Polymerase Chain Reaction (PCR)

Lecture-2

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What is the Polymerase Chain Reaction?

- It's a means of selectively amplifying a particular segment of DNA.
- The segment may represent a small part of a large and complex mixture of DNAs:
  e.g. a specific exon of a human gene.
- It is a molecular photocopier

A Molecular Photocopier

- A photocopier capable of duplicating a part of a sentence:
- "The next day was quite a different day. Instead of being hot and sunny, it was cool and misty. Pooh didn't mind for himself, but when he thought of all the honey the bees wouldn't be making, a cold misty day always made him feel sorry for them." *A.A. Milne, 1928.*
- The words in red must be unique for the copier to locate the correct piece of text to be amplified.

How Powerful is PCR?

- PCR can amplify a usable amount of DNA (visible by gel electrophoresis) in ~2 hours.
- The template DNA need not be highly purified — a boiled bacterial colony.
- PCR can amplify a single DNA molecule, *e.g.* from a single sperm.

Typical PCR mix

In a thin wall Eppendorf tube assemble the following

PCR components	Amount
Template DNA (5-200 ng)	variable
1 mM dNTPs (200 uM final)	10 uL
10 X PCR buffer	5 uL
25 mM MgCl2 (1.5 mM final)	3 uL
20 uM forward primer (20 pmoles final)	1 uL
20 uM reverse primer (20 pmoles final)	1 uL
5 units/uL Taq DNA polymerase (1.5 units)	0.3 uL
Ultra pure Water	Variable
Final Volume	50 uL

PCR

5' CATACGATGCTAGCTAGCTGCTAGTGCTG
ATCGTAGTCGTAGCTAGTCGTACGTACGT
CGATGTAATTCGCATCGATATGCGCTAGC
GATATGCGCATCGATCGATATCGATATGC 3'

Primer 1: 5-CATACGATGCTAGCT-3 Primer 2: 5-GCATATCGATATCG-3

Primer design

- Primer Size: Too small, may bind to more than one site in the genome
- Too Large? Yes. Long primers take a longer time to hybridize and would slow down the PCR cycle. (not > 30 bp)

- There is a $^1/_4$ chance (4^1) of finding an A, G, C or T in any given DNA sequence.
- There is a $^1/_{16}$ chance (4^2) of finding any di-nucleotide sequence (e.g. AG).
- There is a $^1/_{256}$ chance (4^4) of finding a given 4-base sequence (e.g. AGCT).
- So, a 17 base sequence will statistically be present only once in every 4^{17} bases or approximately 17 billion.

http://www.nfstc.org/pdi/Subject04/pdi_s04_m01_02_a.htm

- Primers perform differently at different temperatures
- Annealing temperature is most important
  - Too low = non-specific binding
  - Too high = primer will not bind
- Ideal annealing temperature can be mathematically estimated
- Calculated melting temperatures (T_m) should be from **42-65°C**

- **Melting temperature** (Tm) is temperature at which the primer will dissociate.
- Annealing temperature should be just 1-2 °C below Tm.
- *Tm estimation:*

  Tm = (4 x [G+C]) + (2 x [A+T]) °C

Forward Primer Selection

TAAATTCTTTGGAACAGGGGCATGGATTATAAAAGATGTAAGATAATAAAAAGCATTTGTATTTGACT
TTGGAATGTATTGTACTTACATTTGTCTAGAGGTGTGTCTATTCTGGCTATTCTCTTTAAAGGAGCCA
TTCTATCGTGAACAGATCCTGTTGGAGCTGTTTTCTTGTTCTACCAACCTTCAGCCACCTCTCTGTCT
TTCATATTACTTATTGGCAGGGTTTCAAAAGGTTTTAGTCCTTACTTAATATAAACAAAAATGTACAA
TATTGACAAAGTTTCAGTTAAGCAGATGAAATTCTAAGAGTTAAGCTGGGATTTTCCAAAATAATCCT
GTTAACAGACTTGAAAGCACTTATCAGTTCTGTCTAATGAAGACATT**AGAACACCATAACCTTTCCGG**
CCCATTTTCTTTGTCAATAAGCGTTCTTGCCCTGTCAGCAGCTCACCTCCAGCTTTAGTTTTC**X**CATG
ACAGTAAGTCTATTACCCTCCTGATCTGTCTTCTGGCTCCTCCTACCCAGGATGGGGAAGGTTTTTGA
CTTTACTGATATTCTCAGAACAAATTTTGGGAAGTAAATATAAGGTTTTCCAGTCGGGTGCAGTGGCT
CACGCCTATGATCCCAGCGCTTTGGGAAACCAAGGTGGGTGGATCACCTGAGGTCAGGAGTTTGAGAC
CAGCTTGGCCAATAAGGTGAAACCCCATCTCTACAAAAATTAGTTGGGCGTGGTGGCGGCACCTGTAA
ATCCAGCTACTCAGGAGGCTGAGGCAAGAGGATTGCTTGAATCTGGGAGCCGGAGGTTGAAGTGAACT
GAGATTGGGCCACTGCATTCTAGCCTGGGCGACAAGAGTGAAGCTCCATCTCAAAAAAAAAAAAAAAG
ATGAGGTTTTCCTTAAGAGCACTAACCTAGTATACTGCACAGGTGCCTGTATTCATGCATCCCACACA
GAAAGAGAAAATACTTGTCTGAACTTGTCCATAAATTCAGAATCCTGCCCCTTAAC

Forward Primer: 5'-AGAACACCATAACCTTTCCGGCCC-3'

Reverse Primer Selection

TAAATTCTTTGGAACAGGGGCATGGATTATAAAAGATGTAAGATAATAAAAAGCATTTGTATTTGACT
TTGGAATGTATTGTACTTACATTTGTCTAGAGGTGTGTCTATTCTGGCTATTCTCTTTAAAGGAGCCA
TTCTATCGTGAACAGATCCTGTTGGAGCTGTTTTCTTGTTCTACCAACCTTCAGCCACCTCTCTGTCT
TTCATATTACTTATTGGCAGGGTTTCAAAAGGTTTTAGTCCTTACTTAATATAAACAAAAATGTACAA
TATTGACAAAGTTTCAGTTAAGCAGATGAAATTCTAAGAGTTAAGCTGGGATTTTCCAAAATAATCCT
GTTAACAGACTTGAAAGCACTTATCAGTTCTGTCTAATGAAGACATTAGAACACCATAACCTTTCCGG
CCCATTTTCTTTGTCAATAAGCGTTCTTGCCCTGTCAGCAGCTCACCTCCAGCTTTAGTTTTC**X**CATG
ACAGTAAGTCTATTACCCTCCTGATCTGTCTTCTGGCTCCTCCTACCCAGGATGGGGAAGGTTTTTGA
CTTTACTGATATTCTCAGAACAAATTTTGGGAAGTAAATATAAGGTTTT**CCAGTCGGGTGCAGTGGCT**
CACGCCTATGATCCCAGCGCTTTGGGAAACCAAGGTGGGTGGATCACCTGAGGTCAGGAGTTTGAGAC
CAGCTTGGCCAATAAGGTGAAACCCCATCTCTACAAAAATTAGTTGGGCGTGGTGGCGGCACCTGTAA
ATCCAGCTACTCAGGAGGCTGAGGCAAGAGGATTGCTTGAATCTGGGAGCCGGAGGTTGAAGTGAACT
GAGATTGGGCCACTGCATTCTAGCCTGGGCGACAAGAGTGAAGCTCCATCTCAAAAAAAAAAAAAAAG
ATGAGGTTTTCCTTAAGAGCACTAACCTAGTATACTGCACAGGTGCCTGTATTCATGCATCCCACACA
GAAAGAGAAAATACTTGTCTGAACTTGTCCATAAATTCAGAATCCTGCCCCTTAAC

Reverse Primer: 5'-GCGTGAGCCACTGCACCCGACTGG-3'

Amplified Fragment Will Be 230bp

AGAACACCATAACCTTTCCGGCCCATTTTCTTTGTCAATAAGCGTTCTTGCCCTG
TCAGCAGCTCACCTCCAGCTTTAGTTTTC**X**CATGACAGTAAGTCTATTACCCTCC
TGATCTGTCTTCTGGCTCCTCCTACCCAGGATGGGGAAGGTTTTTGACTTTACTG
ATATTCTCAGAACAAATTTTGGGAAGTAAATATAAGGTTTT**CCAGTCGGGTGCAG
TGGCTCACGC**

Before ordering: Imagine the primers annealing to the DNA

Forward Primer: 5'-AGAACACCATAACCTTTCCGGCCC-3'

TAAATTCTTTGGAACAGGGGCATGGATTATAAAAGATGTAAGATAATAAAAAGCATTTGTATTTGACT
TTGGAATGTATTGTACTTACATTTGTCTAGAGGTGTGTCTATTCTGGCTATTCTCTTTAAAGGAGCCA
TTCTATCGTGAACAGATCCTGTTGGAGCTGTTTTCTTGTTCTACCAACCTTCAGCCACCTCTCTGTCT
TTCATATTACTTATTGGCAGGGTTTCAAAAGGTTTTAGTCCTTACTTAATATAAACAAAAATGTACAA
TATTGACAAAGTTTCAGTTAAGCAGATGAAATTCTAAGAGTTA**5'-AGAACACCATAACCTTTCCGG**
CCC-3' →ACTTGAAAGCACTTATCAGTTCTGTCTAATGAAGACATT**AGAACACCATAACCTTTCCGG**
CCCATTTTCTTTGTCAATAAGCGTTCTTGCCCTGTCAGCAGCTCACCTCCAGCTTTAGTTTTC**X**CATG
ACAGTAAGTCTATTACCCTCCTGATCTGTCTTCTGGCTCCTCCTACCCAGGATGGGGAAGGTTTTTGA
CTTTACTGATATTCTCAGAACAAATTTTGGGAAGTAAATATAAGGTTTT**CCAGTCGGGTGCAGTGGCT**
CACGCCTATGATCCCAGCGCTTTGGGAAACCAAGGTGGGTGG← **3'-GCGTGAGCCACTGCACCCGACTGG-5' Reverse Primer: 5'-GCGTG**
ATCCAGCTACTCAGGAGGCTGAGGCAAGAGGATTGCTTGAATCTGGGAGCCGGAGGTTGAAGTGAACT
GAGATTGGGCCACTGCATTCTAGCCTGGGCGACAAGAGTGAAGCTCCATCTCAAAAAAAAAAAAAAAG
ATGAGGTTTTCCTTAAGAGCACTAACCTAGTATACTGCACAGGTGCCTGTATTCATGCATCCCACACA
GAAAGAGAAAATACTTGTCTGAACTTGTCCATAAATTCAGAATCCTGCCCCTTAAC

Reverse Primer: 5'-GCGTGAGCCACTGCACCCGACTGG-3'

Designing PCR Primers

- Primers should be ~20 (15 - 30) bases long.
- The G/C content should be 40–60%.
- Primer pairs should have T_m within **5°C** of each other
- The 3′-most base should be a G or C.
- The primers must not base pair with each other or with themselves or form hairpins.
- Primers must avoid repetitive DNA regions.

Primers That Form Hairpins

- A primer is self-complementary and able to fold into a hairpin:

```
5´-GTTGACTTGATATTCTCAAG-3´

5´-GTTGACTTGATA
        ||||| T
3´-GAACTCT
```

- The 3′ end of the primer is base-paired, preventing it annealing to the target DNA.

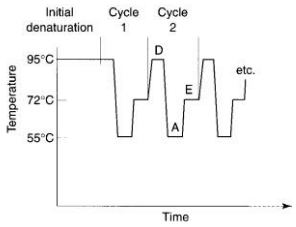
Primers That Form Dimers

- A primer may form a dimer with itself or with the other primer

```
5´-ACCGGTAGCCACGAATTCGT-3´
                ||||||||||
          3´-TGCTTAAGCACCGATGGCCA-5´
```

The Basics of PCR Cycling

- 30–35 cycles each comprising:
  - denaturation (95°C), 30 sec.
  - annealing (55–60°C), 30 sec.
  - extension (72°C), time depends on product size.



What's in the Reaction?

- Template DNA
- Reaction buffer (Tris, ammonium ions and/or potassium ions), magnesium ions, bovine serum albumin
- Nucleotides (dNTPs)
- Primers
- DNA polymerase (usually *Taq*)

PCR In Detail

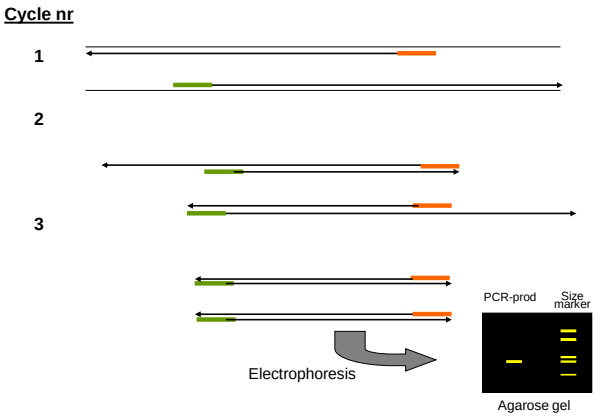
- Denature, anneal, extend and repeat the cycle 30 to 35 times.
- "How does the polymerase know to stop?"
- Most textbooks to not fully explain PCR.

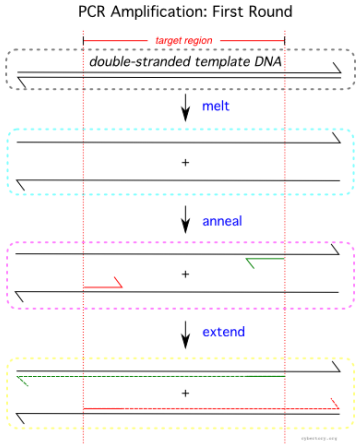
How many copies?

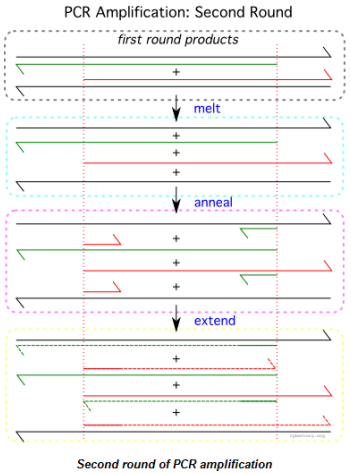
- No target products are made until the third cycle.
- The accumulation is not strictly a doubling at each cycle in the early phase.
- At 30 cycles there are 1,073,741,764 target copies (~1×10^9).

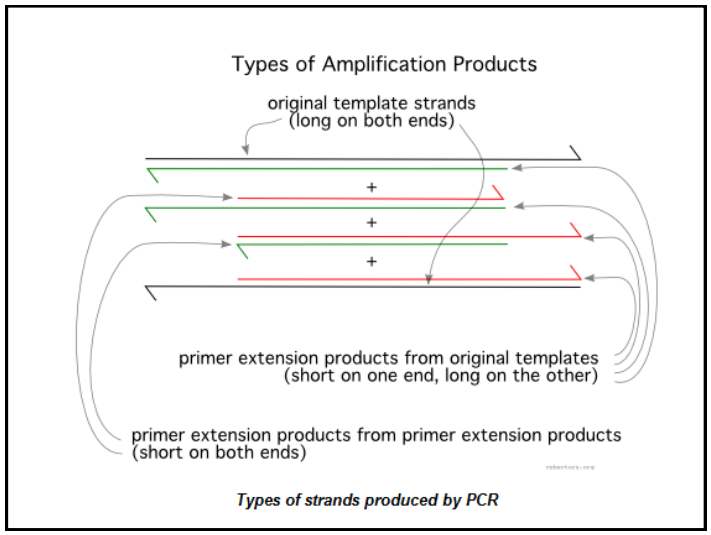
https://static.dna.gov/flash/pdi_s04_m01_01.swf

PCR products (I)





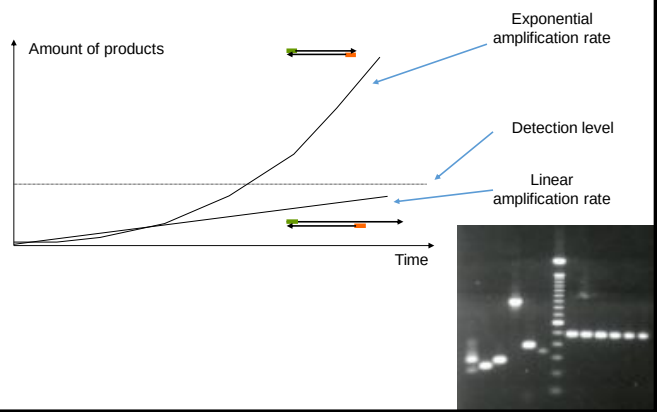




Types of strands produced by PCR

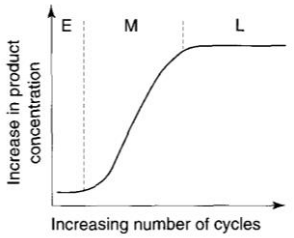
Cycle	Long on both ends	Long on one end, short on the other	Short on both ends	Total strands	Total amplification
0	2	0	0	2	1
1	2	2	0	4	2
2	2	4	2	8	4
3	2	6	8	16	8
4	2	8	22	32	16
5	2	10	52	64	32
6	2	12	114	128	64
7	2	14	240	256	128
8	2	16	494	512	256
9	2	18	1004	1024	512
10	2	20	2026	2048	1024
20	2	40	2097110	2097152	1048576
30	2	60	2147483586	2147483648	1073741824
40	2	80	2199023255470	2199023255552	1099511627776
n	2	2n	$2^n - 2n - 2$	$2^{(n+1)}$	2^n

PCR products (II)



How many cycles?

- Increasing the cycle number above ~35 has little positive effect.
- The plateau occurs when:
  - The reagents are depleted
  - The products re-anneal
  - The polymerase is damaged
- Unwanted products accumulate.



Thermal Cyclers

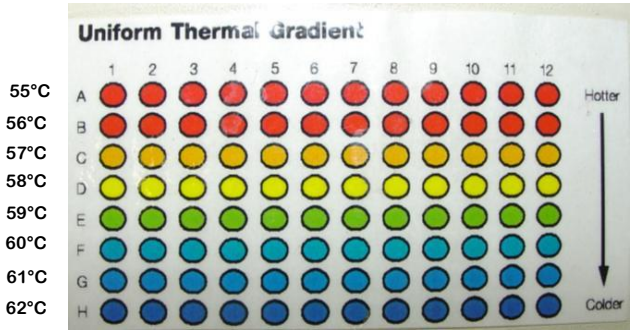
- PCR cyclers available from many suppliers.
- Many block formats and multi-block systems.
- Reactions in tubes or 96-well micro-titre plates.

So Then, it's Easy?

- Cycling performed with three water baths.
- Thermal cyclers introduced in 1986.
- Early polymerases were not thermostable, so had to be replenished each cycle.
- The 37°C temperature caused non-specific priming, resulting in unwanted products.

New Thermocyclers = Gradient Blocks

Gradient thermocyclers allow for optimization of the annealing temperature



Did the PCR work?

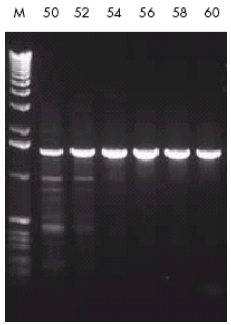
- Check a sample by gel electrophoresis.
- Is the product the size that you expected?
- Is there more than one band?
- Is any band the correct size?
- May need to optimize the reaction conditions.

Optimising the PCR Reaction

- Annealing temperature of the primers.
- The concentration of Mg^{2+} in the reaction.
- The extension time.
- The denaturing and annealing times.
- The extension temperature.
- The amount of template and polymerase

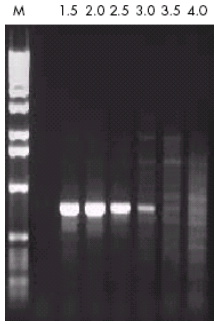
Optimising the Annealing Temperature

- Primers have a calculated annealing temperature (*e.g.* 54°C).
- Temperature must be confirmed practically.
- Temperature steps of 2°C above and below.
- Use gradient cycler.



Optimising the Mg^{2+} Concentration

- The fidelity of the PCR depends on $[Mg^{2+}]$.
- Vary $[Mg^{2+}]$ in steps of 0.5 mM.
- Sometimes a compromise between yield and specificity.
- Magnesium Concentration 1.5-2.0 mM is optimal for *Taq* DNA Polymerase



Fidelity of the Reaction

- *Taq* DNA polymerase lacks the 3′→5′ proof-reading activity commonly present in other polymerases.
- *Taq* mis-incorporates 1 base in 10^4 (10,000)
- A 400 bp target will contain an error in 33% of molecules after 20 cycles.
- Error distribution will be random.

Do Errors Matter?

- Yes, if you want to clone the amplified DNA — an individual molecule may harbour several mutations.
- if you want to sequence the amplified DNA or cut it with restriction enzymes.
- Use a proof-reading thermo-stable enzyme rather than *Taq*.

How Big A Target?

- Amplification products are typically in the size range 100-1500 bp.
- Longer targets are amplifiable — >25 kb.
- Requires modified reaction buffer, cocktails of polymerases, and longer extension times.
- Limited by the integrity of the starting target DNA — > 50 kb.

Can PCR Amplify RNA?

- Not directly — the DNA polymerase requires a DNA template and will not copy RNA.
- mRNA can first be copied into cDNA using reverse transcriptase.
- cDNA is a template for PCR — it need not be double-stranded.