Techniques in Molecular Biology

Polymerase Chain Reaction:

The 'Swiss Army Knife' of molecular biology

Slide materials both original and web-"borrowed"

Basics of PCR

Primers
- 15-60bp (60bp is limit synthesized by IDT)
- Annealing temp ideally >55C (portion that anneals to your template)
- Hairpins Tm<50 ?
- Self dimers---only important if they are 3' annealing dimers
- Silent mutants---better to have them on 5' end than on 3' end

Cycles
- Denature
  - Can be extended in GC rich templates
- Anneal
  - Standard is 5C below Tm of primer (the portion that anneals to your gene)
  - Can do gradient on our thermocycler
- Extend
  - 68-72C

Polymerases
- TAQ—faster and better at amplifying long genes
  - Adds a A to sequence
  - Error prone
- PFU—slow but provides proofreading
  - Used for mutagenesis primarily
- TAQ:PFU mix—qualities of both

PCR Instruments.

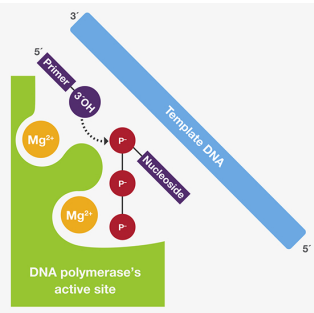
Low profile tube

Regular profile tube

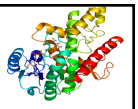
Minimal DNA synthesis components:

- Template DNA (single stranded)
- Oligonucleotide primer
- dNTPs: (dATP, dCTP, dGTP, dTTP)
- DNA Polymerase

[proper conditions – for fidelity and read length]



Heat-stable DNA Polymerase



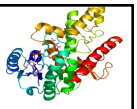
Given that PCR involves very high temperatures, it is imperative that a heat-stable DNA polymerase be used in the reaction.

- Most DNA polymerases would denature (and thus not function properly) at the high temperatures of PCR.

Taq DNA polymerase was purified from the hot springs bacterium *Thermus aquaticus* in 1976

Taq has maximal enzymatic activity at 75 °C to 80 °C, and substantially reduced activities at lower temperatures.

Polymerases used for PCR



The original: *Taq* polymerase

from *Thermus aquaticus*, first isolated from Yellowstone N.P. hotsprings

Some polymerases used for PCR

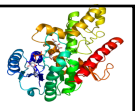


TABLE 6.1. Thermostable DNA polymerases differ in their enzymatic activities

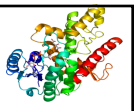
Enzyme	Relative efficiency[a]	Error rate[b]	Processivity[c]	Extension rate[d]	3′ to 5′ exo	5′ to 3′ exo
Taq Pol	88	2×10^{-4}	55	75	no	yes
Tli Pol (Vent)	70	4×10^{-5}	7	67	yes	no
Pfu Pol	60	7×10^{-7}	n.d.	n.d.	yes	no
rTth	n.d.	n.d.	30	60	no	yes

[a] Percent conversion of template to product per cycle.
[b] Frequency of errors per base pairs incorporated.
[c] Average number of nucleotides added before dissociation.
[d] Average number of nucleotides added per second.
n.d = not determined.

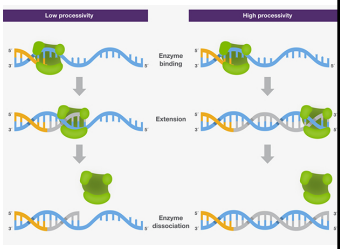
AMG p. 147

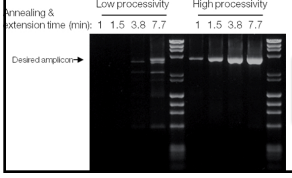
Note: values will vary depending on method of measurement

Properties of DNA Polymerase: (for PCR)



- **Elongation** - Rate of synthesis (e.g., nts synthesized / min)
- **Processivity** (e.g. nts synthesized / initiation event)



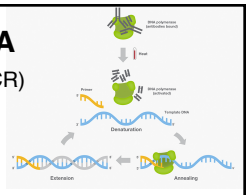


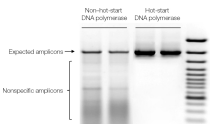
Highly processive DNA polymerases have higher affinity for substrates with longer reads per binding event

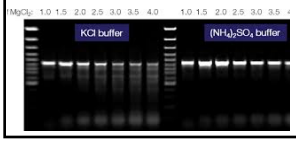
Properties of DNA Polymerase: (for PCR)

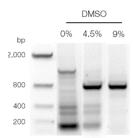
Specificity

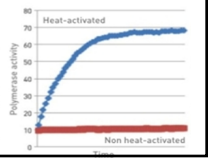
- Nonspecific amplification impacts yield and side reactions.
- ✓ Results from misprimed targets, prestarting pol activity before primer-target dimers form…
- ✓ One way to avoid this is to keep all reactions on ice to reduce pol activity.
- ✓ Using Hot Start, chemically modified pol or salts and additives can help



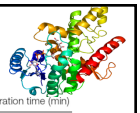






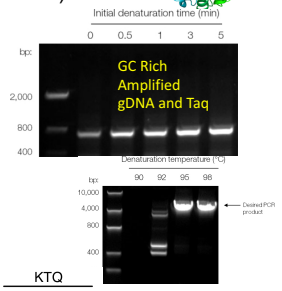


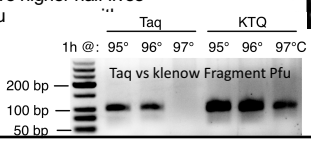
Properties of DNA Polymerase: (for PCR)



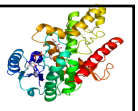
Thermostability

- Half life of Taq limits its usability above 90oC. Important when Tm of primers is high Higher temps are needed for GC rich regions
- ✓ Longer reads (half life issues) or higher temps may need to re-supply Taq
- ✓ Other pol have higher half lives – but other issu fidelity…



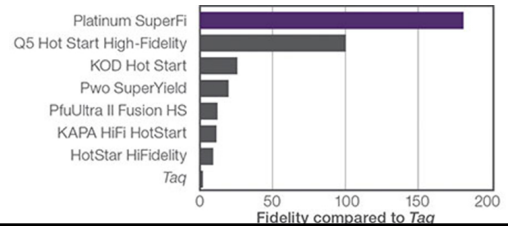


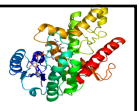
Properties of DNA Polymerase: (for PCR)



Fidelity

- Proofreading increases accuracy and ensures correct bases are incorporated into growing strands





Polymerases also have **exonuclease** activities

Nomenclature reminders:

Exonuclease – removes nucleotides from 5' or 3' end of nucleic acid

Endonuclease – cleaves within nucleic acid chain

DNA Synthesis Reaction (5' - 3' polymerase)

Primer ->

```
5'-AACGTTAACGTAGCT-OH - 3'
   |||||||||||||||
3'-TTGCAATTGCATCGATCGATCCCGATCGTGCGATCTATCGA - 5'
```

DNA Template

```
5'-AACGTTAACGTAGCT  - 3'
   |||||||||||||||   +dATP
3'-TTGCAATTGCATCGATCGATCCCGATCGTGCGATCTATCGA - 5'
```

+ DNA Polymerase

```
5'-AACGTTAACGTAGCTA - 3'
   ||||||||||||||||
3'-TTGCAATTGCATCGATCGATCCCGATCGTGCGATCTATCGA - 5'
```

+ PPi

DNA Synthesis Reaction

```
5'-AACGTTAACGTAGCTA - 3'
   ||||||||||||||||
3'-TTGCAATTGCATCGATCGATCCCGATCGTGCGATCTATCGA - 5'
```

+ DNA Polymerase
+ dNTPs

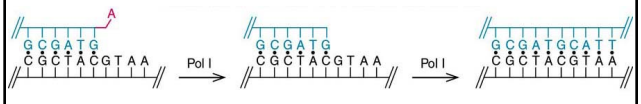
Primer ->

```
5'-AACGTTAACGTAGCTAGCT - 3'
   |||||||||||||||||||
3'-TTGCAATTGCATCGATCGATCCCGATCGTGCGATCTATCGA - 5'
```

DNA Template

Synthesis 5' to 3'

Proof-reading activity (3'-5' exonuclease)

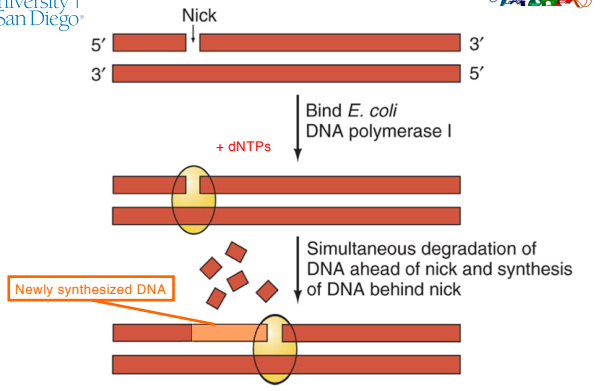


incorrect nucleotide incorporated

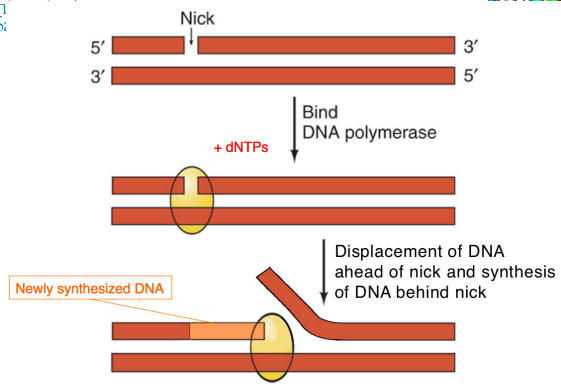
nucleotide removed

5'-3' synthesis continues

5'-3' exonuclease activity



Strand displacement activity



E. coli DNA Pol I activities can be separated:

Klenow fragment:

5' - 3' polymerase (synthesis)

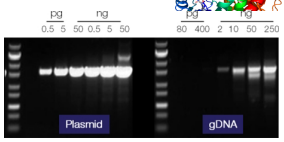
3' - 5' exonuclease (proof-reading)

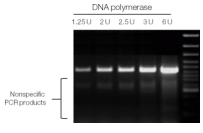
lacks 5' - 3' exonuclease

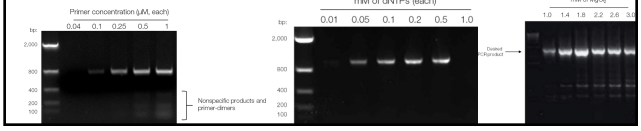
Template DNA and PCR

Composition, sequence, type (gDNA vs cDNA, or plasmid DNA) and mass all impact yield and specificity.

- Higher processive and specific polymerases do not need the same mass of template
- Increasing DNA pol does not ensure better results
- Primer design and concentration is crucial
- dNTP and $MgCl_2$ concentration is important







The three main steps of PCR

The basis of PCR is temperature changes and the effect that these temperature changes have on the DNA.

In a PCR reaction, the following series of steps is repeated 20-40 times

(note: 25 cycles usually takes about 2 hours and amplifies the DNA fragment of interest 100,000 fold)

Step 1: **Denature** DNA

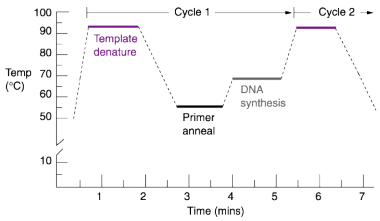
At 95°C, the DNA is denatured (i.e. the two strands are separated)

Step 2: Primers **Anneal**

At 40°C- 65°C, the primers anneal (or bind to) their complementary sequences on the single strands of DNA

Step 3: DNA polymerase **Extends** the DNA chain

At 72°C, DNA Polymerase extends the DNA chain by adding nucleotides to the 3' ends of the primers.



PCR

conceived by Kary Mullis, developed at Cetus Corp.

First publication using PCR in *Science*, Saiki et al., 1985

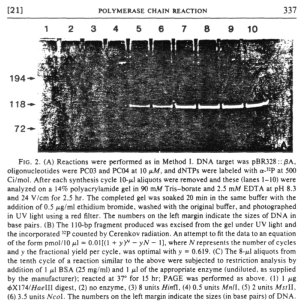


FIG. 2. (A) Reactions were performed as in Method 1. DNA target was pBR328::βA, oligonucleotides were PC03 and PC04 at 10 μM, and dNTPs were labeled with α-^{32}P at 500 Ci/mol. After each synthesis cycle 10-μl aliquots were removed and these (lanes 1–10) were analyzed on a 14% polyacrylamide gel in 90 mM Tris–borate and 2.5 mM EDTA at pH 8.3 and 24 V/cm for 2.5 hr. The completed gel was soaked 20 min in the same buffer with the addition of 0.5 μg/ml ethidium bromide, washed with the original buffer, and photographed in UV light using a red filter. The numbers on the left margin indicate the sizes of DNA in base pairs. (B) The 110-bp fragment produced was excised from the gel under UV light and the incorporated ^{32}P counted by Cerenkov radiation. An attempt to fit the data to an equation of the form pmol/10 μl = $0.01[(1 + y)^x - y/N - 1]$, where N represents the number of cycles and y the fractional yield per cycle, was optimal with $y = 0.619$. (C) The 8-μl aliquots from the tenth cycle of a reaction similar to the above were subjected to restriction analysis by addition of 1 μl BSA (25 mg/ml) and 1 μl of the appropriate enzyme (undiluted, as supplied by the manufacturer); reacted at 37° for 15 hr; PAGE was performed as above. (1) 1 μg ϕX174/*Hae*III digest, (2) no enzyme, (3) 8 units *Hin*fI, (4) 0.5 units *Mn*lI, (5) 2 units *Msp*I, (6) 3.5 units *Nco*I. The numbers on the left margin indicate the sizes (in base pairs) of DNA.

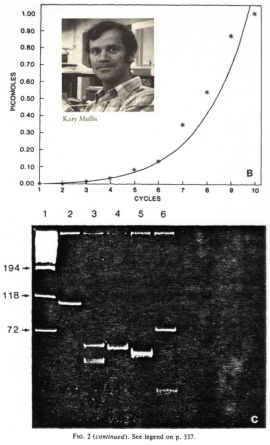
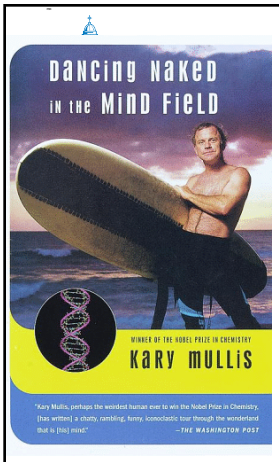


FIG. 2 (*continued*). See legend on p. 337.

Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods in Enzymology 155: 335-50



The Nobel Prize in Chemistry 1993

"for contributions to the developments of methods within DNA-based chemistry"

"for his invention of the polymerase chain reaction (PCR) method"

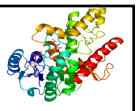
Kary B. Mullis
1/2 of the prize
USA
La Jolla, CA, USA
b. 1944

"for his fundamental contributions to the establishment of oligonucleotide- based, site-directed mutagenesis and its development for protein studies"

Michael Smith
1/2 of the prize
Canada
University of British Columbia
Vancouver, Canada
b. 1932
(in Blackpool, United Kingdom)
d. 2000

University of San Diego

Basic Reaction

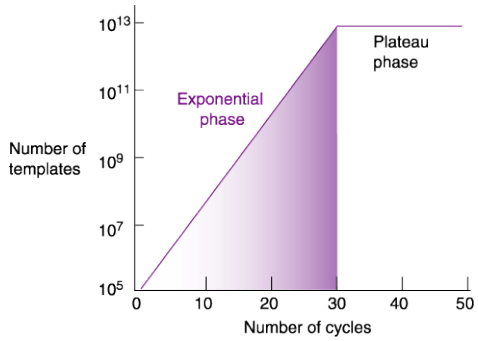


Component	Volume	Final Concentration
10×Taq Buffer	2 μl	1x
dNTPs (10 mM each)	0.4 μl	0.2 mM each
10 pmol/μl Primer #1	0.4 μl	0.2 μM
10 pmol/μl Primer #2	0.4 μl	0.2 μM
Template DNA	X μl	DNA ≤ 500 ng/50 μl
		Bacteria liquid = 1 μl
PCR grade water	Y μl	
Taq DNA Polymerase	0.4 μl	1.0 U / 50 μl
Total reaction volume	20 μl	

Parameters to vary

- Template DNA concentration
- Primer concentration
- Mg^{++} concentration
- Temperature of annealing
- dNTP concentration (may come as a master mix)

PCR exponential amplification is limited



AMG Fig. 6.2b

double-stranded template

```
5'-..TCTCAAACGTTAACGTAGCTAGCTCGGGCTAGCTCGCTAGATAGCTGATCCTCTCTCTGCATCCGT..- 3'
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3'-..AGAGTTTGCAATTGCATCGATCGATCCCGATCGTGCGATCTATCGACTAGGAGAGAGACGTAGGCA..- 5'
```

Denature (e.g., 95°)

```
5'-..TCTCAAACGTTAACGTAGCTAGCTCGGGCTAGCTCGCTAGATAGCTGATCCTCTCTCTGCATCCGT..- 3'

3'-..AGAGTTTGCAATTGCATCGATCGATCCCGATCGTGCGATCTATCGACTAGGAGAGAGACGTAGGCA..- 5'
```

Lower temperature – primers anneal (hybridize)

```
5'-..TCTCAAACGTTAACGTAGCTAGCTCGGGCTAGCTCGCTAGATAGCTGATCCTCTCTCTGCATCCGT..- 3'
                                                  ||||||||||||||||
                                          3'- TAGGAGAGAGACGTA      - 5'

5'-      AACGTTAACGTAGCT - 3'
         ||||||||||||||||
3'-..AGAGTTTGCAATTGCATCGATCGATCCCGATCGTGCGATCTATCGACTCGGAGAGAGACGTAGGCA..- 5'
```

Primer design guidelines & considerations

Primer length: 18 – 30nts
- too short: inadequate specificity
- too long: slow annealing

Primer T_m: 65° – 75° C
- Determined by sequence
- If the Tm is low find more GC content or extend length of primer

crude approximation: GC content

`ATTTATATAAGCGGGCGCCC` 20 nts, 50% GC

`ACTGTGACTAGTGATCTCAG` 20 nts, 50% GC

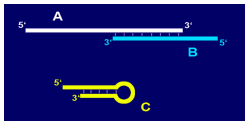
by one calculation method, 4° different

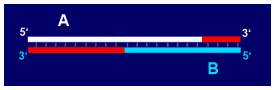
Keep Primer Tm within 5°C of each other
- Tms should match if possible

Aim for GC to be between 40-60% with a 3' ending in a C or G
- 1 or 2 GC bp acts as a "clamp"
- Helps to promote correct binding at 3' end due to 3H bonding hybridization
- Too much GC in the clamp will result in mis-priming of Pol

Primer design guidelines & considerations

Avoid regions of secondary structure
- Hairpin structures
- Primer dimer
- Self complementation





Avoid runs of 4 or more of a single base or dinucleotide repeats

AGC**GGGGG**AT**GGGG**
or AGC**ATATATATAT**GGCG

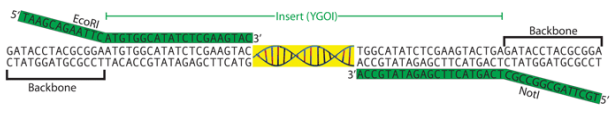
Avoid intra-primer homology (more than 3 bases that complement w/in primer) or inter-primer homology (forward and reverse primers having complementary sequences)

self-dimers or primer-dimers instead of annealing to the desired DNA sequences

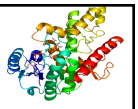
Don't forget – primer design adding restriction site!

Primer should add 3-6 random bp upstream of restriction site.

- watch for addition of hairpin structure. TAAGCA often works.



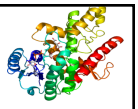
Selectivity of Primers



Primers bind to their complementary sequence on the target DNA

- A primer composed of only 3 letter, ACC, for example, would be very likely to encounter its complement in a genome.
- As the size of the primer is increased, the likelihood of, for example, a primer sequence of 35 base letters repeatedly encountering a perfect complementary section on the target DNA become remote.

Probability in Genetics

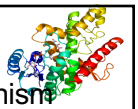


- There are 4 bases in the DNA molecule A,C,G,T
- The probability of encountering any of these bases in the code is 0.25 (1/4)
- So let us look at the probability of encountering a particular sequence of bases

Event	Probability	
A	0.25	= 0.25
A,T	0.25 x 0.25	= 0.0625
A,T,A	0.25 x0.25 x 0.25	= 0.015625
A,T,A,G,G	$(0.25)^5$	= 0.0009765
A,T,A,G,G,T,T,T,A,A,C	$(0.25)^{11}$	= 0.000002384
A,T,A,G,G,T,T,T,A,A,C,C,T,G,G,T	$(0.25)^{16}$	=0.0000000002384

So it become increasing unlikely that one will get 16 bases in this particular sequence (1 chance in 4.3 billion). In this same way, one can see that as the primer increases in size, the chances of a match other than the one intended for is highly unlikely.

RFLP



Restriction Fragment Length Polymorphism

Cutting a DNA sequence using restriction enzymes into pieces → specific enzymes cut specific places

Starting DNA sequence:
5' -TAATTCCGTTAGTCAAGCGTTAGGACC
3' -ATTAAGGCAATCAGTTCGCAATAATGG

Enzyme X
5' -TTC-
3"-AAG-

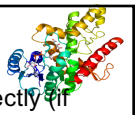
Enzyme X
5' -TTC-
3"-AAG-

5' -TAATTT
3' -ATTAAA

5' -CCGTTAGTT
3' -GGCAATCAA

5' -CAAGCGTTAGGACC
3' -GTTCGCAATAATGG

RFLP



DNA can be processed by RFLP either directly (if you can get enough DNA from an environment) or from PCR product

T-RFLP (terminal-RFLP) is in most respects identical except for a marker on the end of the enzyme

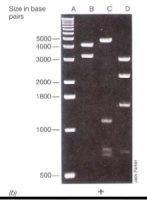
Works as fingerprinting technique because different organisms with different DNA sequences will have different lengths of DNA between identical units targeted by the restriction enzymes

- specificity can again be manipulated with PCR primers

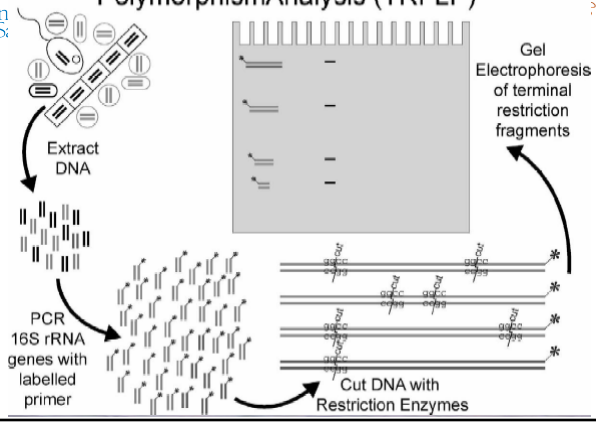
Liu et al. (1997) Appl Environ Microbiol 63:4516-4522

Electrophoresis

Fragmentation products of differing length are separated – often on an agarose gel bed by electrophoresis, or using a capilarry electrophoretic separation



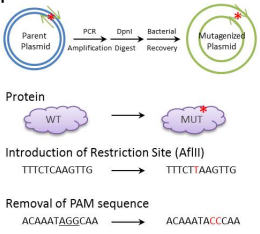
Terminal Restriction Fragment Length PolymorphismAnalysis (TRFLP)



Mutagenesis

Purpose
- Study regulatory regions of the genes
- Study structure-function relationship of protein
- Alter activity of enzymes or proteins

Types
- Random Mutagenesis
- Site-directed Mutagenesis



Error Prone PCR

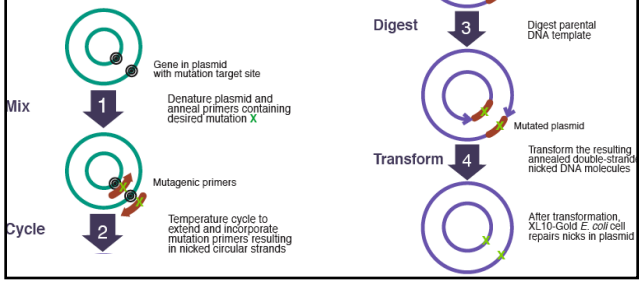
- Non-proofreading polymerase, i.e. Taq
- Low annealing temperature
- Low/unequal dNTP concentration
- High Mg^{2+}
- High cycle number 40-80
- Incorporation of Mn^{2+} ion (0.5-.5 mM)

Site-Directed Mutagenesis

SnapGene Video

PCR-based mutagenesis
- stratagene Quickchange

Addgene Website



Real Time PCR

Real-time (rt) or quantitative (q)PCR is a sensitive and reliable method for detection and quantitation of nucleic acids (DNA, cDNA & RNA) levels.

- Based on detection and quantificaiton of fluorescence emitted from reporter molecule at real time during thermocycling (PCR) process
- Detection Occurs during accumulation of PCR product at EACH CYCLE of amplification during early and exponential phase where the first significant increase is the amount of PCR product correlates to the initial amount of target template
- Differs from traditional "endpoint" assays – gel of PCR product/Southern blot or mRNA blotting (northern blot)

What is rtPCR used for?

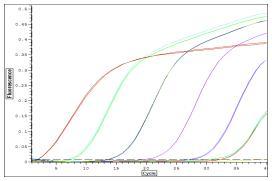
Real-Time PCR has become a cornerstone of molecular biology:

Gene expression analysis
- Cancer research
- Drug research

Disease diagnosis and management
- Viral quantification

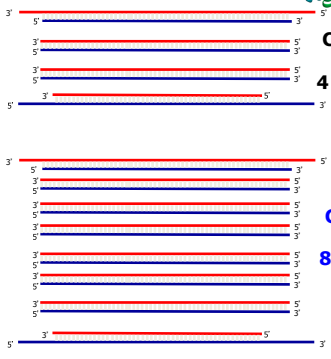
Food testing
- Percent GMO food

Animal and plant breeding
- Gene copy number



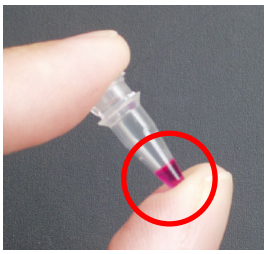
How does rtPCR Work?

Consider traditional PCR



Imagining Real-Time PCR

To understand real-time PCR, let's imagine ourselves in a PCR reaction tube at cycle number 25…



Imagining Real-Time PCR

What's in our tube, at cycle number 25?

A soup of nucleotides, primers, template, amplicons, enzyme, etc.

1,000,000 copies of the amplicon right now.

Imagining Real-Time PCR

How did we get here?

What was it like last cycle, 24?

Almost exactly the same, except there were only 500,000 copies of the amplicon.

And the cycle before that, 23?

Almost the same, but only 250,000 copies of the amplicon.

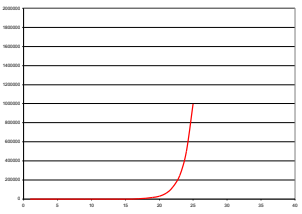
And what about cycle 22?

Not a whole lot different. 125,000 copies of the amplicon.

Imagining Real-Time PCR

How did we get here?

If we were to graph the amount of DNA in our tube, from the start until right now, at cycle 25, the graph would look like this:

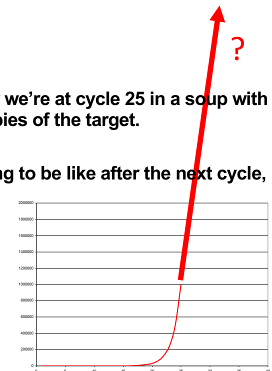


Imagining Real-Time PCR

How did we get here?

So, right now we're at cycle 25 in a soup with 1,000,000 copies of the target.

What's it going to be like after the next cycle, in cycle 26?



Imagining Real-Time PCR

So where are we going?

What's it going to be like after the next cycle, in cycle 26?
Probably there will be 2,000,000 amplicons.
And cycle 27?
Maybe 4,000,000 amplicons.
And at cycle 200?
In theory, there would be
1,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000 amplicons…
Or 10^35 tonnes of DNA…
To put this in perspective, that would be equivalent to the weight of ten billion planets the size of Earth!!!!

Imagining Real-Time PCR

So where are we going?

A clump of DNA the size of ten billion planets won't quite fit in our PCR tube anymore.

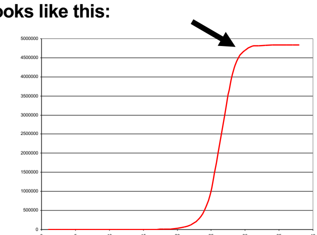
Realistically, at the chain reaction progresses, it gets exponentially harder to find primers, and nucleotides. And the polymerase is wearing out.

So exponential growth does not go on forever!

Imagining Real-Time PCR

So where are we going?

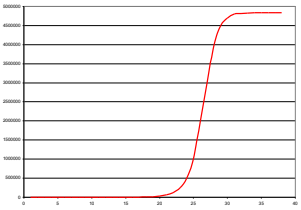
If we plot the amount of DNA in our tube going forward from cycle 25, we see that it actually looks like this:



Imagining Real-Time PCR

Measuring Quantities

How can all this be used to measure DNA quantities??



Imagining Real-Time PCR

Measuring Quantities

Let's imagine that you start with <u>four</u> times as much DNA as I do…picture our two tubes at cycle 25 and work backwards a few cycles.

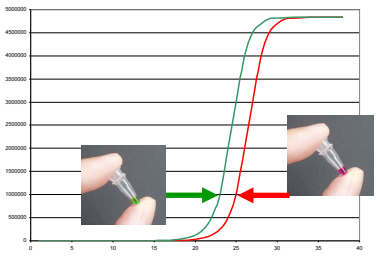
Cycle	Me	You
23	250,000	1,000,000
24	500,000	2,000,000
25	1,000,000	4,000,000

Imagining Real-Time PCR

Measuring Quantities

So, if YOU started with FOUR times as much DNA template as I did…

…Then you'd reach 1,000,000 copies exactly TWO cycles earlier than I would!



Imagining Real-Time PCR

Measuring Quantities

What if YOU started with EIGHT times LESS DNA template than I did?

Cycle	Me	You
25	1,000,000	125,000
26	2,000,000	250,000
27	4,000,000	500,000
28	8,000,000	1,000,000

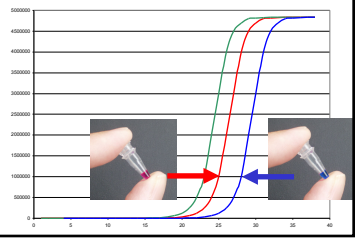
Imagining Real-Time PCR

Measuring Quantities

What if YOU started with EIGHT times LESS DNA template than I did?

You'd only have 125,000 copies right now at cycle 25…

And you'd reach 1,000,000 copies exactly THREE cycles later than I would!



Imagining Real-Time PCR

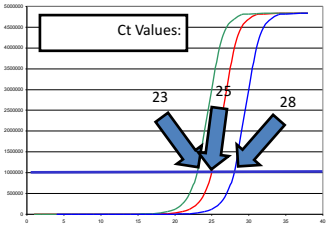
Measuring Quantities

We describe the position of the lines with a value that represents the cycle number where the trace crosses an arbitrary threshold.

This is called the "Ct Value".

Ct values are directly related to the starting quantity of DNA, by way of the formula:

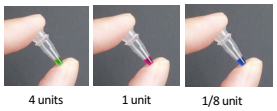
Quantity = $2^{\wedge Ct}$



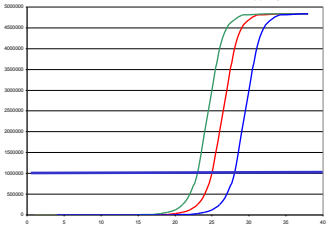
Imagining Real-Time PCR

Measuring Quantities

Let's recap…



4 units Ct=23 | 1 unit Ct=25 | 1/8 unit Ct=28

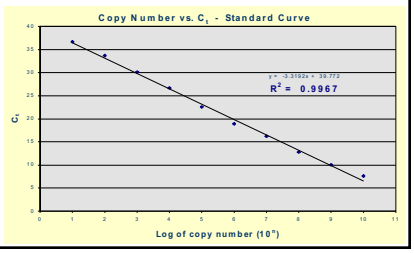


Imagining Real-Time PCR

Measuring Quantities

There's a DIRECT relationship between the starting amount of DNA, and the cycle number that you'll reach an arbitrary number of DNA copies (Ct value).

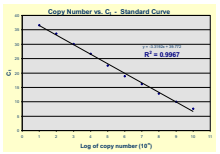
DNA amount ≈ 2 Cycle Number



Imagining Real-Time PCR

Measuring Quantities

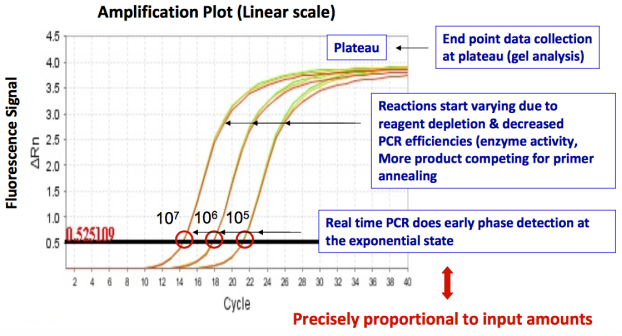
How sensitive is Real-Time PCR?



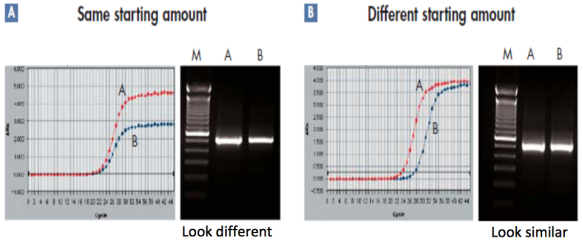
Ultimately, even a single copy can be measured! In reality, typically about 100 copies is around the minimum amount.

One hundred copies of a 200-bp gene is equivalent to just twenty attograms (2×10^{-17} g) of DNA!

Understanding Kinetics of rtPCR



End Point Detection

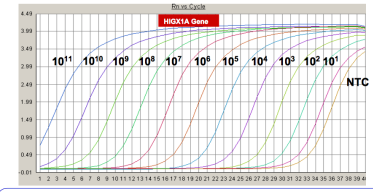


The RNA was reversed transcribed to cDNA followed by real-time or end point analysis by gel electrophoresis. (A) same starting amounts of RNA; (B) Different starting amounts. Y, in linear scale; M: DNA marker.

Wide Dynamic Range

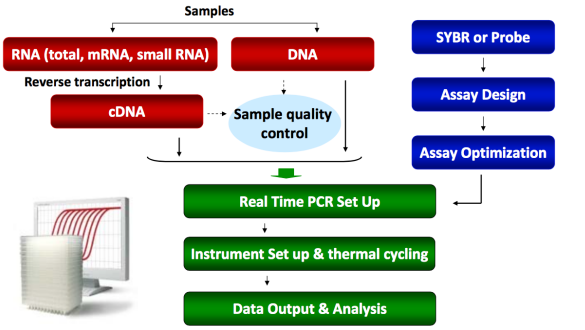
- Detect the reporter signals at early and exponential phase
- Precisely detect the amplicon during each cycle proportional to the initial template amounts
- Increased dynamic range of detection over traditional PCR
- Increased sensitivity: detect as small as a 2-fold change
- No-Post PCR processing necessary.

DEFINITION: The range of initial template concentrations over which accurate measurement can be achieved

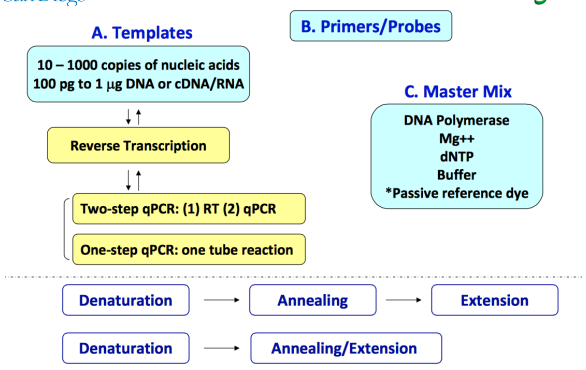


At least 7-log difference range can be detected to accommodate varying initial sample input amounts/gene expression levels, in contrast to <2-log difference by traditional PCR (End point analysis by gel electrophoresis)

How do we measure DNA in rtPCR?



Rt Steps – An Overview



Detection

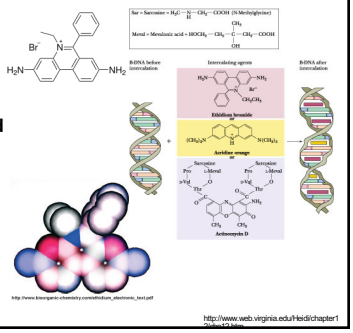
We use reagents that fluoresce in the presence of amplified DNA!

Ethidium bromide and SYBR Green I dye are two such reagents.

They bind to double-stranded DNA and emit light when illuminated with a specific wavelength.

SYBR Green I dye fluoresces much more brightly than ethidium.

Ethidium Bromide

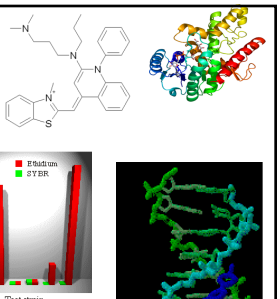


http://www.web.virginia.edu/Heidi/chapter1

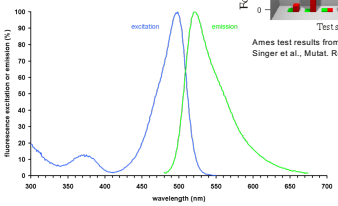
SYBER Green I

Dye binds to DNA in Major groove. Only after interaction (intercalation) with pi bond orbitals will the dye fluoresce, excitation of blue light (lambda max=497nm) and emission of green (520 nm)

- Non mutagenic (Ames test)

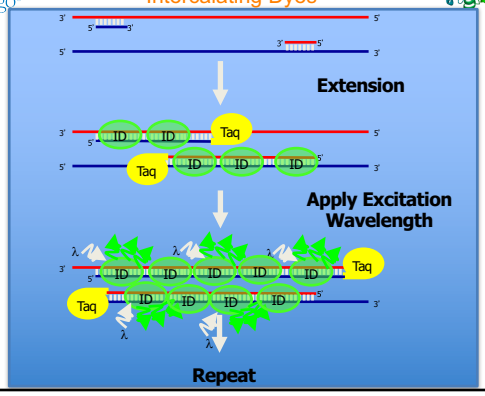


Ames test results from Molecular Probes
Singer et al., Mutat. Res. 1999, 439: 37- 47



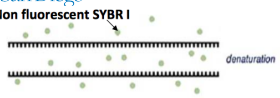
Fluorescent Dyes in PCR

Intercalating Dyes

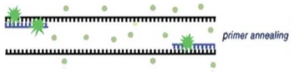


Fluorescent DNA Binding Dye

Non fluorescent SYBR I

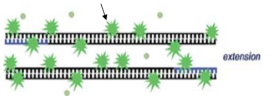


SYBR I binds to double-strand DNA but not single strand DNA. Little fluorescence emitted from SYBR I in solution.



SYBR I upon binding to double-strand DNA emits fluorescence very brightly
Simple & cost saving

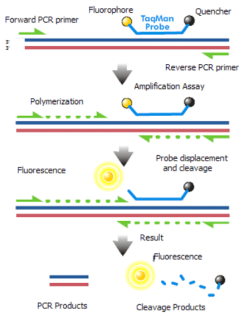
Fluorescent SYBR I



The SYBR I signal intensities correlate with DNA amplified (amplicon amount) thus the initial **sample** input amounts

High Specificity Is Required when using SYBR Green
since SYBR I binds all double-strand DNA (non-specific or primer dimmer).

Hydrolysis Based Probe Taqman Probe Assay



The fluorescence of the reporter dye is suppressed by the quencher

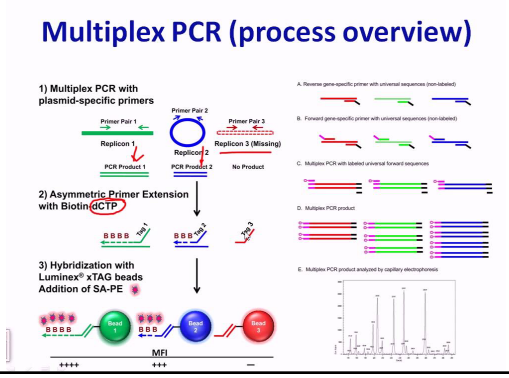
Primer binding followed by extension

Probe cleavage by Taq to free the reporter dye thus the fluorescence intensity correlates with the initial sample input amounts.
Taq has 5'→ 3' exonuclease activity

Each amplicon needs a sequence-specific probe (cost & time)

Multiplexing – Read several genes at one time.

Multiplex PCR (process overview)



Matching Dye and Quencher for Multiplexing is not Trivial

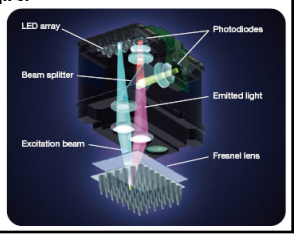
Instrument	Dye 1	Dye 2	Dye 3	Dye 4	Dye 5
Applied Biosystems 7300	FAM	HEX™ or JOE			
Applied Biosystems 7500	FAM	HEX™ or JOE	Cy3 or Tye™ 563	TEX 615™	Cy5 or Tye™ 665
Applied Biosystems 7900	FAM	HEX™ or JOE			
Applied Biosystems StepOne™	FAM	HEX™ or JOE			
Applied Biosystems StepOnePlus™	FAM	HEX™ or JOE	TAMRA		
Applied Biosystems ViiA™ 7	FAM	HEX™ or JOE	TAMRA		
Bio-Rad CFX384™	FAM	HEX™ or JOE	Cy3 or Tye™ 563	TEX 615™	Cy5 or Tye™ 665
Bio-Rad CFX96™	FAM	HEX™ or JOE	Cy3 or Tye™ 563	TEX 615™	Cy5 or Tye™ 665
Bio-Rad iCycler iQ™	FAM	HEX™ or JOE	Cy3 or Tye™ 563	TEX 615™	Cy5 or Tye™ 665
Bio-Rad Miniopticon™	FAM	HEX™ or JOE			
Bio-Rad MyiQ™2	FAM	HEX™ or JOE			
Bio-Rad MyiQ™5	FAM	HEX™ or JOE	TAMRA	TEX 615™	Cy5 or Tye™ 665
Roche Lightcycler® 480	FAM	HEX™ or JOE			
Stratagene Mx3000P™	FAM	HEX™ or JOE	C		
Stratagene Mx3005P™	FAM	HEX™ or JOE	C		

Dye	Excitation Wavelength	Emission Wavelength	Dark Quencher
FAM	495	520	Iowa Black® FQ/ZEN™
HEX™	538	555	Iowa Black® FQ/ZEN™
Cy3	550	564	Iowa Black® RQ
TEX 615™[2]	596	613	Iowa Black® RQ
LC Red 640[1]	620	635	Iowa Black® RQ
Tye™ 665	645	665	Iowa Black® RQ
Cy5	648	668	Iowa Black® RQ

What Type of Instruments are used with Real-Time PCR?

Real-time PCR instruments consist of THREE main components:

1. **Thermal Cycler (PCR machine)**
2. **Optical Module (to detect fluorescence in the tubes during the run)**
3. **Computer (to translate the fluorescence data into meaningful results)**

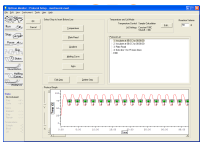


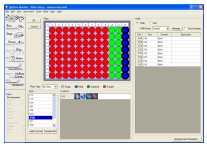
What Type of Software is used with Real-Time

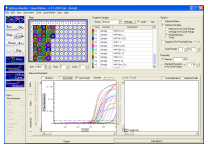
The real-time software converts the fluorescent signals in each well to meaningful data.

1. **Set up PCR protocol.**
2. **Set up plate layout.**
3. **Collect data.**
4. **Analyze data.**

1 2 3,4



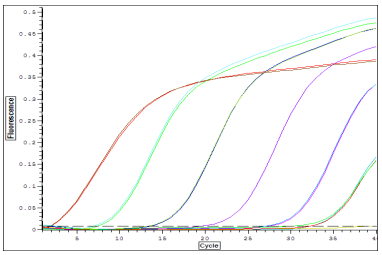




Real-Time PCR Actual Data

This is some actual data from a recent real-time PCR run.

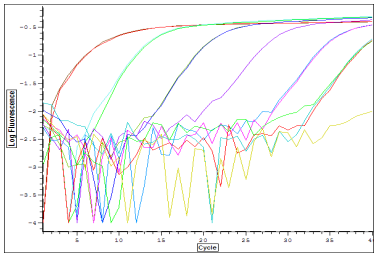
Data like this can easily be generated by preparing a dilution series of DNA.



The same data set in log view

Real-Time PCR

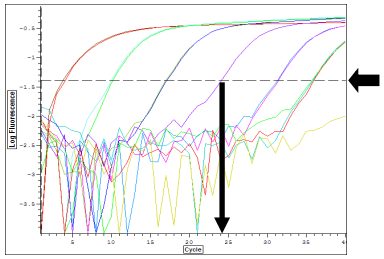
Actual Data



Once threshold is set, Ct values can be calculated automatically by software.

Real-Time PCR

Setting Thresholds



- Ct values can then be used to calculate quantities of template DNA.

Amplification Plots and Terms

Before nucleic acid level can be qualified, the raw data must be analyzed and baseline & threshold values set

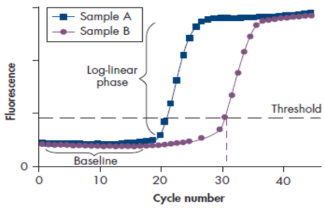
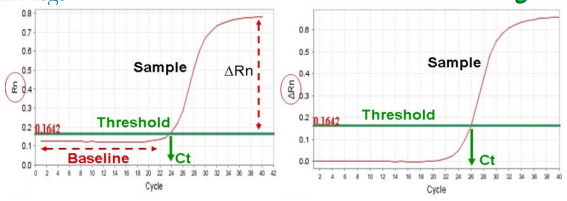


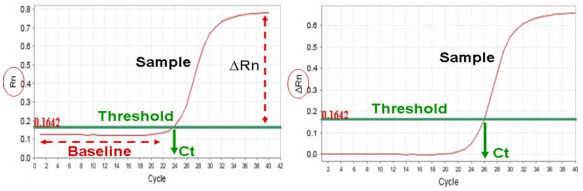
Figure 8. Typical amplification plot. Amplification plots showing increases in fluorescence from 2 samples (A and B). Sample A contains a higher amount of starting template than sample B. The Y-axis is on a linear scale.

Amplification Plots and Terms



- **Rn:** Fluorescence signal normalized with passive reference dye signal (Fluorescence/dye signal)
- **Passive Reference Dye**: An internal reference to correct well to well non-PCR fluctuations. **ROX** is the most commonly used reference dye.
- **ΔRn**: Fluorescence signal with baseline subtracted (background signal subtracted)
- **Baseline**: background noise level before a significant amplification occurs (3-15 cycles)

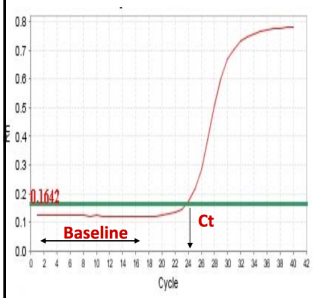
Amplification Plots and Terms



- **Threshold**: At which significant & specific amplification occurs (auto or manual set)
  - exponential phase (linear doubling phase of amplification)
  - above the highest baseline values.
  - 10 x Rn standard deviations
- **C_t (Threshold cycle)**: The cycle number at which the fluorescence signal crosses threshold. Also called **Cp (Cross point cycle)** for LightCycler terminology.
  - **inversely correlates with initial template concentrations (amounts)**

Setting up Baseline

Linear Amplification Plot



-Automated Baseline Option

if an instrument has a adaptive baseline function

-Define manually:

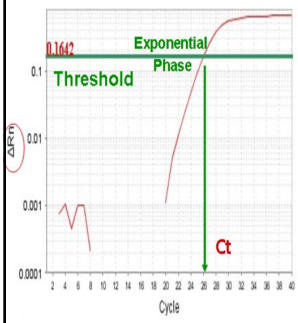
(1) Use linear view of the plot

(2) Set up the baseline reading from cycle #2 to the cycle that 2 cycles before the earliest visible amplification

(3) Usually a baseline falls in **3-15** cycles

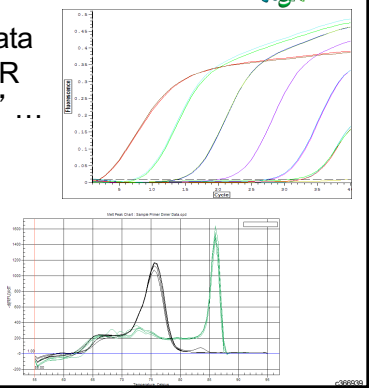
Defining Threshold

Log View Amplification Plot



- Use **log view** of amplification plot
- Threshold should be higher than baseline (higher than the noise level)
- Threshold should at **LOWER** 1/3 or 1/2 of the linear phase of amplification
- **Linear phase = exponential phase**
- Different runs across samples for the same experiments should have **the same threshold** for comparison

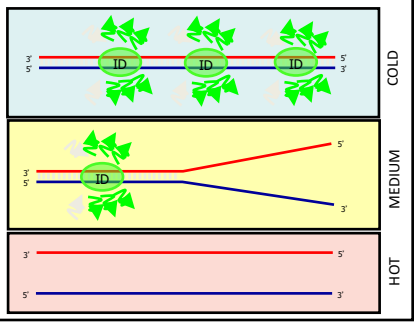
The fluorescence data collected during PCR tells us "how much" … but there is another type of analysis we can do that tells us "what"!



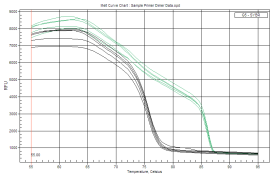
Real-Time PCR – the Concept of MELT CURVES…

Melt curves can tell us what products are in a reaction.

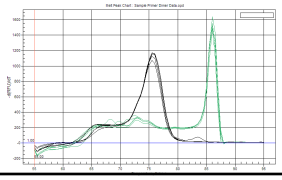
Based on the principle that as DNA melts (becomes single stranded), intercalating dyes will no longer bind and fluoresce.



Melt curves can tell us what products are in a reaction.



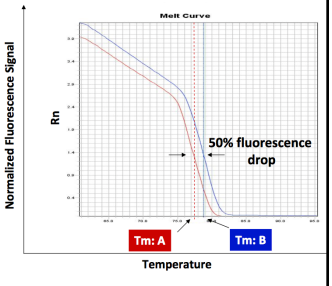
RFU vs T



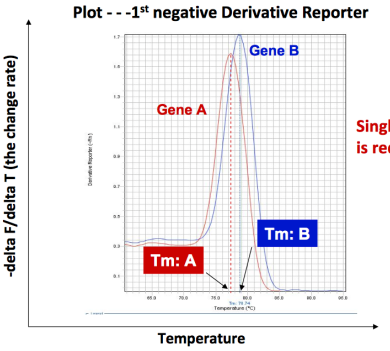
dRFU/dT

Tm & Melt Curve

- This is only for SYBR rtPCR – Taqman is not fluorescent
- When 50% of DNA is melted = Tm. Inflection point on curve
- Best analyzed by derivative (1st negative dF/dT)
- Tm varies based on length, sequence and GC Content.
- Heat to 94oC to denature
- Cool to 60oC to anneal.
- Slowly heat at 0.2oC/sec
- As temp increases DNA melt and the F signal should decrease



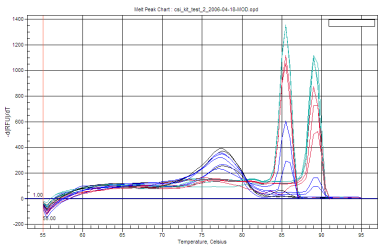
1st Negative Derivative Plot



Single melt curve of each amplicon is required for specificity validation!

Different amplicons will have different melt peaks.

Primer-Dimers will have a very different melt peak.

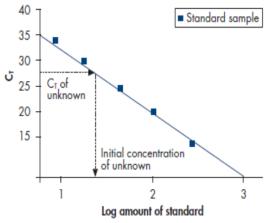


Color key: Green=100X, Red=10000X, Blue=1000000X , Black=NTC.

Absolute Quantification

Typical standard curves showing determination of concentration of sample of interest

Goal: absolute/exact concentration of a sample is desired.



- The known standard & an unknown target should have very similar characteristics:
  - *Amplicon & adjacent sequences
  - *Primer binding sites
  - *PCR efficiencies
  - One standard curve ---- One GOI
  - &
- At least 5 serial dilution points needed for the known standard

Limited Throughput - - - Often applied to assays of single to a small number of genes (For example, viral load and copy number counting)

Comparative Ct Method Quantification

Gene Expression Profiling Analysis
Focused on Changes in Gene Expression Across Samples

Any changes upon treatment?

Gene of interest A in untreated cells — **Ct-untreated**

GOI A in drug treated cells — **Ct-treated**

by relative measurement

A Fold Change of Expression = $2^{\text{delta (Ct-treated - Ct-untreated)}}$
(Up or down regulation)
$= 2^{\Delta Ct\ (Treated - Untreated)}$

Housekeeping/Reference Genes for Normalization

Any changes?

Gene of interest A in untreated cells — **GOI A in drug treated cells**

Reference Gene B in untreated cells — **Ref Gene B in drug treated cells**

The expression level of a reference gene remain consistent under experimental conditions or different tissues

- **A Reference Gene** is aimed to normalize possible variations during:
  - Sample prep & handling (e.g use the same number of cells from a start)
  - RNA isolation (RNA quality and quantity)
  - Reverse transcription efficiency across samples/experiments
  - PCR reaction set up
  - PCR reaction amplification efficiencies

Normalization Calculation

Any changes?

Target Gene A in control cells	Target Gene A in drug treated cells
Reference Gene B in control cells	Ref Gene B in drug treated cells

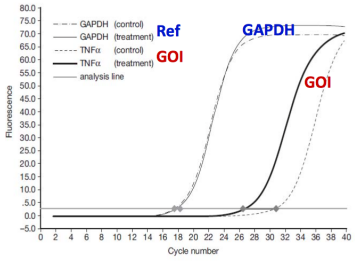
→ ΔCt1 = Ct (Target A -treated) – Ct (Ref B-treated)

→ ΔCt2 = Ct (Target A-control) – Ct (Ref B-control)

→ Δ Δ Ct = ΔCt1 (treated) – ΔCt2 (control)

Normalized target gene expression level = $2^{\Delta\Delta Ct}$

DD Ct Analysis



$\Delta\Delta Ct = \Delta Ct\ (TNF\alpha_{treat}\text{-}GAPDH_{treat}) - \Delta ct\ (TNF\alpha_{control}\text{-}GAPDH_{control})$

The fold change = $2^{\Delta\Delta Ct}$

Gene	Gene symbol: Human	Gene symbol: Mouse	Relative expression level*: Human	Relative expression level*: Mouse
18S ribosomal RNA	RRN18S	Rn18s	++++	++++
Actin, beta	ACTB	Actb	+++	+++
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Gapdh	+++	+++
Phosphoglycerate kinase 1	PGK1	Pgk1	+++	++
Peptidylprolyl isomerase A	PPIA	Ppia	+++	+++
Ribosomal protein L13a	RPL13A	Rpl13a	+++	+++
Ribosomal protein, large, P0	RPLP0		+++	
Acidic ribosomal phosphoprotein PO		Arbp		+++
Beta-2-microglobulin	B2M	B2m	++ – +++	++ – +++
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ	Ywhaz	++ – +++	+
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	SDHA	Sdha	++	+
Transferrin receptor	TFRC	Tfrc	++	+
Aminolevulinate, delta-, synthase 1	ALAS1	Alas1	+	+
Glucuronidase, beta	GUSB	Gusb	+	+
Hydroxymethylbilane synthase	HMBS	Hmbs	+	++ – +++
Hypoxanthine phosphoribosyltransferase 1	HPRT1	Hprt1	+	+
TATA box binding protein	TBP	Tbp	+	+
Tubulin, beta	TUBB		+	
Tubulin, beta 4		Tubb4		+

* "+" indicates relative abundance of the transcripts.