Session 4

qPCR – Signals and Probes

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qPCR - in More Detail

- History & Applications
- ABI 7000 Instrumentation
- Detection Chemistries
- Selected Forensic Assays

qPCR: Some History

1985 – PCR first published. Mullis and others at Cetus (Emeryville, CA)

Late1980's through present– PCR used for quantification, but required electrophoresis to detect product at end-point of PCR

1991 – Use of 5'-> 3' exonuclease activity of *Taq* polymerase to detect specific PCR activity ("TaqMan" approach). Gelfand and others at Cetus

1992 – Discovery that EtBr (dsDNA probe) can be added to the PCR mix and that the fluorescence of EtBr will increase at each cycle of PCR due to product formation. No need to cool to room temperature or to open tubes to measure the increased EtBr fluorescence. First real-time experiments – in closed PCR tubes on UV-trans-illuminator. Higuchi and others at Roche and Chiron .

qPCR: Some History

1993 – First real-time PCR detection experiments to show utility for DNA quantitation (log Co v. Ct). Improved EtBr detection (illumination, CCD camera detection). Higuchi and others at Roche (Higuchi, et al., BioTechnology 1993, *11*, 1026-1030).

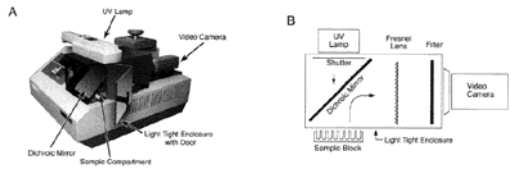
1996 – TaqMan detection methods used, instead of EtBr, for real-time detection of PCR. Improved specificity. Heid and others at ABI and Genentec.

1996-7 – ABI introduces first real-time qPCR ("Sequence Detection System") instrument (the ABI 7700).

Since then – many more instrument manufacturers (Roche, BioRad, Stratagene, Corbett, Cepheid, MJ) and many more detection chemistries – big business (and mainly **not** forensic)

qPCR: Some History

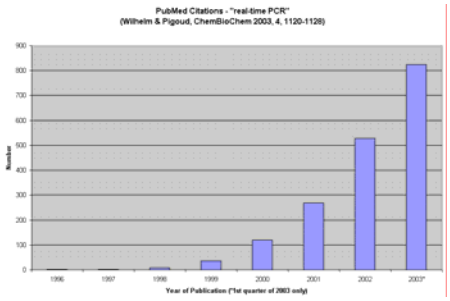
An Early "Home-Built" Real-Time qPCR Instrument (Higuchi, et al.)
- a modified ABI 9600 thermocycler for detecting EtBr in real-time



NOTE: A dichroic mirror is a semi-transparent "two color" mirror – in this example, it transmits the short wavelength excitation energy from the light source, but reflects the longer wavelength fluorescence emission from the samples into the detection optics.

qPCR

Real-time PCR Methods – no longer "novel"



qPCR: **Principal Applications**

Are not in forensic DNA analysis !

Quantification of mRNA, and thus gene expression (research & gene therapy diagnostics)

mRNA -Rev.Transcriptase→ cDNA -qPCR→ quantitation of gene expression

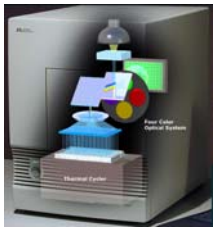
Detection (and/or quantification) of pathogens

Single Nucleotide Polymorphism (SNP) genotyping

Detection (and/or quantification) of GMO's in food

qPCR: **Detection - Instrumentation**

How are the PCR products ("amplicons") detected in real time?
by fluorescence associated with the PCR



ABI 7000 SDS Instrument

- by combining a PCR thermal cycler with a fluorimeter
- qPCR instruments are commonly configured to detect 2-5 different "colors" (or "channels")
- multiple detection channels allow for quantification of more than one target (multiplex qPCR) in a single tube

qPCR: **Detection - Instrumentation**

dichroic mirror

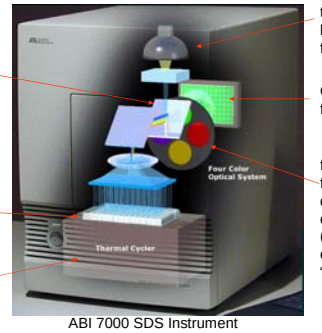
tungsten-halogen lamp for excitation

CCD array for detection

filter wheel for 4-color detection of emission (also software color comp. "matrix")

sample tubes/plate

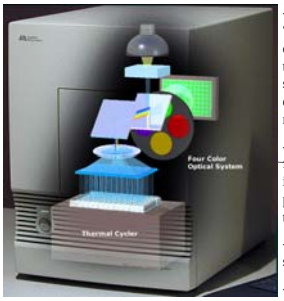
thermal cycler ("9700")



ABI 7000 SDS Instrument

qPCR: **Detection - Instrumentation**

- in ABI instruments, the optics does not always allow every sample in the 96well plate to be equally excited and detected
- in addition, there can be intra-plate volume differences due to evaporation and/or pipetting



ABI 7000 SDS Instrument

- to correct for (or "normalize") these differences across the plate, ABI spikes all of their qPCR master mixes with ROX
- the ROX signal from each sample is used to improve precision across the plate
- R = fluorescence signal of reporter
- Rn – normalized flourescence signal

qPCR - Instruments

ABI 7000 – validated by ABI for Quantifiler (QF) qPCR kits

ABI 7300 – 4-color detection – QF-validated by ABI (?)

ABI 7500 – 5-color detection (Cy5) – just QF-validated by ABI

ABI 7900 – uses laser-induced fluorescence, high throughput

Stratagene Mx3000P – "inexpensive" ($25K), some labs validating

Corbett Rotorgene 3000 – validated in Vermont using ALU assay

Cepheid SmartCycler

Roche LightCycler – under development here

BioRad iCycler

MJ Opticon

Clearly, qPCR is not used primarily for forensic DNA analysis !

qPCR: **Detection - Chemistry**

How are the PCR products ("amplicons") detected in real time? by increased fluorescence due to the PCR

Two General Approaches for Detection

1. Fluorophore is not sequence-specific – detects any double-stranded PCR product at each cycle; specificity of detection and quantification is due to specificity of primers.
   - fluorophore typically SYBR Green
2. Fluorophore is sequence-specific – detects only specific double-stranded PCR product at each cycle; specificity of detection and quantification is due to specificity of primers AND to specificity of reporter fluorophore
   - fluorophore commonly a "TaqMan" probe
   - many others

qPCR: **Detection – SYBR Green**

What is SYBR Green (SG) ?

- a proprietary fluorophore (Molecular Probes)

- binds to dsDNA (in minor groove); binding is NOT sequence-dependent (binds to "primer dimer" and non-specific product)

- upon binding to dsDNA, shows greatly enhanced fluorescence (similar, in principle, to EtBr, except >10x greater fluorescence)

unbound SG = "dark" dsDNA-bound SG = "FAM-like"

- SYBR Green is typically a pre-added ingredient in so-called "SYBR Green Master Mixes"; such a Master Mix will typically contain: rxn buffer, *Taq* Gold, dNTP's, ROX, and SG. The primers of interest (and template) are added by the user.

qPCR: **Detection – SYBR Green**

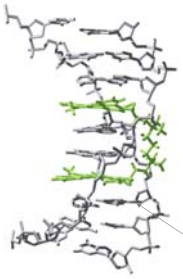
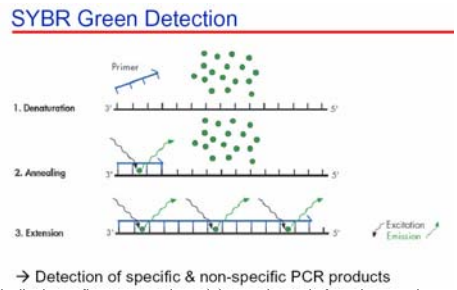


Figure 8.6 NMR solution structure of the TOTO-1 dye (T3600) bound to DNA; the image was derived from data submitted to the Protein Data Bank (number PDB 108D, www.rcsb.org/pdb/,). The NMR structure shows that TOTO-1 binds to DNA through intercalation.

from
http://www.probes.com/handbook/figures/1557.html

TOTO-1 fluorophore
(similar to SYBR Green)

qPCR: **Detection – SYBR Green**



→ Detection of specific & non-specific PCR products

Typically detect fluorescence in real time at the end of each extension step in PCR

qPCR: **Detection – SYBR Green**

Advantages of SYBR Green Detection:

- simple to design – just need to find good, specific primers for the target sequence of interest

- sensitive - produces >1 reporter per amplicon

- inexpensive, relative to "TaqMan" detection, because dye-labeled oligo-nucleotides are not required

- (can use melt curve to assess specificity of PCR)

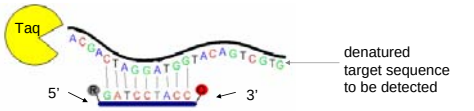
qPCR: **Detection – SYBR Green**

Disadvantages of SYBR Green Detection:

- SYBR Green detects ALL double-stranded DNA, so if PCR is poorly designed, "primer-dimer" product will be detected and quantified

- cannot multiplex SYBR Green qPCR assays

qPCR: **Detection - TaqMan Chemistry**

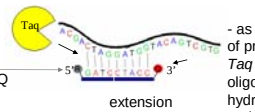


TaqMan detection probe = a dual-labeled oligonucleotide
- complimentary to target sequence (anneals between primers)
- designed to anneal ~8-10 degrees C higher than primer
- 5' end of probe = a Reporter fluorophore
  (e.g., FAM, VIC, NED, Cy5, etc.)
- 3' end of probe = a Quencher – a chemical group that
  will quench the fluorescence of the Reporter
  (e.g., Tamra, "BHQ," or "NFQ")
- quenching occurs only if R and Q are sufficiently proximate so
  that excitation energy is transferred from R to Q (rather
  than emitted by R as fluorescence)
- ideally, an "intact" TaqMan probe is not fluorescent ("dark")

qPCR: **Detection - TaqMan Chemistry**

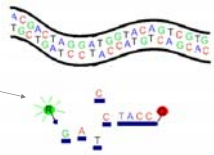
- detection relies on 5' nuclease activity of Taq polymerase

- R is "dark" or "quenched" due to proximity to Q in intact probe



- as *Taq* extends from 3' end of primer (not shown), if the *Taq* encounters a bound oligonucleotide, it will hydrolyze the oligo from its 5' end

- 5' hydrolysis separates R from Q so that fluorescence of R is no longer quenched



- qPCR instruments are typically configured to detect fluorescence at the end of each extension cycle; get one unquenched R for each PCR cycle

qPCR: **TaqMan Chemistry**

More Detail…

Annealing/Extension Step

- TaqMan probe hybridizes to denatured DNA (sequence specific)
- reporter fluorescence is quenched due to proximity to quencher (reporter starts ~dark)

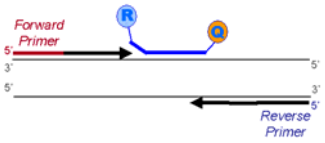
- Why is temp for probe annealing ~8-10 C higher than for primers ?
- Why doesn't *Taq* polymerase extend from 3' end of probe ?

qPCR: **TaqMan Chemistry**

More Detail…

Annealing/Extension Step

- Lengthening strand displaces 5'end of probe

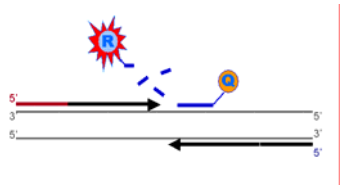


qPCR: **TaqMan Chemistry**

More Detail…

Annealing/Extension Step

-*Taq* polymerase mediates hydrolysis of probe from 5' end ("5' exo-nuclease activity")
-Reporter fluorophore is no longer quenched
-Hydrolyzed TaqMan probe eventually dissociates

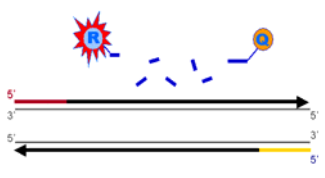


qPCR: **TaqMan Chemistry**

More Detail…

End of Annealing/Extension Step

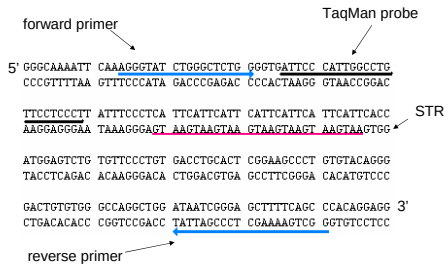
- Extension is completed
- Fluorescence is detected by qPCR instrument
- Ready for next cycle of PCR



qPCR: **TaqMan Chemistry**

An Example…

Target Sequence of CA DOJ in-house qPCR Assay at TH01 STR Locus



qPCR: **TaqMan Detection Chemistry**

Advantages:

-Very specific, because combines specificity of primers and specificity of the TaqMan probe – typically do not detect non-specific PCR product

- Can multiplex qPCR assays to simultaneously amplify and detect different target sequences in the same tube

e.g., use FAM-labeled probe for nuclear target sequence and VIC-labeled probe for mitochondrial target (or Y-specific target, or Internal PCR control target, etc.)

qPCR: **TaqMan Detection Chemistry**

Some Disadvantages: (relative to SYBR Green)

- More difficult to design because of need for efficient amplification AND efficient probe hydrolysis (and *possibility* that amplification and hydrolysis chemistries inhibit differently)

- More difficult to design because some TaqMan probes do not quench efficiently => large background fluorescent and lower signal-to- noise

- For some target sequences, AT-rich sequences make probe design difficult (see "MGB" probes)

- More expensive, due to cost of dual-labeled oligonucleotide

qPCR: **a Variation**

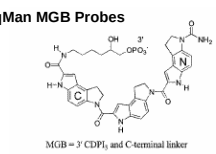
TaqMan MGB Probes
- MGB = Minor Groove Binder
  - functional group chemically attached to the 3' end of theTaqMan probe
  - group binds to minor groove of DNA so as to increase the annealing temperature of the probe (by ~10 degrees C)

- invented by Epoch Pharmaceuticals; licensed exclusively to ABI

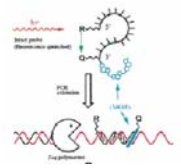
- advantages are mainly due to shortening of probe:
  - better quenching of reporter by quencher ("dark" is darker, so less background fluorescence)
  - shorter sequences allow for more easily designed probes
    e.g., typical TaqMan probe – 20-30 basepairs
    typical TaqManMGB probe – 12-18 basepairs

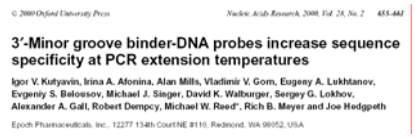
qPCR: **a Variation**

TaqMan MGB Probes



structure of MGB group





qPCR- Other Detection Chemistries

Fluorescence detection of amplicons in real time

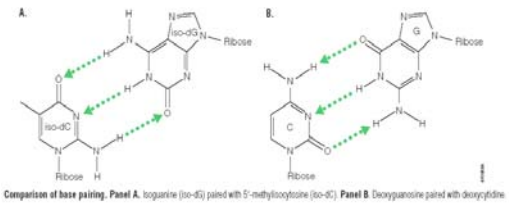
by any number of methods:

- FRET Hybrids (Roche)
- Molecular Beacons (NJ Dept of Public Health)
- Scorpions
- Light Upon Extension (LUX) primer
- EraGen, a.k.a., "Plexor" (licensed by Promega)

EraGen qPCR Detection Chemistry

(J.Am.Chem.Soc., 2004, v.126, 4550-6)

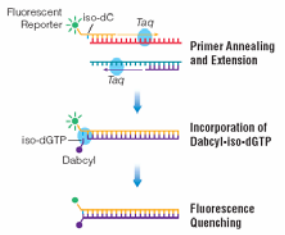
- uses Watson-Crick pairing of synthetic (non-natural) dNTPs



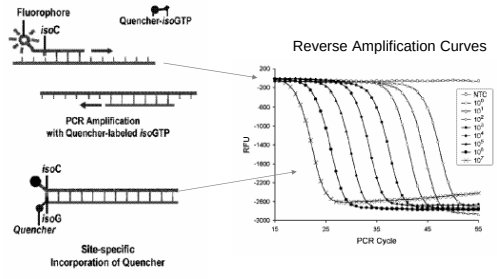
Comparison of base pairing. Panel A. Isoguanine (iso-dG) paired with 5'-methylisocytosine (iso-dC). **Panel B.** Deoxyguanosine paired with deoxycytidine.

EraGen qPCR Detection (cont.)

- one primer is labeled on 5'-end with fluorophore (e.g., FAM) linked to a terminal iso-CTP
- fluorophore is NOT quenched before PCR
- PCR is done with standard dNTPs AND iso-GTP linked to a quencher



EraGen qPCR Detection (cont.)



EraGen qPCR Detection (cont.)

Advantages:

- Can also multiplex multiple target sequences
- proposed to give good sensitivity

Disadvantages:

- not as widely used as TaqMan or SYBR Green, so less experimental history to rely on

Comments:

- licensed to Promega (for many applications, not just forensic typing)

Some Forensic qPCR Assays

Vermont ALU Assay (J.Nicklas, E. Buel – NIJ funded)

– 2002 – amplifies a primate specific, 124bp sequence at ALU target (>100,000 copies per genome !)

- •uses SYBR Green
- •not commercialized
- •Orange County, CA has a different SG-detected ALU assay on-line

ABI Quantifiler Human and Y DNA Quantitation Assays – see ABI website

- • released late 2003/early 2004
- • human target is 62 bp sequence at htert (intronic), detected using FAM- labeled TaqManMGB probe
- •Y target is 62 bp sequence at SRY (intronic), detected using FAM-labeled TaqManMGB probe
- •FAM-labeled assays are each run as duplex qPCR with VIC-labeled Internal PCR Control (IPC) assay to detect for PCR inhibition

Some Forensic qPCR Assays

Cal DOJ DNA Nuclear/Mitochondrial Duplex Assay

•(M.Timken, K.Swango, C.Orrego, M.Buoncristiani, funded by NIJ)

- • human nuclear target – amplifies the TH01 STR target sequence (~170-190 bp in length, depending upon genotype of source)
  - • uses FAM-labeled TaqMan probe
- • human mitochondrial target – amplifies 69 bp target sequence in the
  - •ND1 region of the mitochondrial genome
    - •used VIC-labeled TaqManMGB probe

•Reliagene – presumptive test for male DNA based on real-time qPCR

•Promega – duplex (human/IPC and/or male/IPC) using EraGen/Plexor chemistry

•Orchid-Cellmark – duplex human/male using TaqManMGB chemistry

- •(TPOX flanking sequence for human, SRY for male)

Why do qPCR?

- simple protocol; PCR setup, then walk-away (~2 hours); simple data analysis
- dynamic range up to 7 orders of magnitude
- detection down to single copy number is possible; quantification down to ~10 copy numbers
- assays are target specific (human, mt, Y) and can be multiplexed
- set-up can be automated
- quantitation based on PCR, same as DNA analysis methods
  - quantitates "amplifiable" DNA (better than QB for degraded DNA)

qPCR is Not Perfect

- qPCR is subject to inhibition (=> internal PCR control (IPC) assays are useful)
- the exponential relationship between Ct and quantity means that small variations in Ct lead to larger variations in quantity
- qPCR quantitation precision suffers at low copy numbers (e.g., below ~30 pg about a factor of 2)
- qPCR quantitates specific target sequences, it does not quantify "DNA"
  - in highly degraded samples, assays that amplify short target sequences will detect and measure more DNA than assays that amplify long target sequences => implications for STR amps
- accurate qPCR quantitation assumes that each unknown sample is amplified at the same efficiency as the standard samples in the dilution series – not necessarily true
- there is no NIST-certified quantification standard (yet?)