Dyes for Multiplex Assays*

Cycler	Reference dye	Dye 1†	Dye 2†	Dye 3†	Dye 4†
ABI PRISM® 7700	ROX	FAM	HEX, JOE, VIC®	—	—
ABI PRISM 7000 and 7900, Applied Biosystems® 7300	ROX	FAM	HEX, JOE, VIC	Bodipy® TMR, NED	—
Applied Biosystems 7500	ROX	FAM	HEX, JOE, VIC	Bodipy TMR, NED	Alexa Fluor® 647, Cy®5
iCycler iQ® and iQ5	Not required	FAM	HEX, JOE, TET, VIC	Texas Red®, ROX	Cy5
LightCycler® 2.0	Not required	FAM	HEX, JOE, VIC	Texas Red, ROX	Alexa Fluor 660, Bodipy 630/650, Pulsar® 650
Mx3000P™, Mx3005P™	Not required	FAM	HEX, JOE, VIC	Texas Red, ROX	Cy5
Rotor-Gene™ 3000	Not required	FAM	HEX, JOE, VIC	Texas Red, ROX	Cy5

* Visit www.qiagen.com/multiplex to view dye combinations for other cyclers.

† Preferably, select Dye 1 for the least abundant target, Dye 2 for the second least abundant target, and Dyes 3–4 for the most abundant targets.

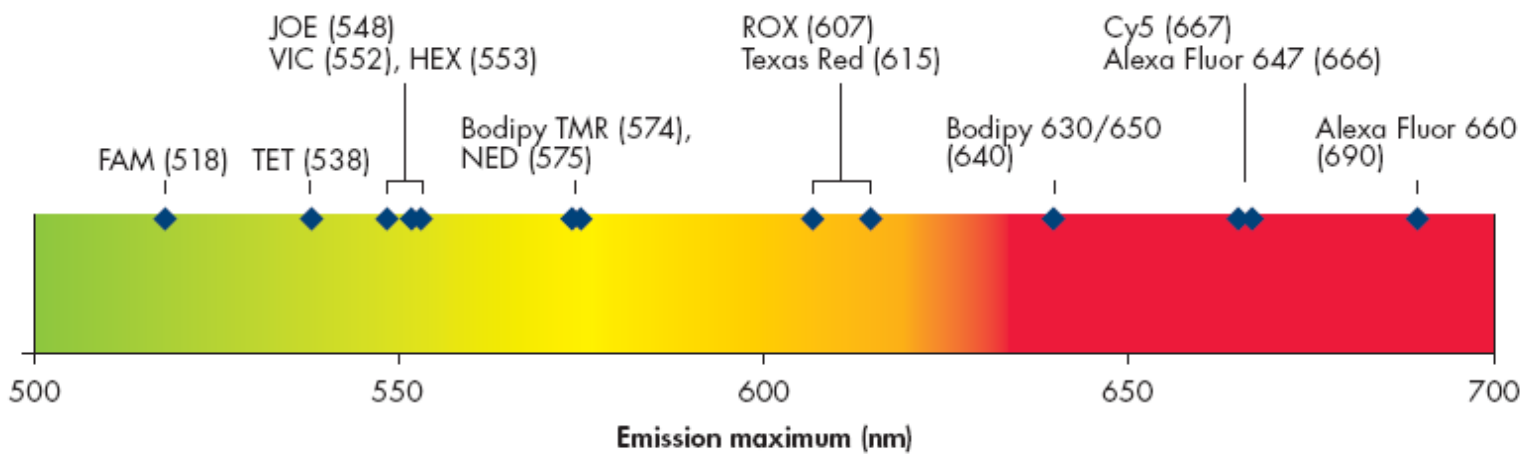


Figure 1 The emission maximum (nm) of selected reporter dyes are displayed in parentheses. Emission maximum may vary depending on buffer conditions. Other dyes with similar wavelengths may not be suitable for multiplex assays due to low fluorescence and/or stability.

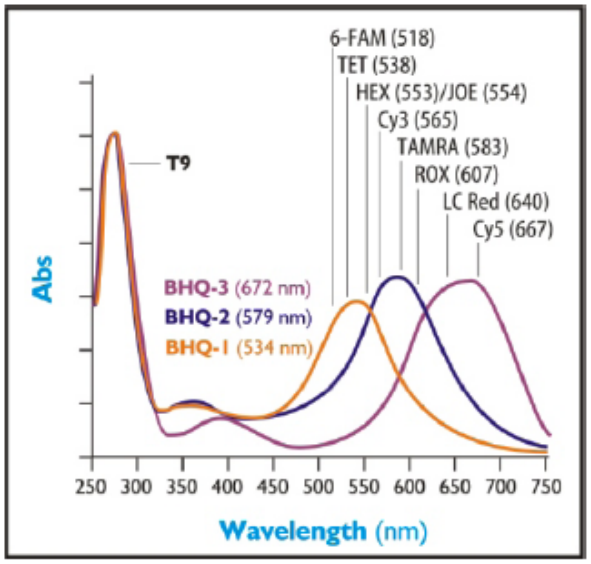
What are Black Hole Quenchers™?

Some commonly used quenchers–such as DABCYL and TAMRA–suffer from a number of drawbacks, including poor spectral overlap between the fluorescent dye and quencher molecule (DABCYL) or inherent fluorescence of the quencher (TAMRA)–resulting in a relatively poor signal-to-noise ratio. Black Hole Quencher (BHQ™) molecules have been developed to overcome these drawbacks.

Features

- No native fluorescence, resulting in lower background fluorescence
- Increases signal-to-noise ratios, providing higher sensitivity
- Accesses the visible spectrum into near-IR for reporting (480 to 730 nm)
- Maximizes spectral overlap, increasing efficiency of quenching
- Enables wider choice of reporter dyes for multiplexing assays

Absorption Spectra of BHQ dyes among the most commonly used dyes.



BHQ Dye Absorption Maxima and Quenching Range

Quencher	Abs max	Quenching Range (nm)
BHQ-1	534	480 - 580
BHQ-2	579	550 - 650
BHQ-3	672	620 - 730

BHQ Dye / Reporter Combinations

Quencher	Suggested Fluorophores
BHQ-1	FAM, TET, JOE, HEX, Oregon Green®
BHQ-2	TAMRA, ROX, Cy3, Cy3.5, CAL Red™, Red 640
BHQ-3	Cy5, Cy5.5

(www)

TaqMan Primers

* equal Tm (58 - 60^0 C)

* 15 - 30 bases in length

* G+C content 30 - 80%

* no runs of four or more Gs (any nucleotide)

* no more than two G+C at the 3’ end

* no G at the 5' end (A or C preferred)

* amplicon size 50 - 150 bp (max 400)

* span exon-exon junctions in cDNA

ABI Primer Express Software Tutorial

(www)