UNCC Biotechnology and Bioinformatics Camp

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Summer 2010

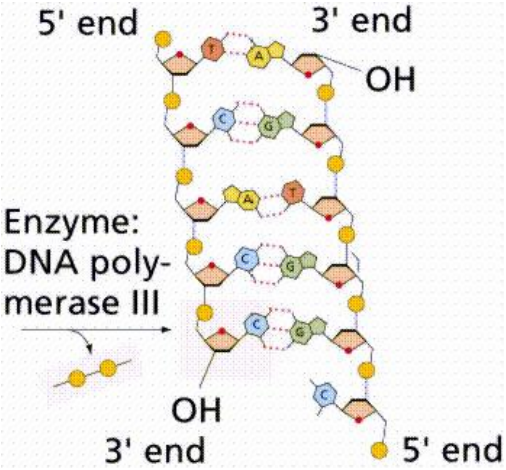
PCR

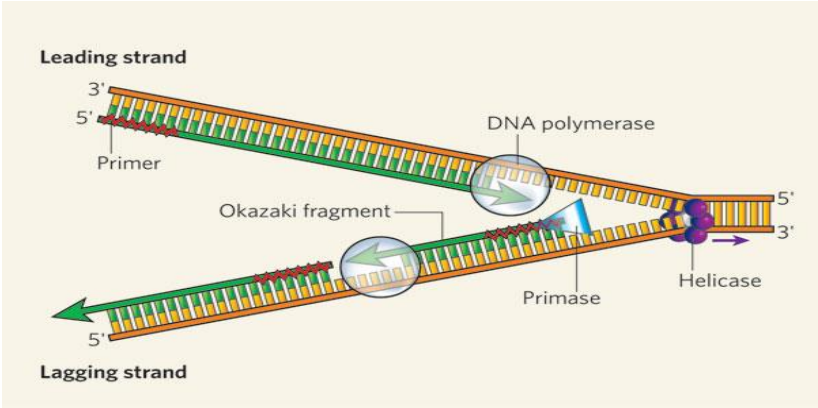
- Topic: the Polymerase Chain Reaction (PCR)
  - 1988, Saiki, Mullis et al. proposed using a heat stable polymerase to carry out a method outlined years earlier
    - Purified from an organism characterized by Brock from a hot spring in Yellowstone N.P.
- Problem addressed: how do you get enough genetic material to characterize it in the lab?
  - Tens of thousands of specific sequences and millions to billions of bases in any genome
    - How do you pull out and manipulate the 3000 nucleotides of interest?

DNA Polymerases

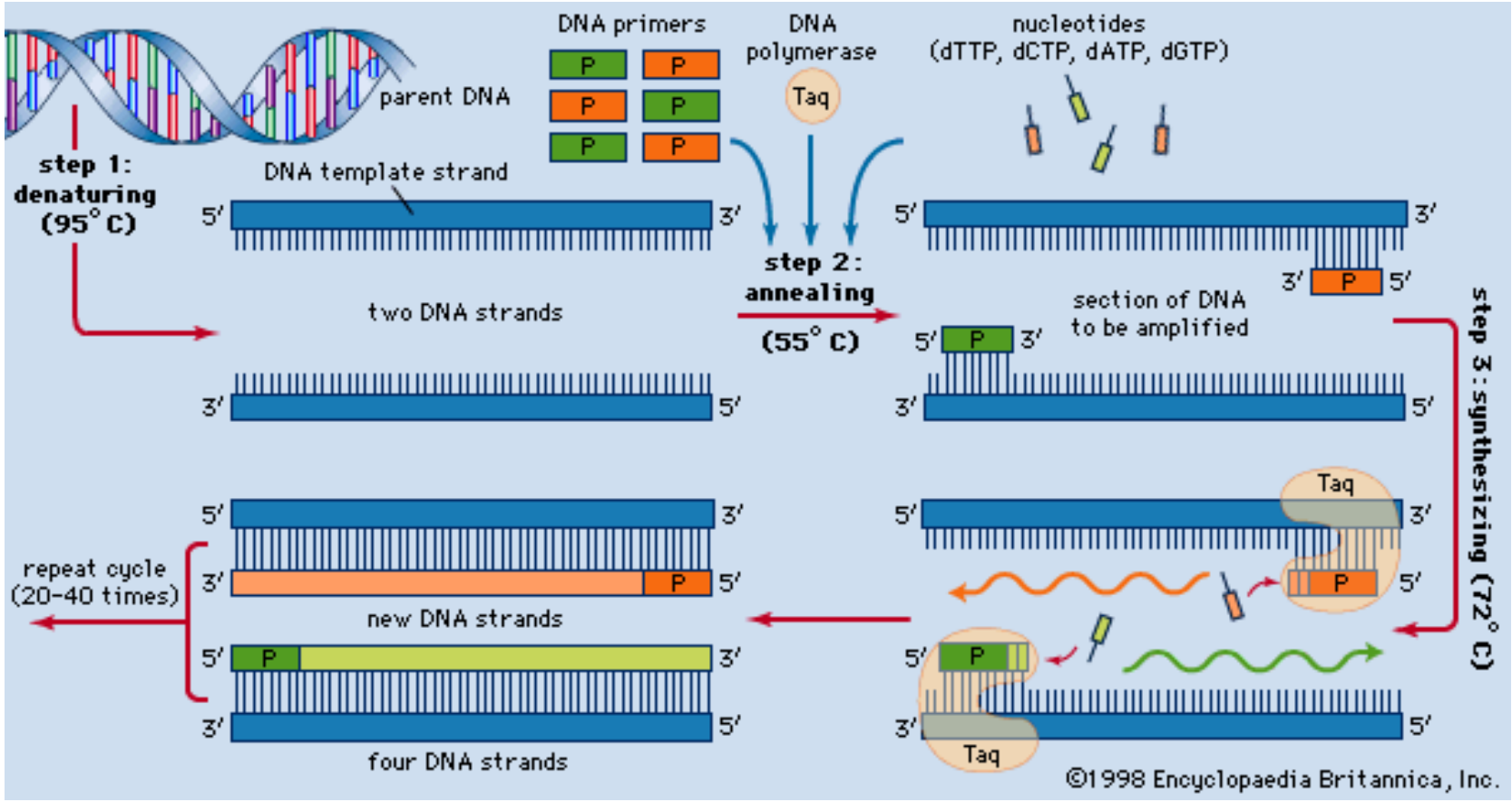
- Polymerases are enzymes that make polymers: attach subunits (monomers) to each other covalently to make a long chain.
- Some nucleotide polymerases are 'template free' and randomly string together nucleotides.
- Most polymerases make a complementary copy of a strand of existing nucleic acid.
- Functionally: the polymerase adds subunits that are complementary to the 3' strand, forming covalent phosphodiester bonds as it goes.
  - As the monomer is added it forms H-bonds

  Across the center of the helix





PCR steps


DNA primers
DNA polymerase
Taq
nucleotides
(dTTP, dCTP, dATP, dGTP)
parent DNA
step 1:
denaturing
(95° C)
DNA template strand
5′
3′
P
two DNA strands
step 2:
annealing
(55° C)
section of DNA
to be amplified
step 3: synthesizing (72° C)
repeat cycle
(20–40 times)
new DNA strands
four DNA strands
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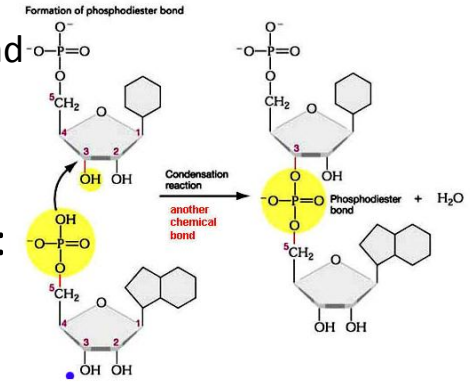
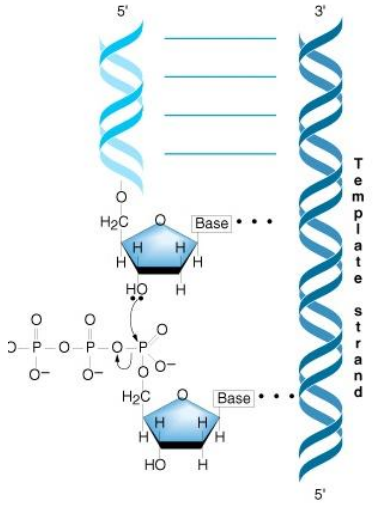
Polymerase steps

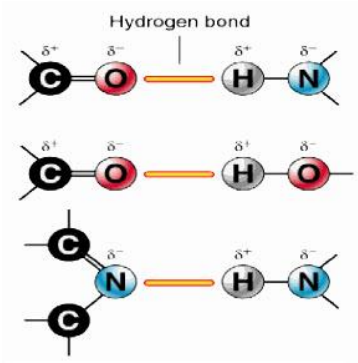
- Template preparation:
  - Template strands must be separated (H-bonds in the center disrupted and base stacking eliminated)
  - Possibilities are: thermal, enzymatic, chemical.
- A place for the polymerase to start must be provided: short complementary sections of DNA are added (6-20nt in length): Primers
- Monomers must be provided to build the new material (nuclecotides)
- Initiation: the polymerase binds to the double-stranded primer-template complex
- Bond catalysis: The polymerase catalyzes the phosphodiester linkage of two initial dNTPs to primer ends: extension
- Elongation: polymerase advances 3' → 5' down template strand, making new duplex DNA
- Termination: at the end of the template the polymerase dissociates from the completed ds molecule.

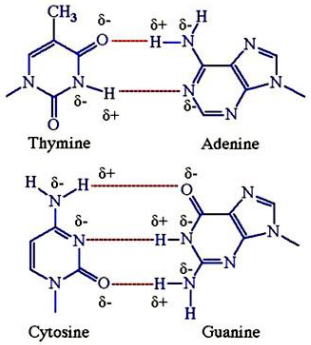


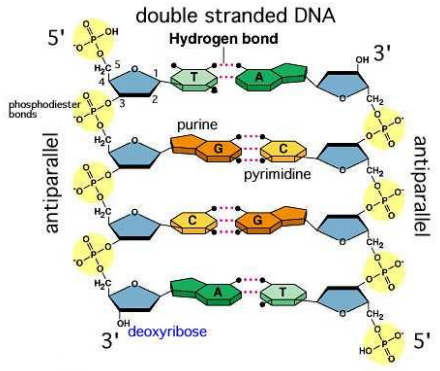


Thermo-cycling

- A polymerase is able to unwind dsDNA, but this happens only during replication or repair.
- How do we force the presence of ssDNA?
  - The easiest way to separate strands and not cause template damage is to use heat. The concept :
    - Thermo-melt duplex DNA so the strands separate, cool the reaction quickly so they don't re-anneal, add short primers that complement the single strand somewhere, add polymerase and the necessary factors, and extend







Taq

- Heat-stable polymerase:
  - Microorganism *Thermus aquaticus* (isolated from a hot spring mat in Yellowstone Park by Brock. Great Fountain in the West Thumb Geyser Basin (photos at Flickr, Russ Finley, James Neeley)
  - The organism and its enzymes are stable at close to 100C, so it survives the repeated rounds of heating.
  - This allows the cycling part of the process to be a one-tube, one-step set-up procedure.

A thermocycler is the instrument in which PCR is performed.

A carefully engineered, *programmable* heating/cooling block
holes for sample tubes and an insulated lid.
The rate of heating or cooling and final temperature are carefully regulated so that results are reproducible.

http://sentrabd.com/_borders/T1b.jpg

Techne
TC-512

Programs

Program
defaults

Data
Logs

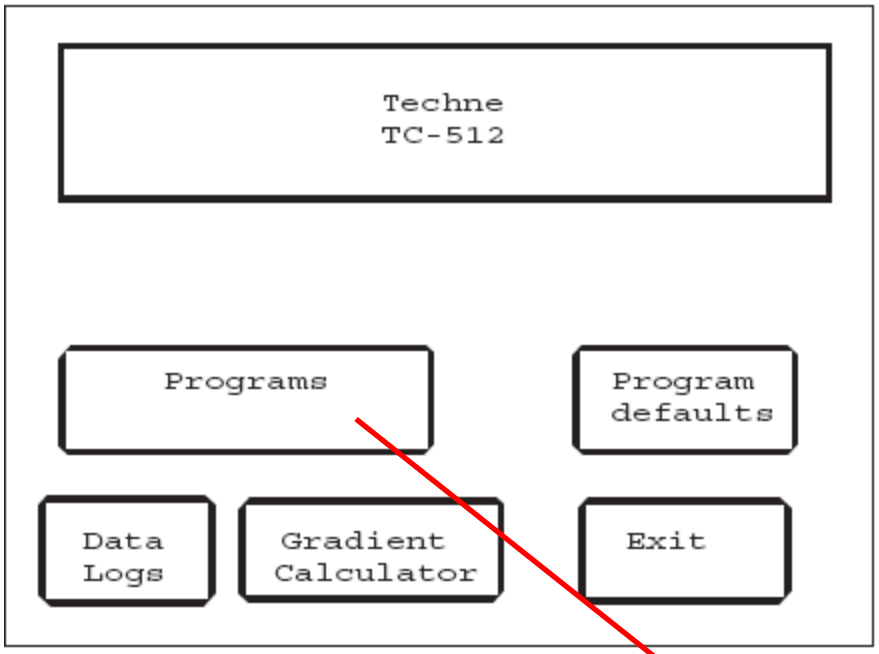
Gradient
Calculator

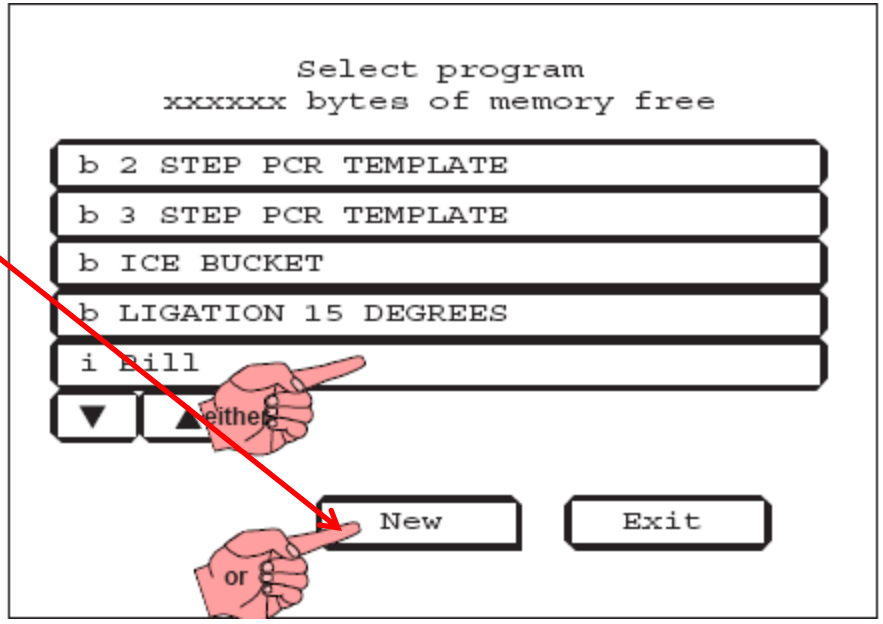
Exit

Set program Defaults

Heated Lid	Off
Heated Lid before program	Off
Pause before program	Off
Initial denaturation 05m00s	94.0°c
Hot Start	94.0°c

Exit


Techne
TC-512
Programs
Program defaults
Data Logs
Gradient Calculator
Exit


Select program
xxxxxx bytes of memory free
b 2 STEP PCR TEMPLATE
b 3 STEP PCR TEMPLATE
b ICE BUCKET
b LIGATION 15 DEGREES
i Bill
either
New
Exit
or

Editing "Your Own"

Initial denaturation	05m00s	94.0°c
Hot start		Disabled
Next step		
Final extension	05m00s	72.0°c
Final hold		10.0°c

▼ ▲

Ins Del Edit

PWord Save As Save Exit

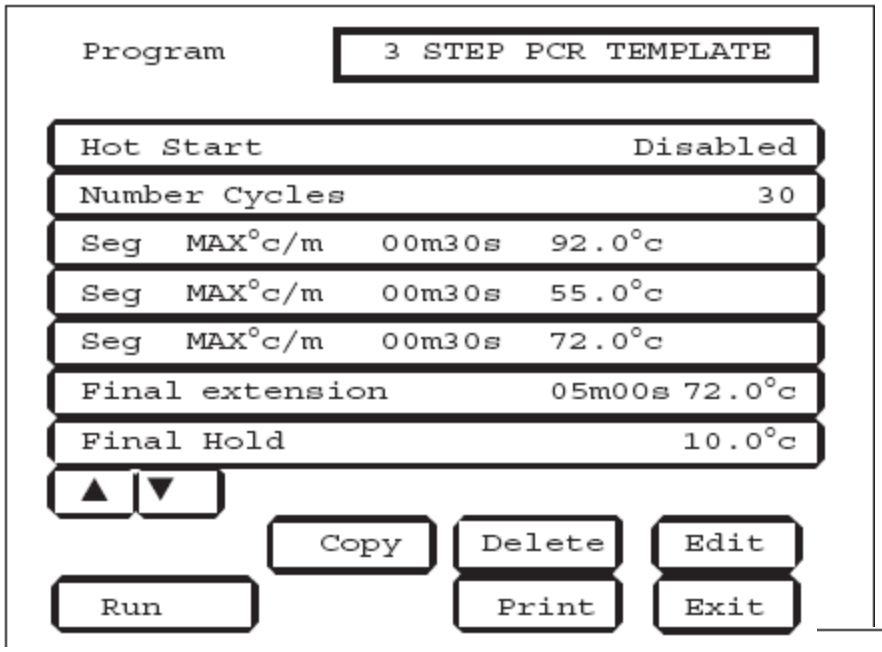
Select program
xxxxxx bytes of memory free

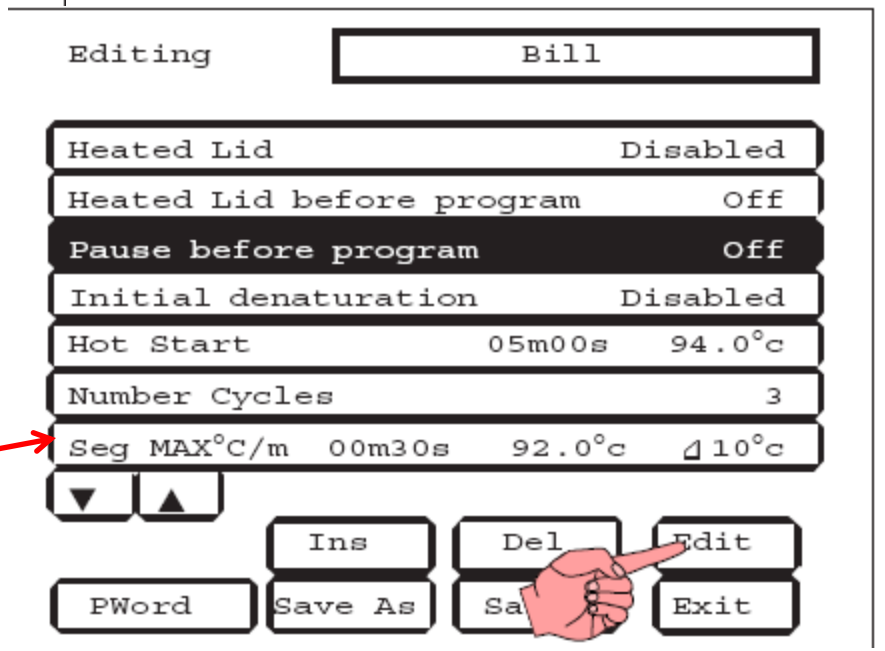
b 2 STEP PCR TEMPLATE
b 3 STEP PCR TEMPLATE
b ICE BUCKET
b LIGATION 15 DEGREES
i Bill

▼ ▲ either

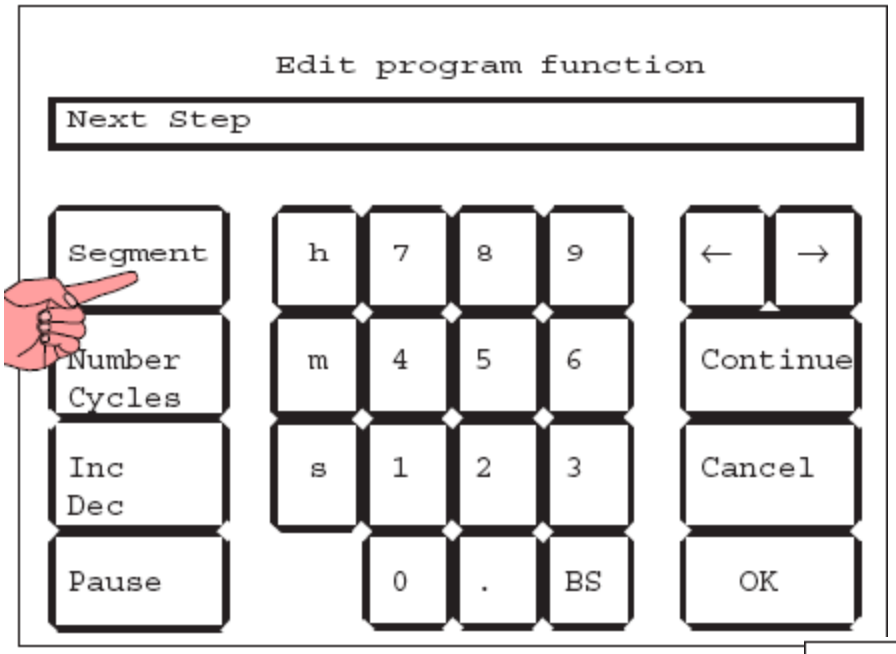
New Exit

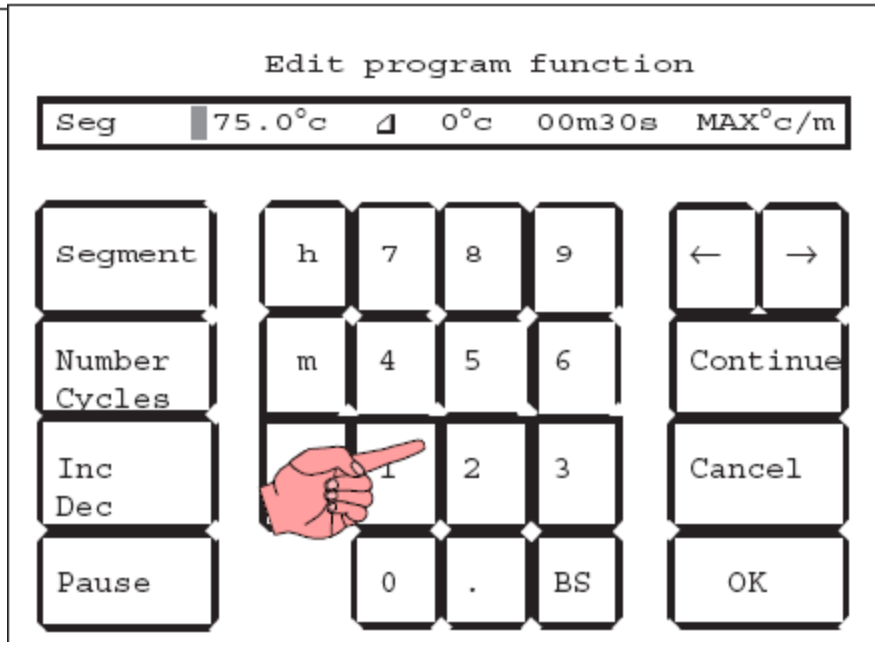
or





Interpret the Segment parts:
Time, temperature and gradient


Edit program function
Next Step
Segment
Number Cycles
Inc Dec
Pause
h
m
s
7
8
9
4
5
6
1
2
3
0
.
BS
←
→
Continue
Cancel
OK


Edit program function
Seg
75.0°c
0°c
00m30s
MAX°c/m
Segment
Number Cycles
Inc Dec
Pause
h
m
7
8
9
4
5
6
1
2
3
0
.
BS
←
→
Continue
Cancel
OK

Edit program function

Next Step

Segment	h	7	8	9	←	→
Number Cycles	m	4	5	6	Disable	
Inc Dec	s	1	2	3	Cancel	
Pause		0	.	BS	OK	

Editing "Your own"

Heated Lid			Disabled
Heated Lid before program			Off
Pause before program			Off
Initial denaturation			Disabled
Hot Start		05m00s	72.0°c
Number Cycles	3		
Seg MAX°c/m	00m35s	75.0°c	⊿ 0°c

▼ ▲

	Ins	Del	Edit
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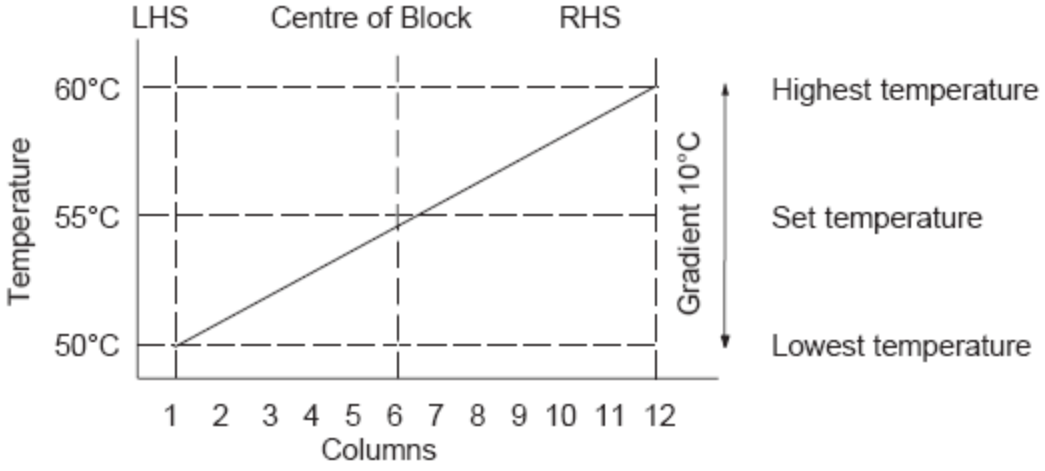
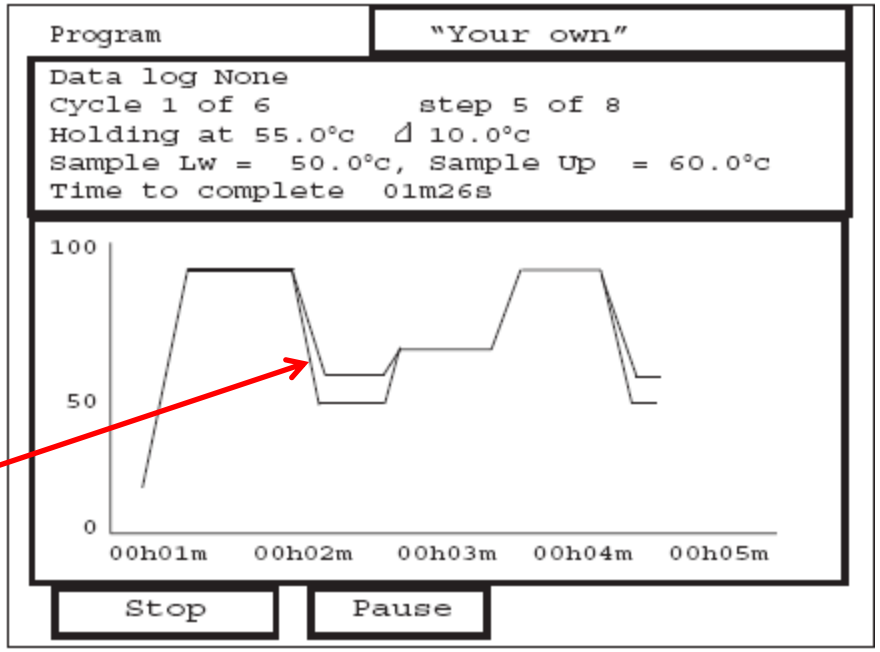
side being the hottest.



Gradient versus ramp rate.
Gradient has the set temperature in the Middle and the lowest temp on the left Side, highest on the Right side.

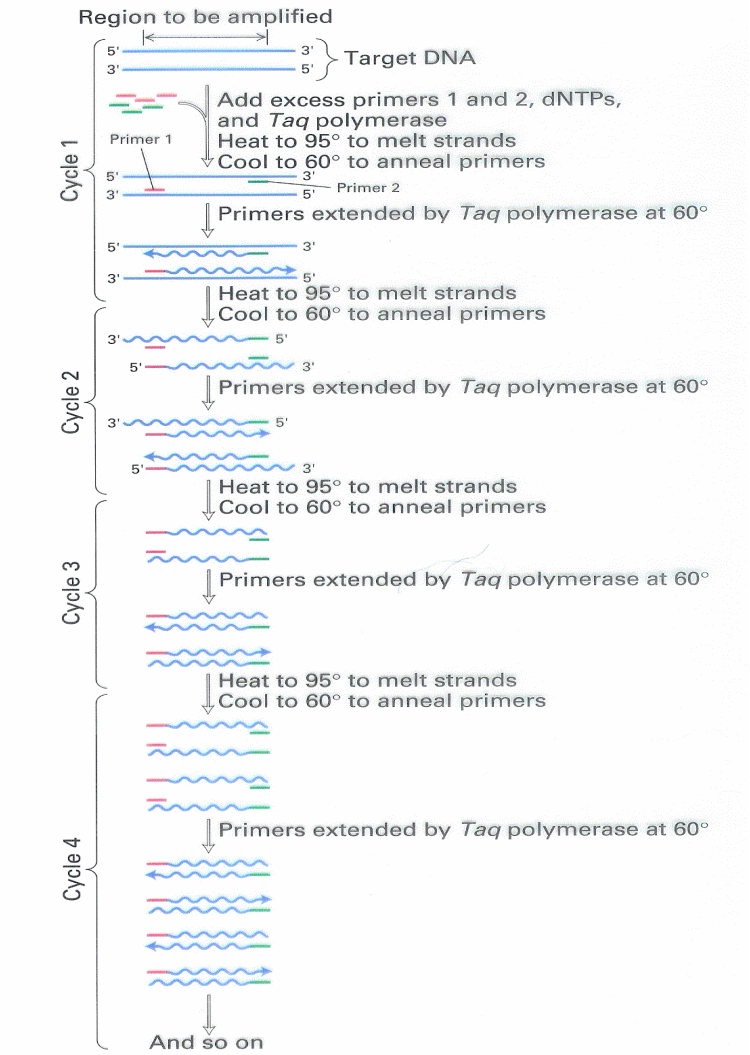
Ramp is how fast you go between temps.



PCR methodology

- What is needed to perform PCR?
  - A template: DNA that has sequences complementary to the primers you will add to the reaction
  - Primers: primers of defined sequence that are complementary to specific regions of the template
  - Subunits (dNTPs) to build the new polymers
  - A thermostable DNA polymerase, such as Taq
  - Conditions for the reactions: Mg++, buffer to stabilize the enzyme and the template
  - A thermocycler
  - A detection method for the product (gel and liquid techniques)

PCR


Region to be amplified
5'
3'
3'
5'
Target DNA
Add excess primers 1 and 2, dNTPs, and *Taq* polymerase
Heat to 95° to melt strands
Cool to 60° to anneal primers
Primer 1
Primer 2
Primers extended by *Taq* polymerase at 60°
Heat to 95° to melt strands
Cool to 60° to anneal primers
Primers extended by *Taq* polymerase at 60°
Heat to 95° to melt strands
Cool to 60° to anneal primers
Primers extended by *Taq* polymerase at 60°
Heat to 95° to melt strands
Cool to 60° to anneal primers
Primers extended by *Taq* polymerase at 60°
And so on
Cycle 1
Cycle 2
Cycle 3
Cycle 4

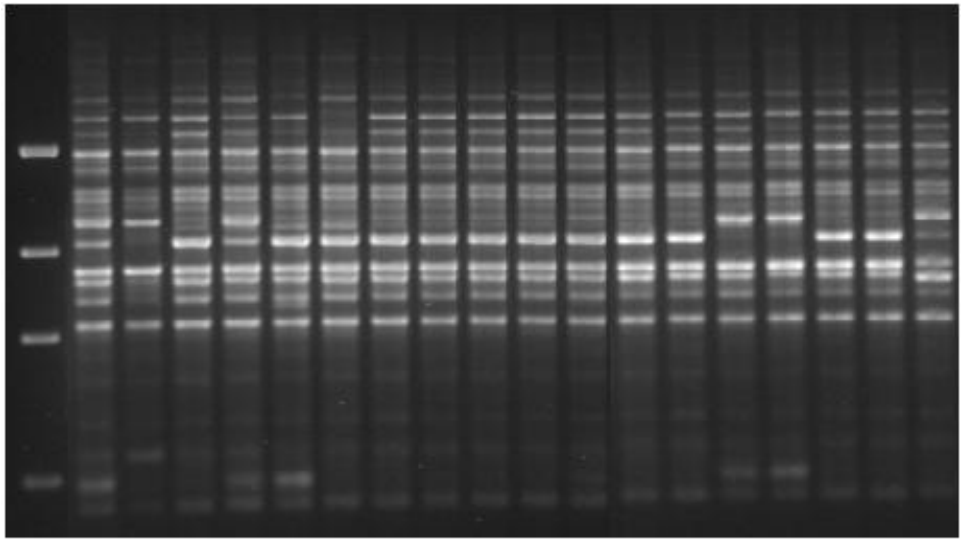
Product Yields

PCR amplification of DNA fragment

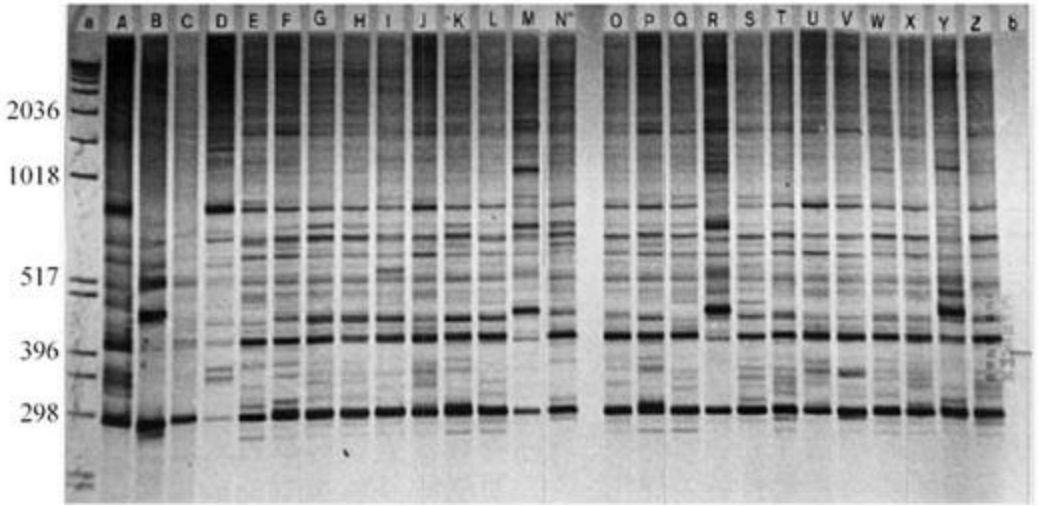
Cycle number	Number of ds target molecules	Cycle number	Number of ds target molecules
1	1	16	32,768
2	2	17	65,536
3	4	18	131,072
4	8	19	262,144
5	16	20	524,288
6	32	21	1,048,576
7	64	22	2,097,152
8	128	23	4,194,304
9	256	24	8,388,608
10	512	25	16,777,216
11	1024	26	33,544,432
12	2048	27	67,108,864
13	4096	28	134,217,728
14	8192	29	268,435,456
15	16,384	30	536,870,912

Theoretically, one can **double** the number of target DNA molecules for each cycle performed (2^n, where n=#cycles). In reality, various factors (*e.g.* decrease in [nucleotides] and [primers], loss of enzyme activity) mean that there is not a perfect doubling of DNA copy numbers with each cycle.

Gel Analysis



Agarose gel



Acrylamide gel

PCR yields non-gel method

- As product increases, primer and dNTPs are used up.
  - The reaction saturates and no more product is made
  - The amount of product depends on the amount of input template and the number of amplification cycles

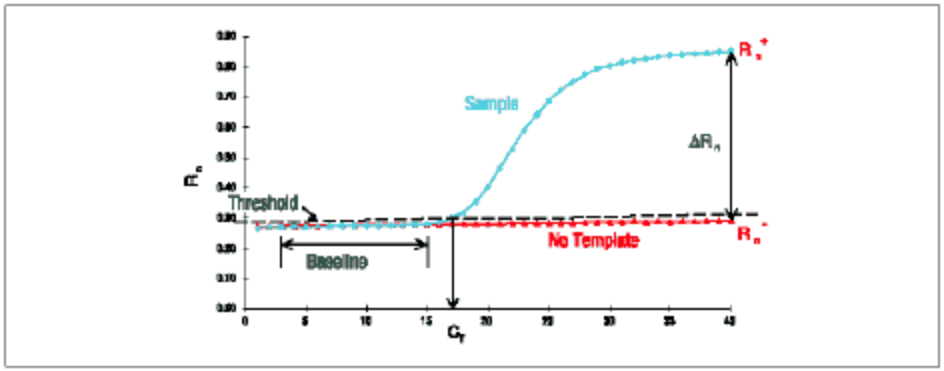


Figure 2. Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR Figure from Applied Biosystems' DNA/RNA Real-Time Quantitative PCR bulletin).

http://dna-9.int-med.uiowa.edu/images/Amplotfig2.gif