

B3-Olympic High School Summer Science Camp

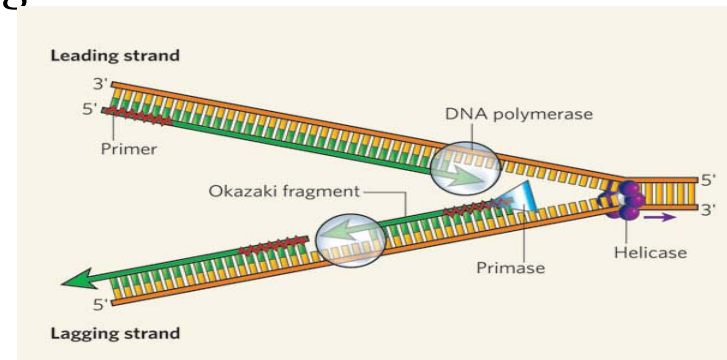
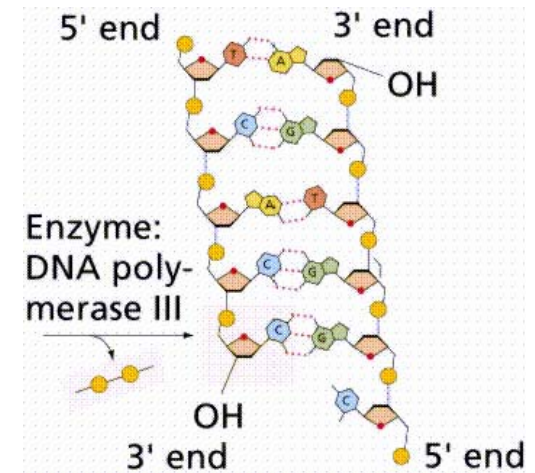
Dr. Jennifer Weller
Summer 2010

PCR

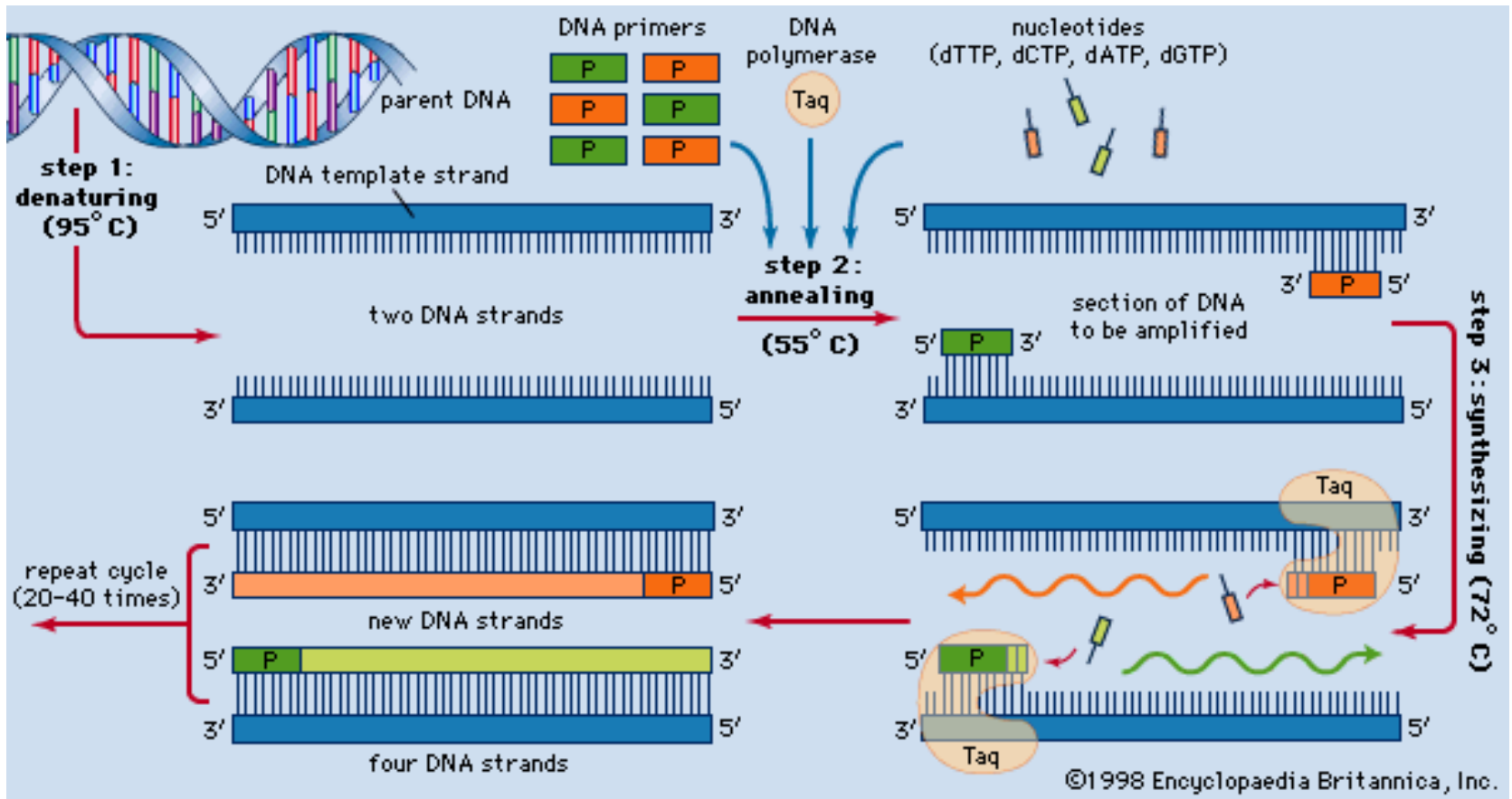
- ▶ **Topic: the Polymerase Chain Reaction (PCR)**
 - In 1988 Saiki, Mullis et al. proposed using a heat stable DNA polymerase to carry out a method that was outlined several years earlier
 - The enzyme was purified from an organism, characterized by Brock, from a hot spring in Yellowstone N.P.
 - Since 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.
 - As the monomer is added it forms H-bonds Across the center of the helix

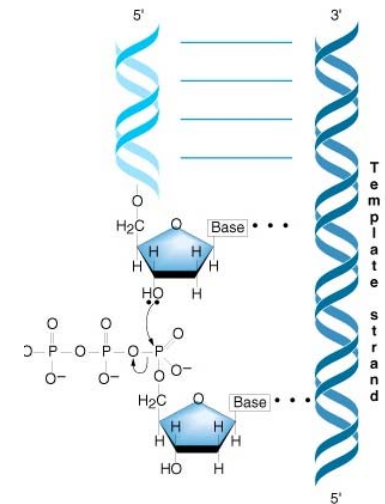
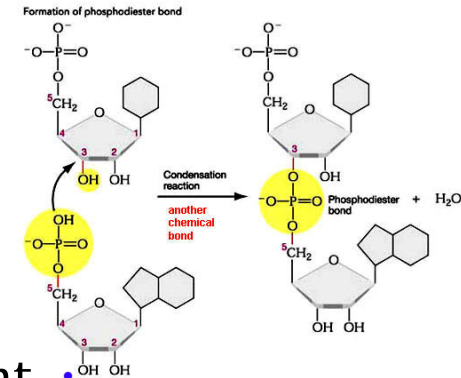


PCR steps



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 (nucleotides)

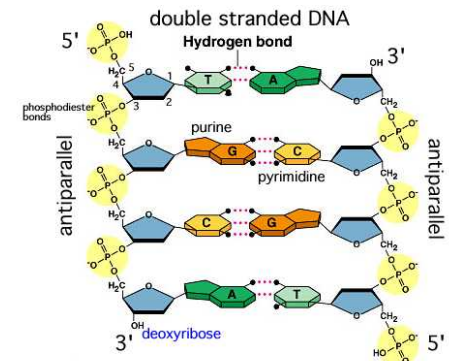
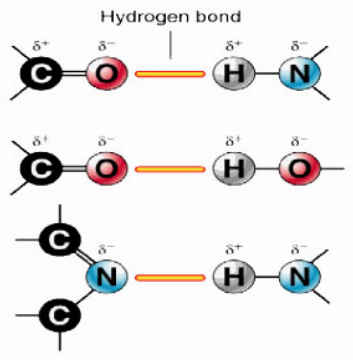


Three basic steps in Polymerization

- ▶ 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.

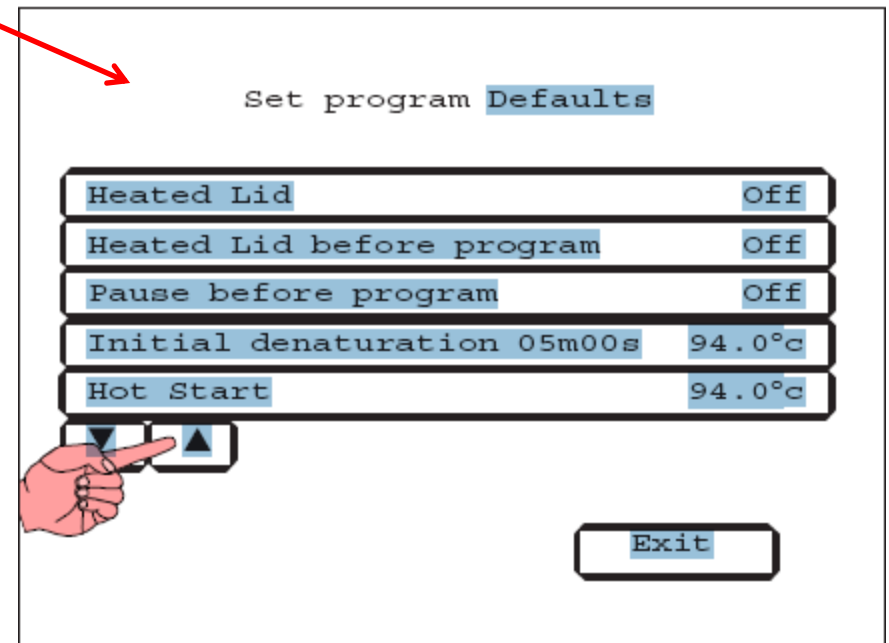
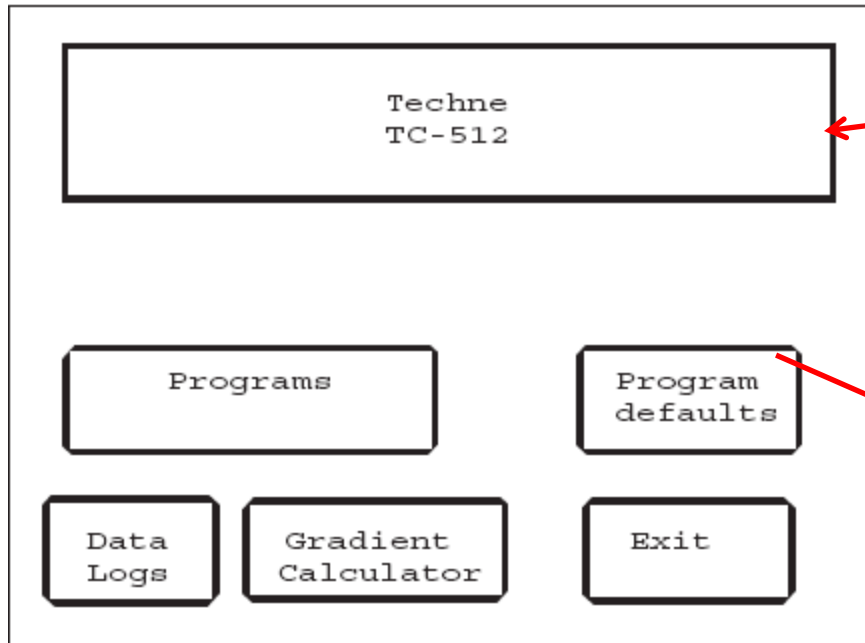
A model thermocycler

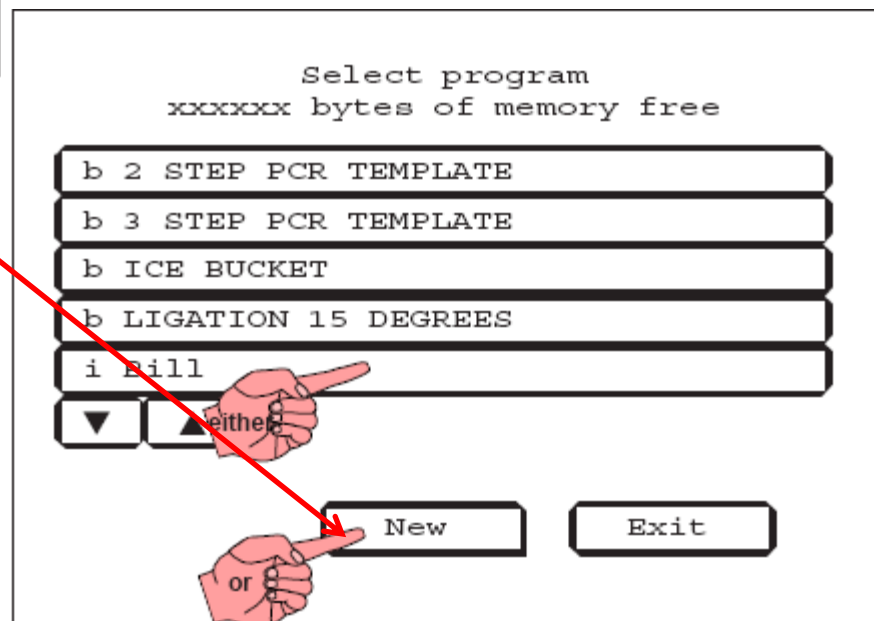
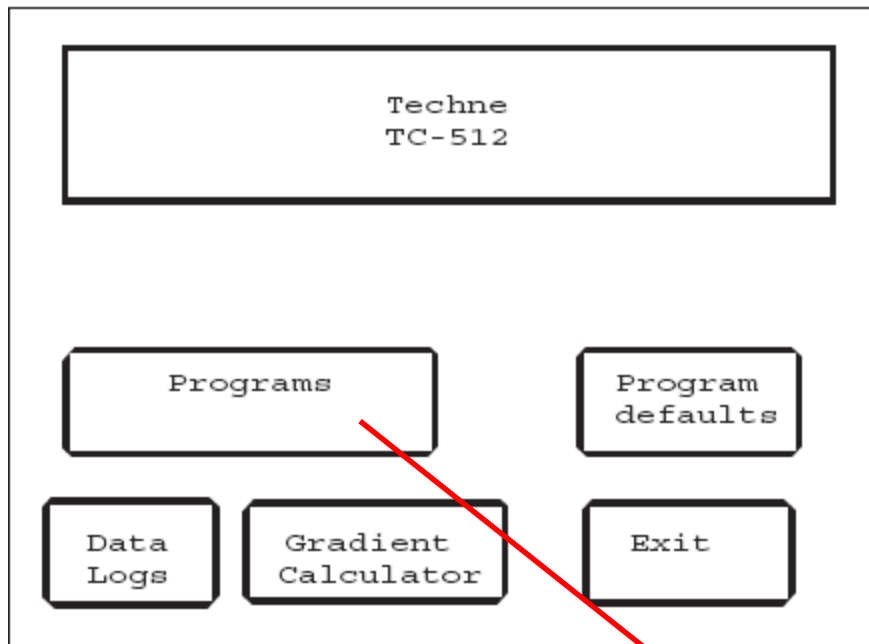
A carefully engineered, *programmable* heating/cooling block has drilled holes for sample tubes. The lid is heated.

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



Example programming







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
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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

▼ ▲

Ins Del Edit

PWord Save As Sa Exit

Interpret the Segment parts:
Time, temperature and gradient





Edit program function

Next Step

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



Edit program function

Seg █ 75.0°C ▽ 0°C 00m30s MAX°C/m


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

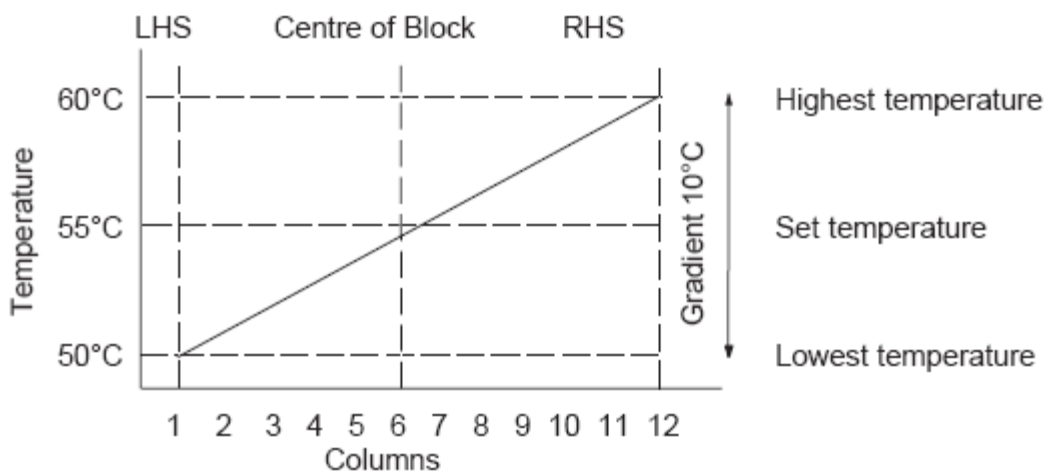
Heated Lid	Disabled
Heated Lid before program	Off
Pause before program	Off
Initial denaturation	Disabled
Hot Start	05m00s 72.0°C
Number Cycles	3
Seg MAX°C/m	00m35s 75.0°C Δ 0°C

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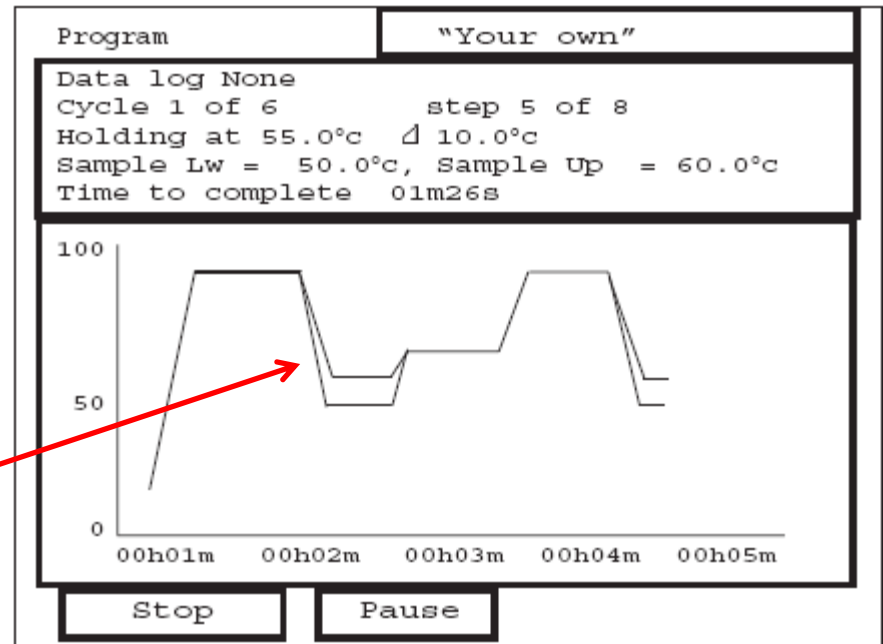
side being the hottest.



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.

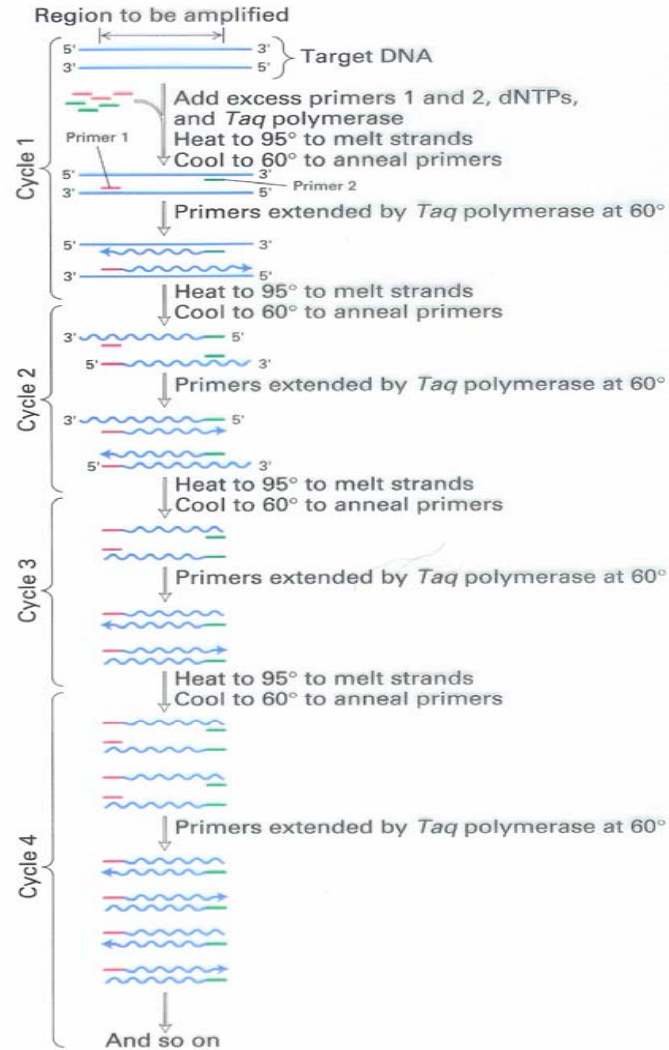
Ramp is how fast you go between temps.



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



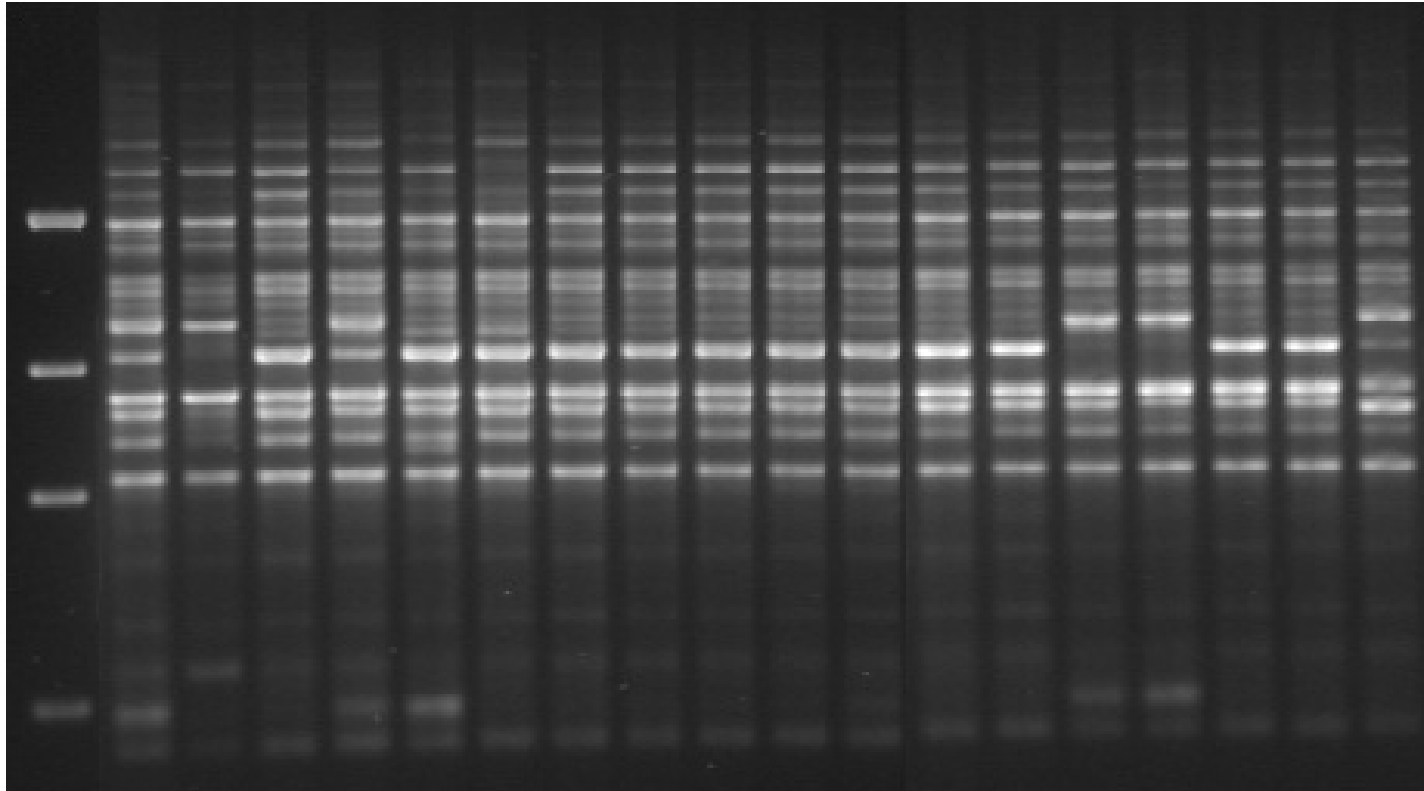
How much DNA will I get from a PCR reaction?

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.