Cryphonectria parasitica tendrils on chestnut tree bark (Photo: Ministry of Agriculture and Regional Development Archive, Ministry of Agriculture and Regional Development, Bugwood.org)

6/22/2014

The Polymerase Chain Reaction

B3 Summer Science Camp at Olympic High School

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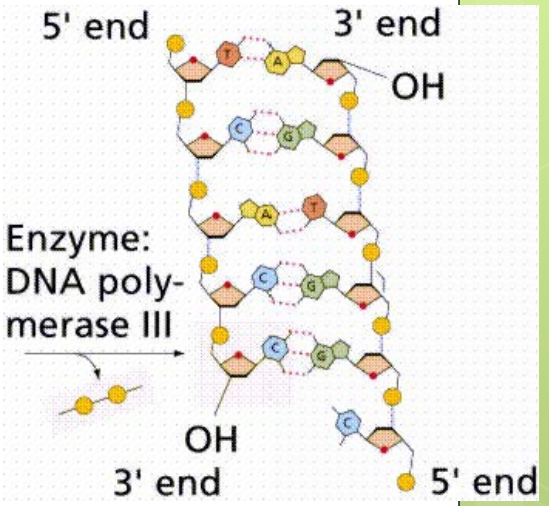
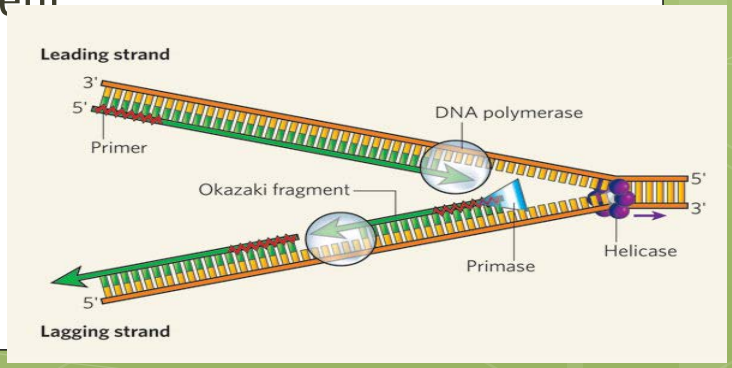
PCR

- Topic: the Polymerase Chain Reaction (PCR)
  - In 1988 Saiki, Mullis et al. proposed using a heat stable DNA polymerase to carry out a method that was outlined several years earlier
    - The enzyme was purified from an organism, characterized by Brock, from a hot spring in Yellowstone N.P.
    - Since it could live at high temperatures its enzymes had to be stable at high temperatures
- Experimental Problem this addressed: how do you get enough DNA to measure it in the lab?
  - There are tens of thousands of genes in a sea of millions to billions of bases in any genome - how do you pull out and manipulate the 3000 nucleotides you care about?

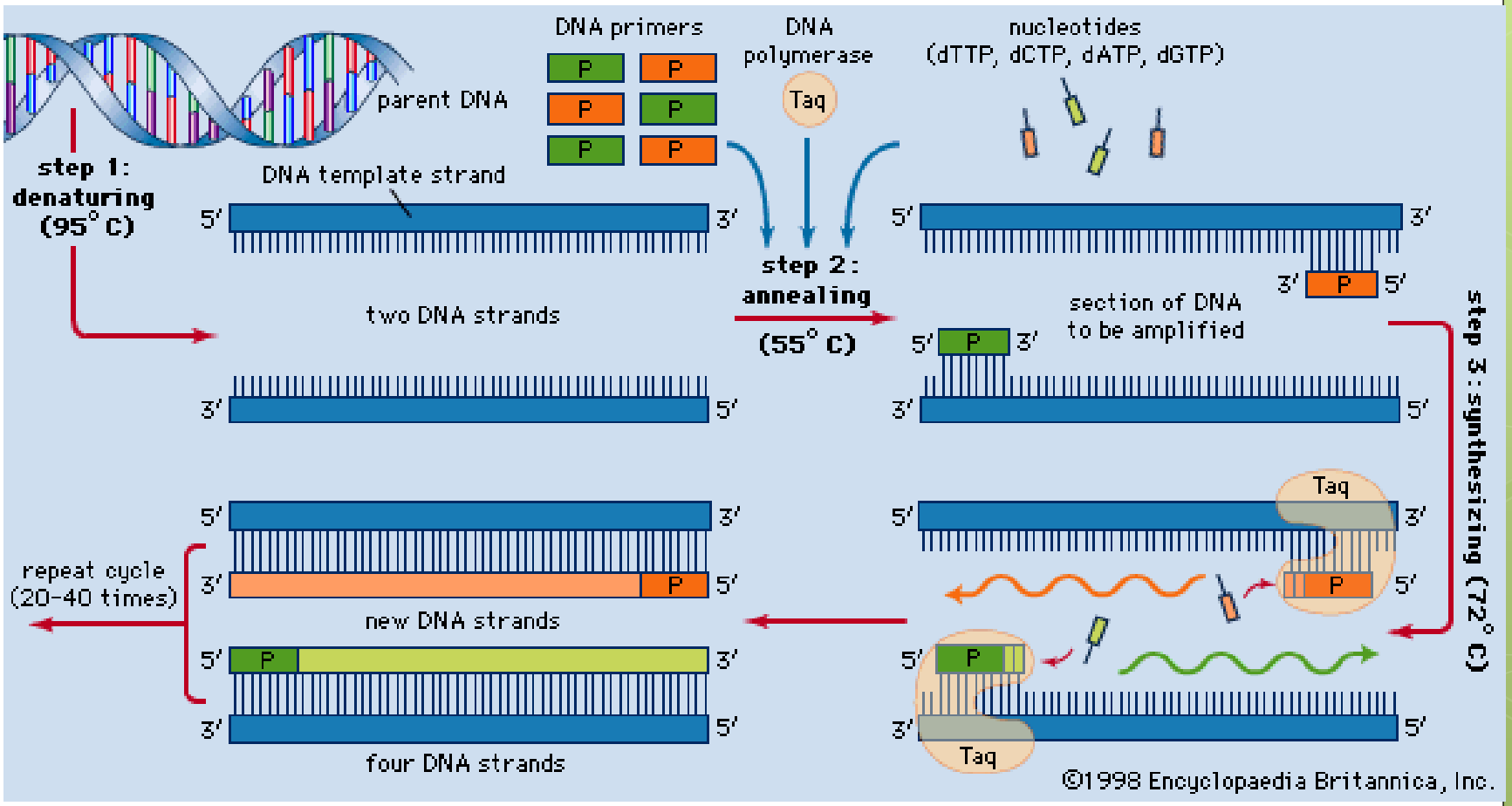
DNA Polymerases



- Polymerases are enzymes that make polymers: they 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 (not an exact 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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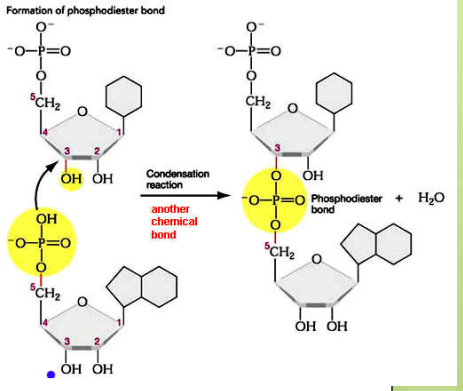
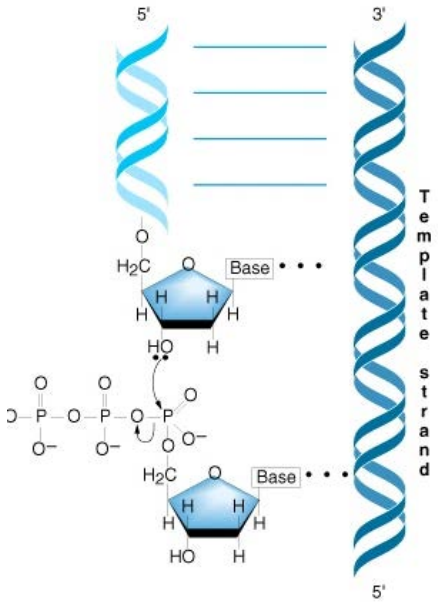
Polymerase steps



- Template (your DNA) 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)

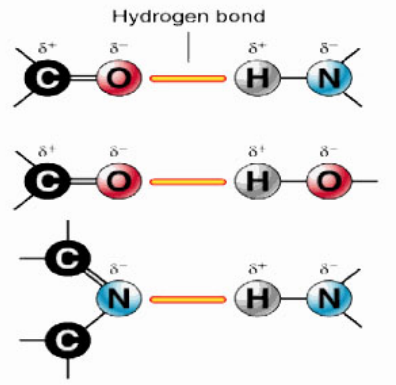


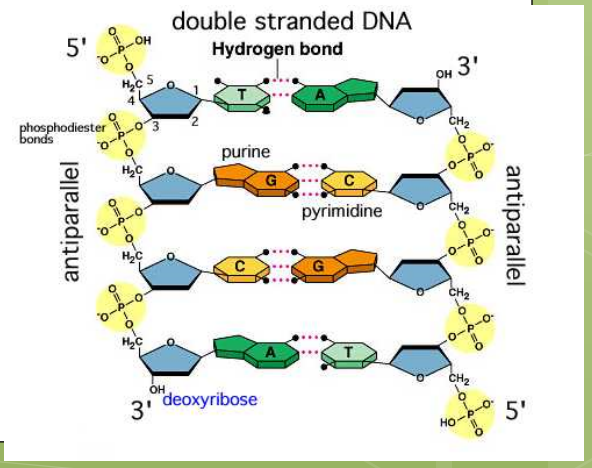
Three basic steps in Polymerization

- 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, which leads to extension of the chain by 1 subunit
- 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 in a test tube?
  - 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 can't find each other to re-anneal, add short primers that complement the single strand somewhere.





Taq

- Heat-stable polymerases: the grand-daddy is Taq
  - Microorganism *Thermus aquaticus*
    - isolated from a hot spring mat in Yellowstone Park by Brock.
    - At the 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 100°C, so it survives the repeated rounds of heating.
  - Experimentally, this allows the cycling part of the process to be a one-tube, one-step set-up procedure.

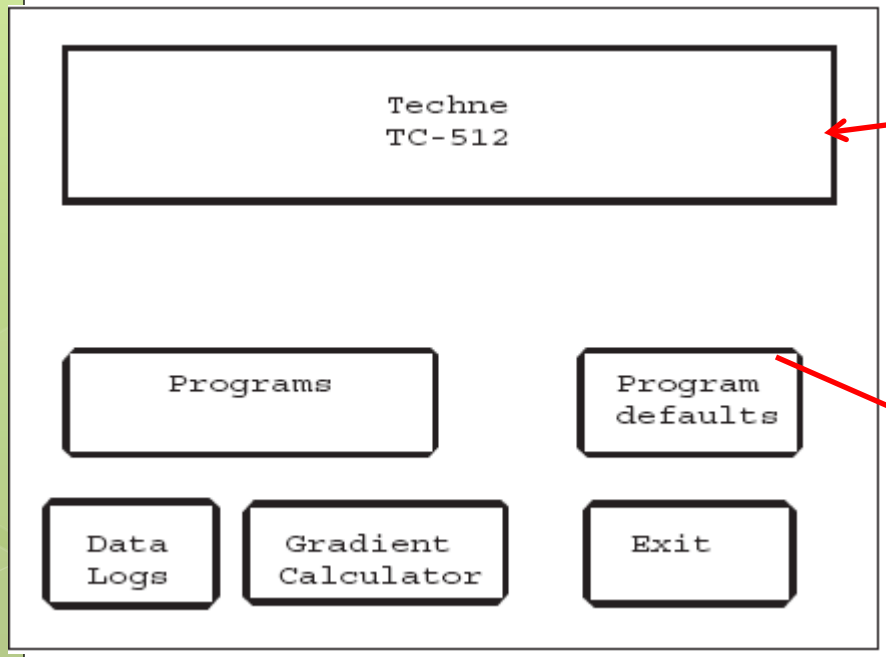
A model thermocycler

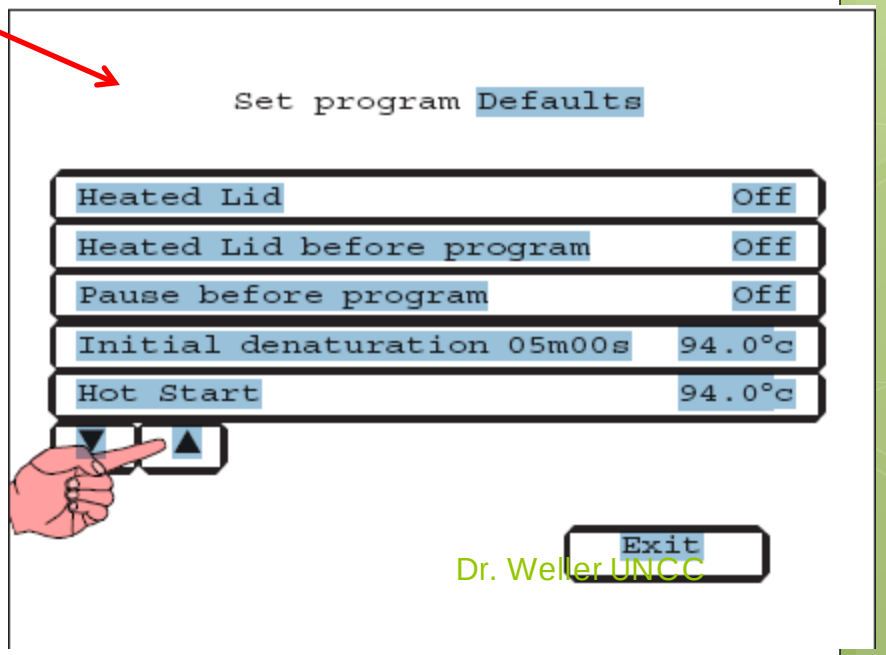
A carefully engineered, *programmable* heating/cooling block has drilled holes for sample tubes. The lid is heated.

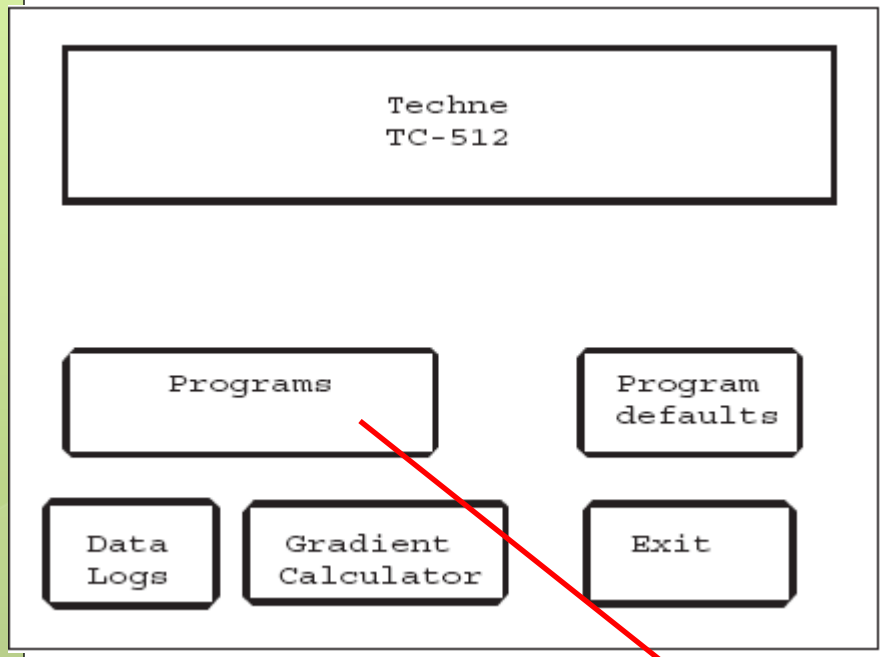
The rate of heating or cooling and final temperature are carefully regulated so that results are reproducible.

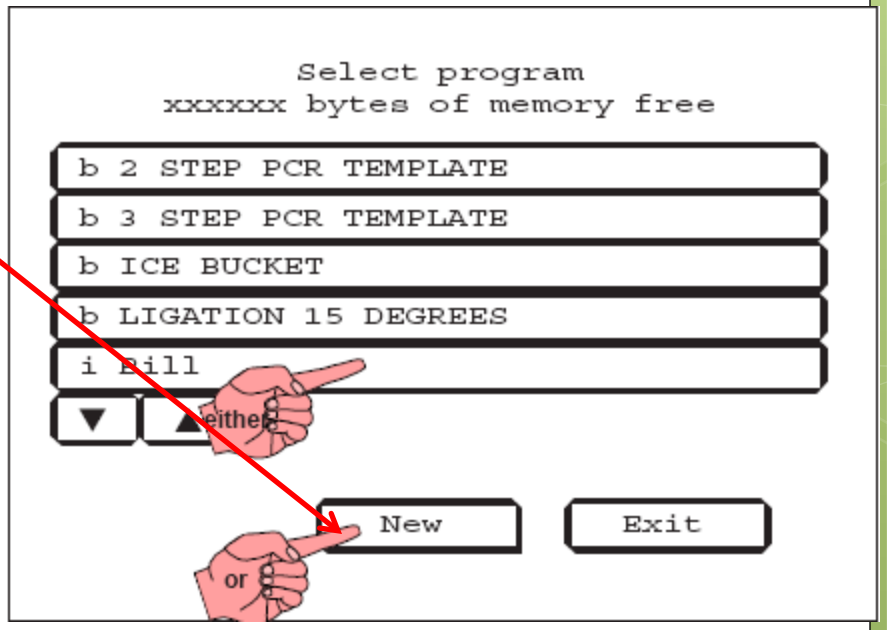
http://sentrabd.com/_borders/T1b.jpg

Example programming






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
or
New
Exit

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

Program | 3 STEP PCR TEMPLATE

Hot Start			Disabled
Number Cycles			30
Seg	MAX°c/m	00m30s	92.0°c
Seg	MAX°c/m	00m30s	55.0°c
Seg	MAX°c/m	00m30s	72.0°c
Final extension		05m00s	72.0°c
Final Hold			10.0°c

▲ ▼

Copy | Delete | Edit

Run | Print | Exit

Editing | Bill

Heated Lid			Disabled
Heated Lid before program			Off
Pause before program			Off
Initial denaturation			Disabled
Hot Start		05m00s	94.0°c
Number Cycles			3
Seg	MAX°C/m	00m30s	92.0°c ⊿10°c

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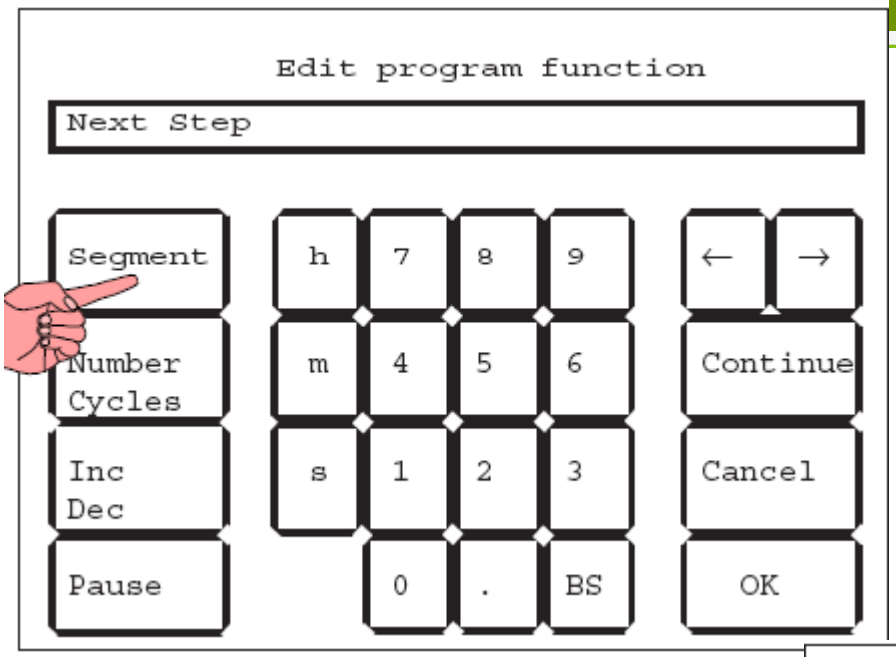
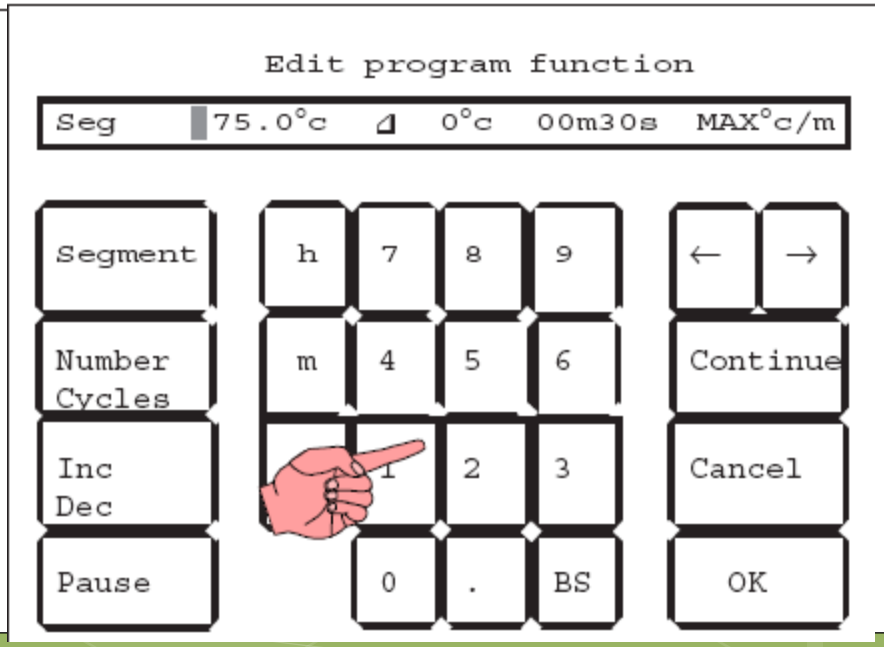
Ins | Del | Edit

PWord | Save As | Sa | Exit

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	

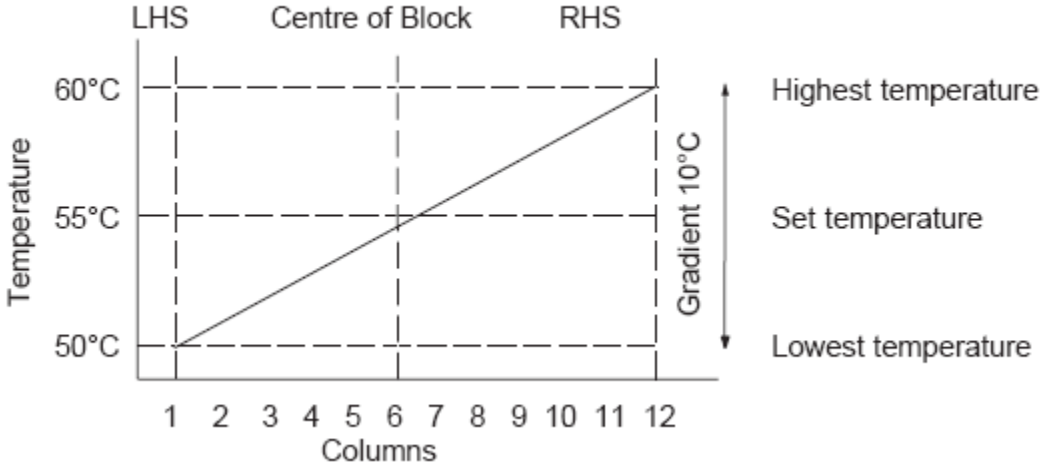
Editing "Your own"

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

▼ ▲

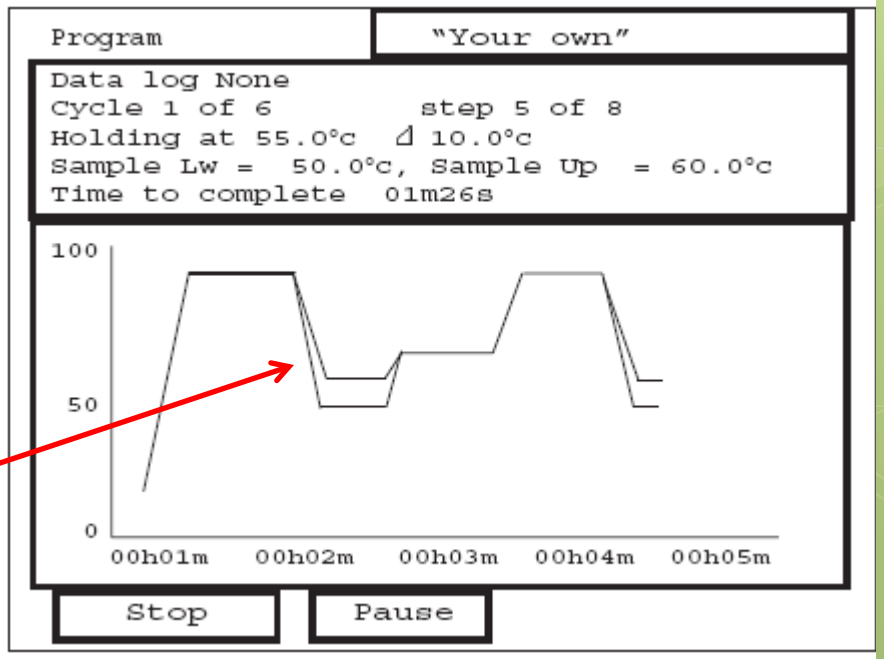
	Ins	Del	Edit
PWord	Save As	Save	Exit

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.

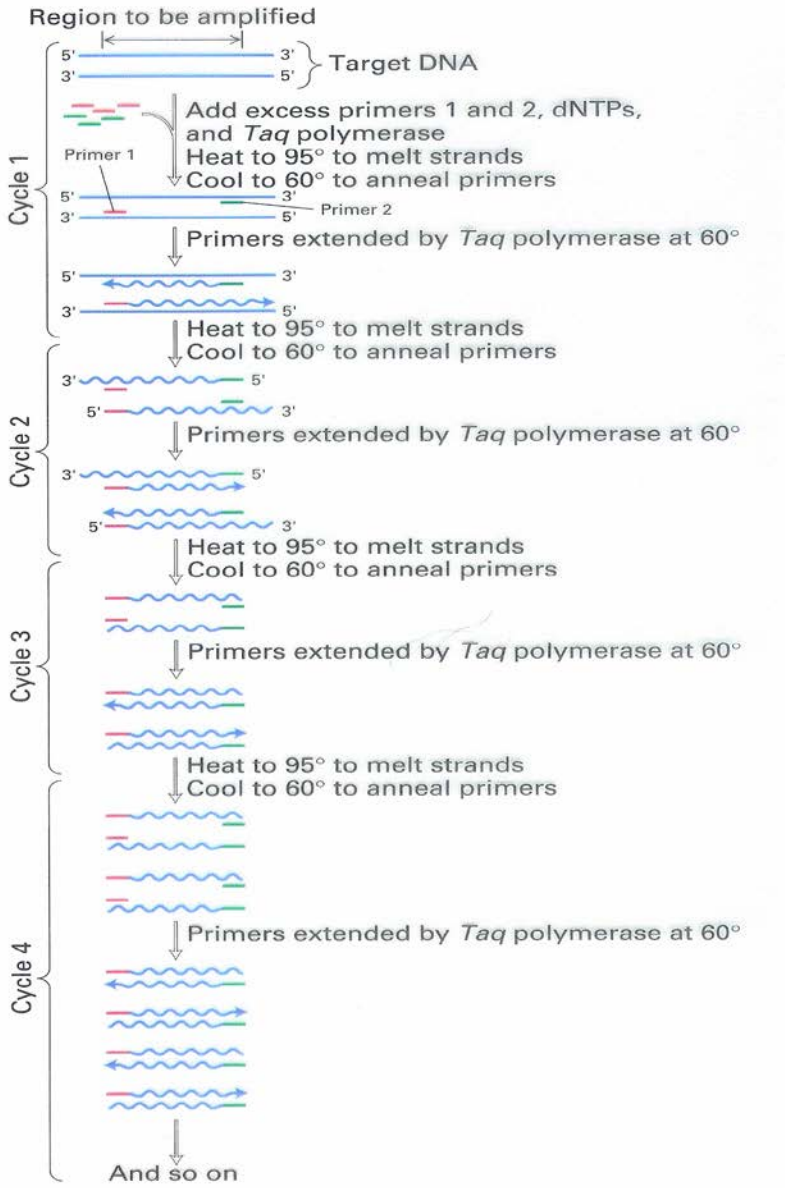
Ramp is how fast you go between temps.



Experimental methodology - PCR

- What is needed to perform PCR?
  - A template: the DNA that has sequences you want. They have to have parts 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'
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°
Cycle 1
Cycle 2
Cycle 3
Cycle 4
And so on

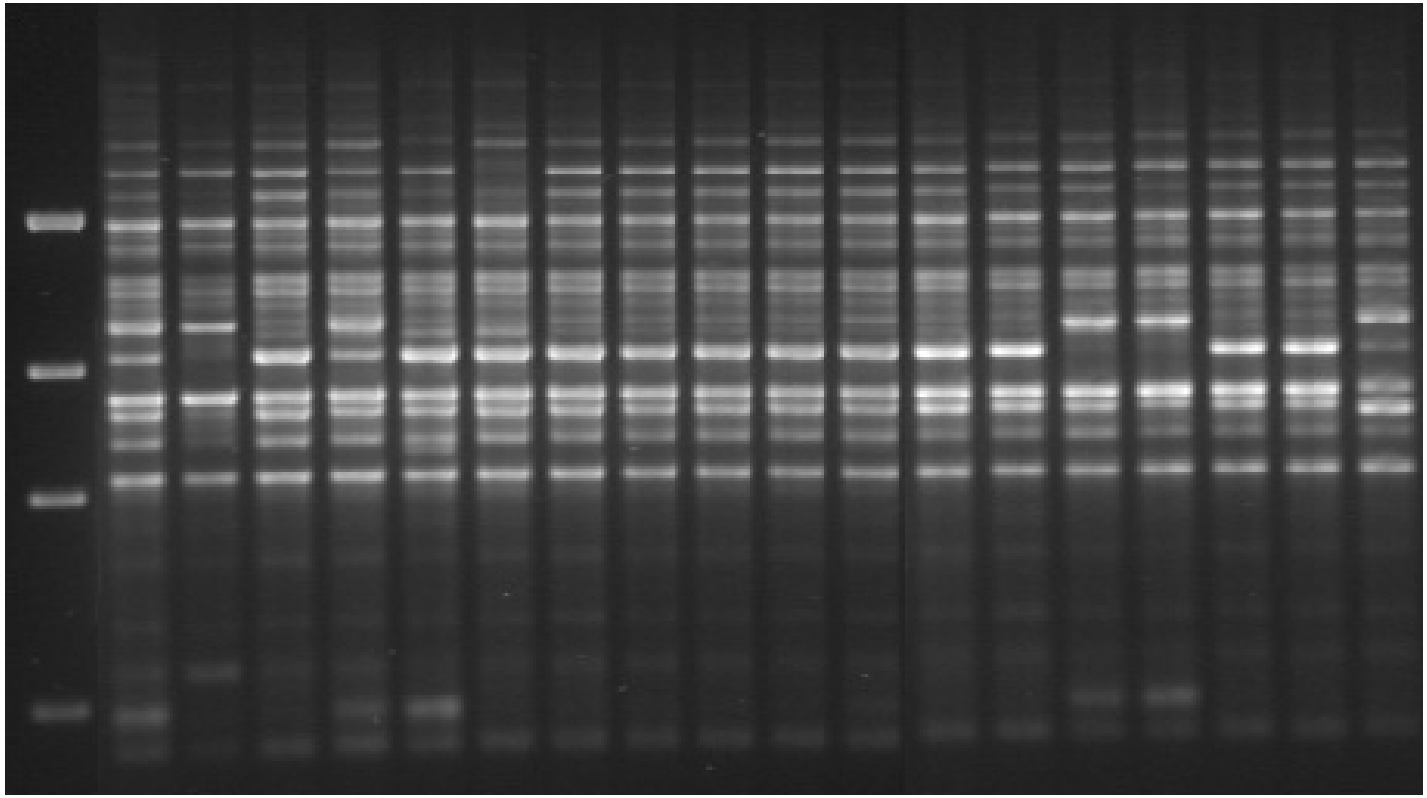
How much DNA will I get from a PCR reaction?

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.

How do I quantify and compare PCR products?



2% Agarose gel, stained with Ethidium bromide.
5ul of a 25 ul reaction was loaded per lane.