Direkt and indirekt mutasyon analizi I: PZR

Yrd. Doç. Dr. Pinar Tulay

Tıp Fakültesi

Hücrede DNA Replikasyonu

- DNA replikasyonu DNA'nın kopyalanmasıdı
- DNA replikasyonu yarı korumalıdır (bir DNA ipliği yeni DNA ipliğinin sentezi için şablon olarak kullanılır)
- Bu işlem çok az hata ile gerçekleşir (avaraj olarak 1 bilyon nukleotid kopyalandığında bir hata olur)
- DNA replikasyonunda bir düzüneden fazla enzim ve protein görev alır.

DNA Replikasyonunda görev alan enzimler:
DNA Ligaz

- Two strands of DNA in a double helix are **antiparallel**
  - i.e. they are oriented in opposite directions with one strand oriented from 5' to 3' and the other strand oriented from 3' to 5'
    - *5' and 3' refer to the numbers assigned to the carbons in the 5 carbon sugar*
- DNA ploymerases can only add nucleotides to the 3' end, thus:
  - one strand (referred to as the **leading strand**) of DNA is synthesized continuously
  - the other strand (referred to as the **lagging strand**) in synthesized in fragments (called **Okazaki fragments**) that are joined together by **DNA ligase**.

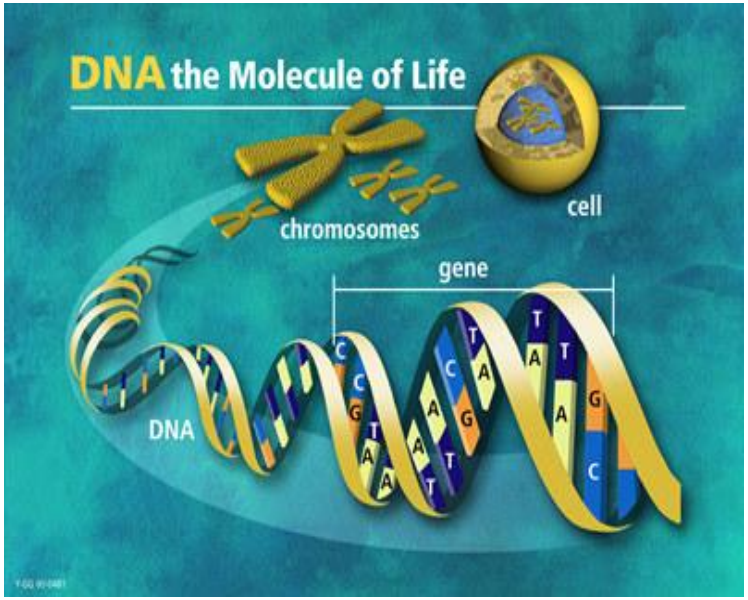
DNA Replication enzymes:

Helicase, Topoisomerase and Single-strand binding protein

- **Helicase** separates two parallel DNA strands
- **Topoisomerase** regulate the overwinding or underwinding of DNA and relieves the stress of this twisting
- **Single-strand binding protein** binds to and stabilizes the unpaired DNA strands

Polymerase chain reaction (PCR)

- *in vitro* version of DNA Replication
- Multiple copies of specific DNA sequence
  - 'Molecular photocopying'



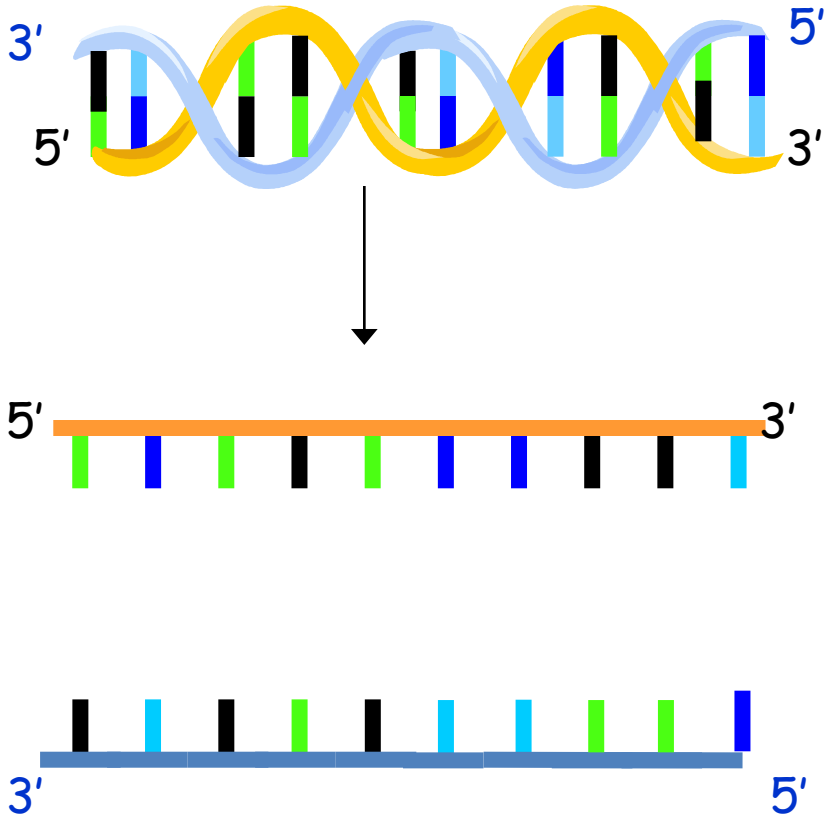
Polymerase chain reaction

- “discovered” in 1983 by Kary Mullis

- 1993: Nobel Prize for Chemistry

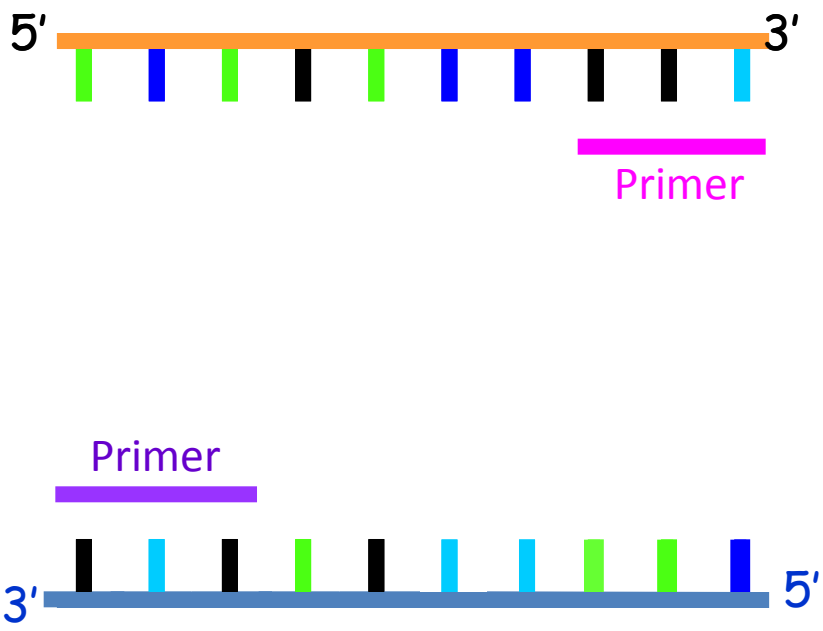
Stages in PCR

- Denaturation
  - Heat to separate double strands
  - This occurs at 95 ºC
  - Mimicks the function of helicase in the cell.



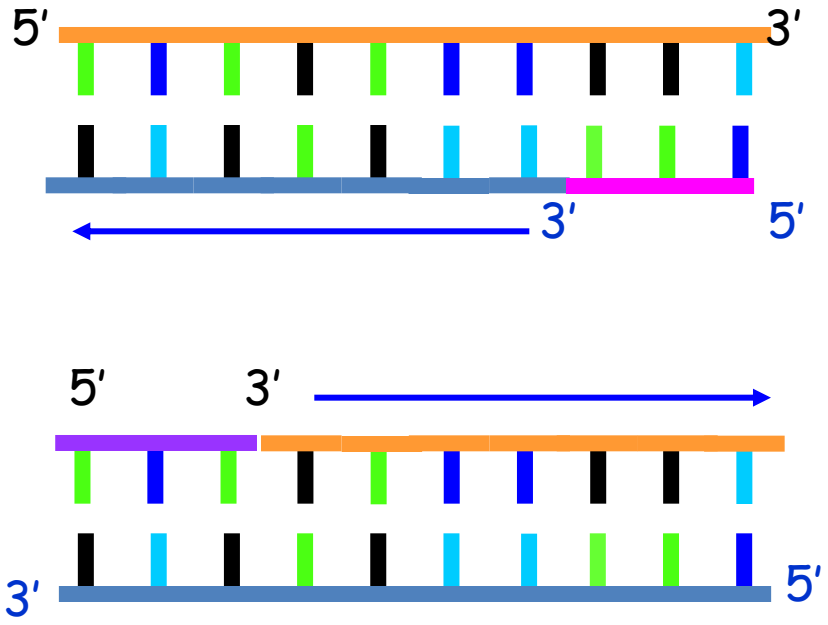
Stages in PCR

- Annealing
  - Primer binds to template sequence
  - Primers are chosen such that one is complimentary to the one strand at one end of the target sequence and that the *other* is complimentary to the *other* strand at the other end of the target sequence.



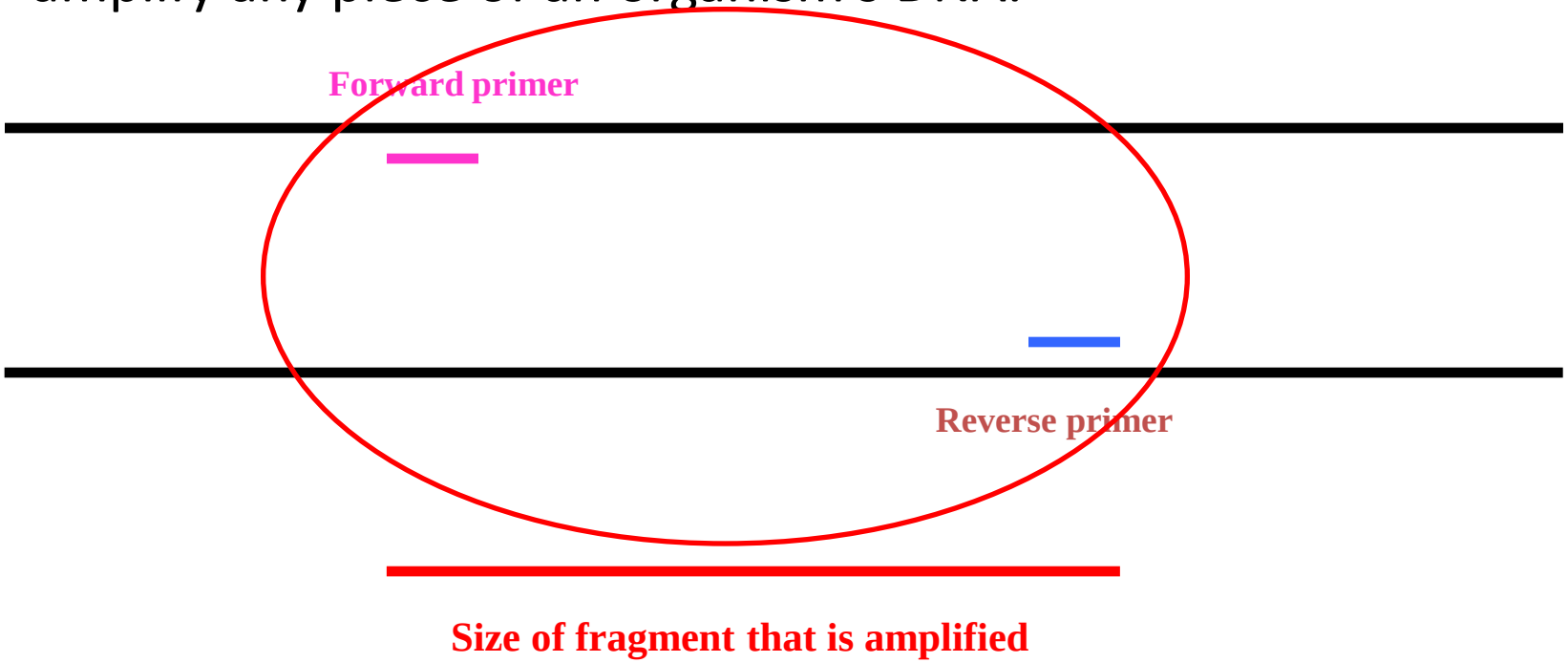
Stages in PCR

- Elongation
  - Primer is extended with addition of dNTPs with Taq polymerase
  - the extension of the strand in the 5-3 direction starting at the primers attaching the appropriate nucleotide (A-T, C-G)

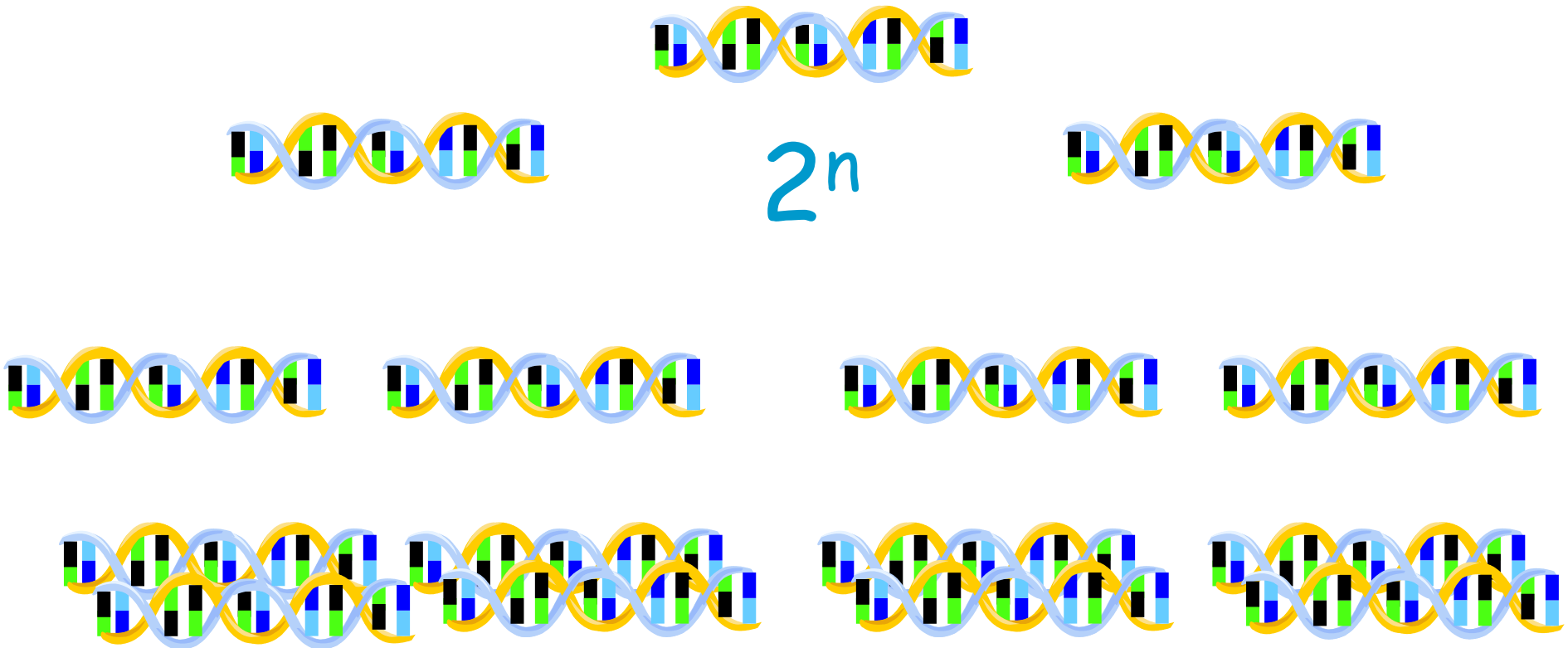


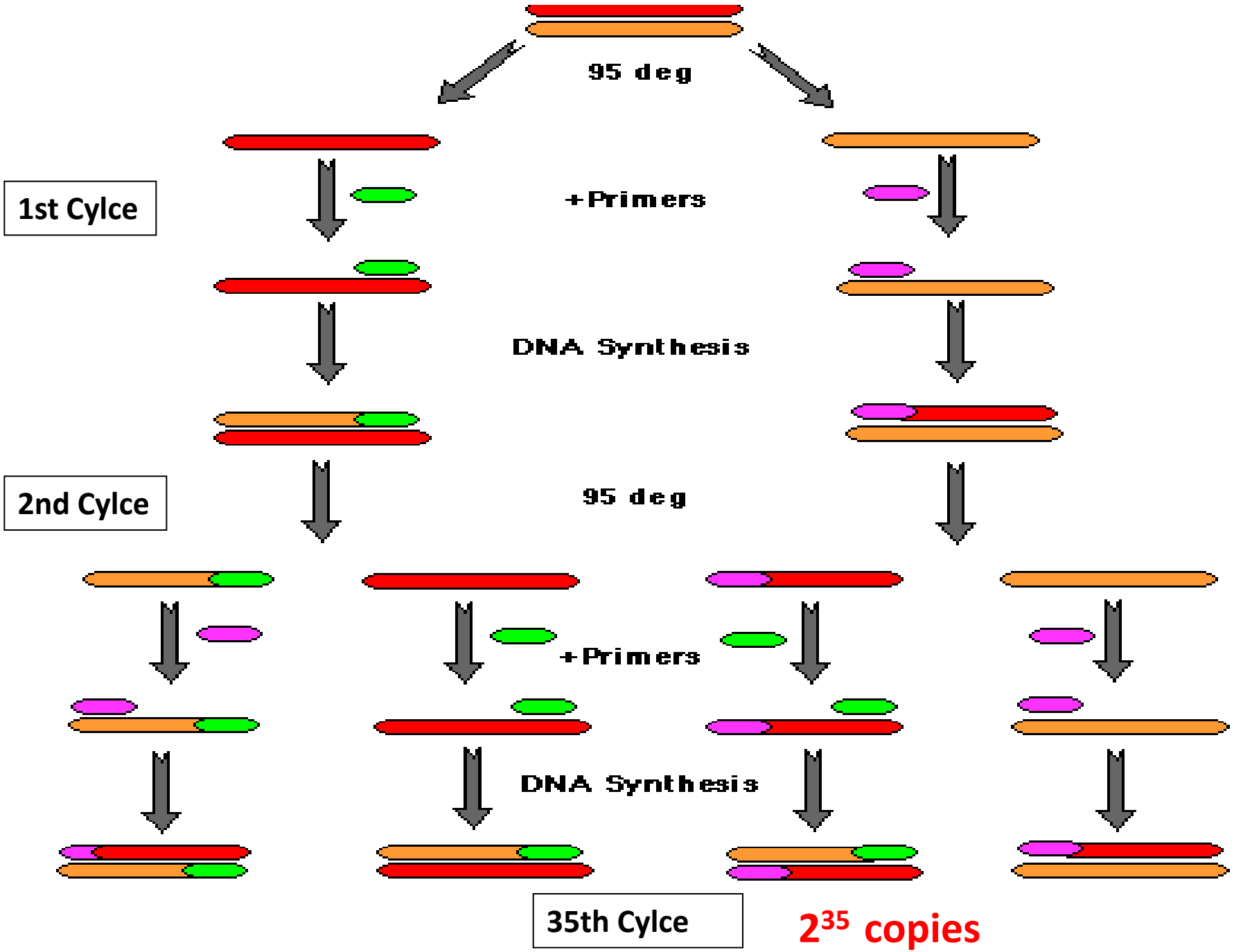
The Size of the DNA Fragment Produced in PCR is Dependent on the Primers

- The PCR reaction will amplify the DNA section between the two primers.
- If the DNA sequence is known, primers can be developed to amplify any piece of an organism's DNA.



PCR amplification




95 deg
1st Cylce
+Primers
DNA Synthesis
2nd Cylce
95 deg
+Primers
DNA Synthesis
35th Cylce
2^35 copies

Reagents for PCR

What we need in the laboratory:

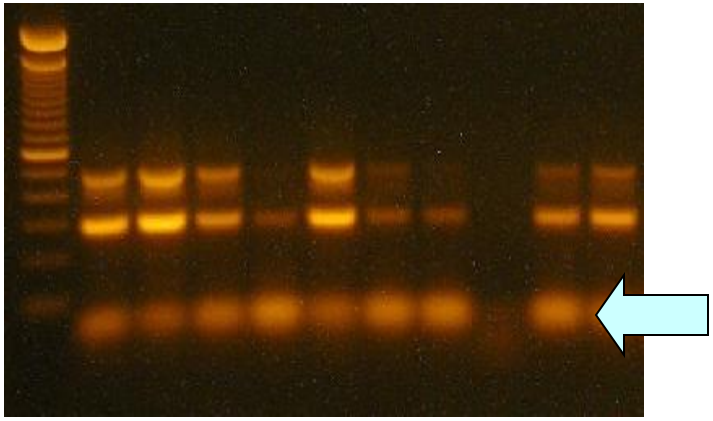
- DNA template
- Primers
- DNA polymerase
- Buffer
- dNTPS (bases)
- $MgCl_2$

Reagents for PCR

- **Primers**
  - ssDNA sequences flanking region of interest
  - Present in excess – up to 0.5μM
    - (too high – primer dimers)
- **Primer Design**
  - Homology of primers
  - Length (18-28bp)
  - GC content (45-60%) Tm=2X(A+T)+ 4X(C+G)

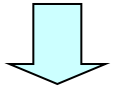
Primer-Dimer

- Results from primers annealing each other at 3’ ends



- Extended primers are no longer available to prime target for PCR

```
atcggactatcga
             gctatacttatggcca
```



```
atcggactatcgatatgaataccgga
tagcctgatagctatacttatggcca
```

Reagents for PCR

- **DNA polymerase**
- Enzyme responsible for copying the sequence starting at the primer from the single DNA strand by adding nucleoside triphosphates to the 3’ end of the growing strand
- DNA polymerase uses each strand as a template to synthesize new strands of DNA, complementary order of nucleotides.
- This enzyme is heat-tolerant → thermally tolerant (survives the melting temperature of DNA denaturation) which also means the process is more specific, higher temperatures result in less mismatch – more specific replication
- Many types available
    - Some modified to allow hot start
    - Some have long half life / stable at high temperature
    - Some have high rate of processivity

Reagents for PCR

- $MgCl_2$
- required for enzyme activation and amplification
- It stabilizes **dsDNA** and raises the Tm
- Mg^{2+} concentration controls the specificity of the reaction.

Reagents for PCR

- **Buffer -** providing a suitable chemical environment for optimum activity and stability of the DNA polymerase

- **dNTPs (bases)**

PCR - before the thermocycler

95º C
5 min

55º C
3 min

72º C
5 min

35 times

8 BORING hours per PCR!

PCR

PCR tube with all the reagents

THERMOCYCLER

•The thermal cycler allows heating and cooling of the reaction tubes to control the temperature required at each reaction step.

•Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration.

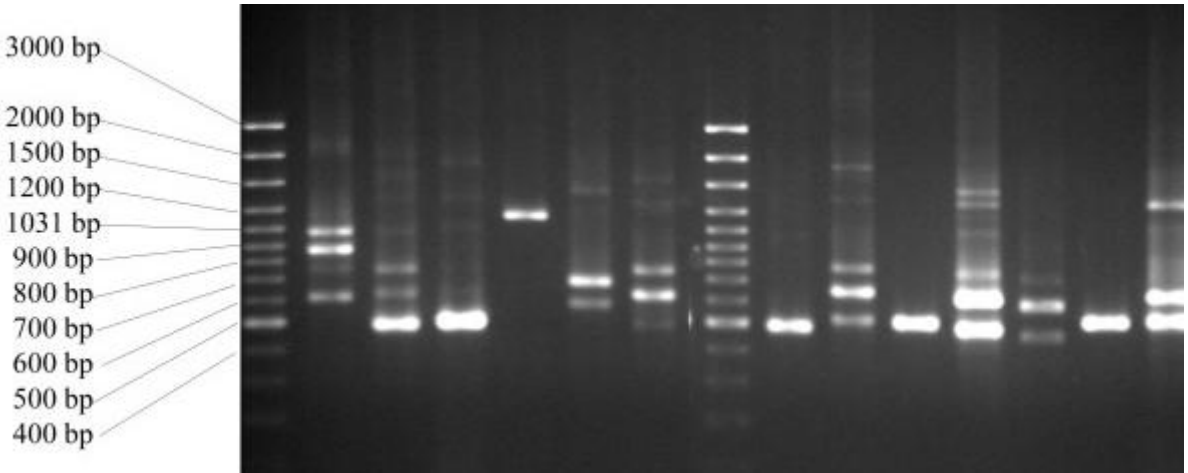
•Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube.

How do we analyse the PCR products?

- Gel electrophoresis
- Fluorescent PCR
- Sequencing
- Blotting
- Short tandem repeat (STR) analysis

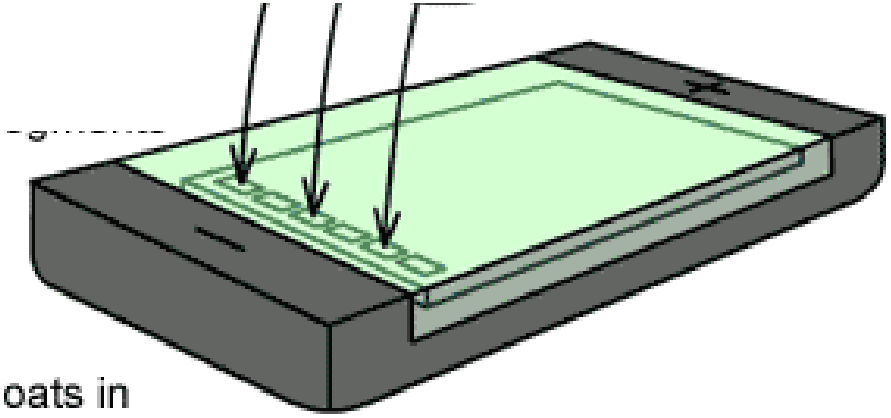
Gel Electrophoresis

- Fragmentation products of differing length are separated
- Separation of DNA fragments
- Separation based (mostly) on length
  - longer molecules move slower.
- Size and shape

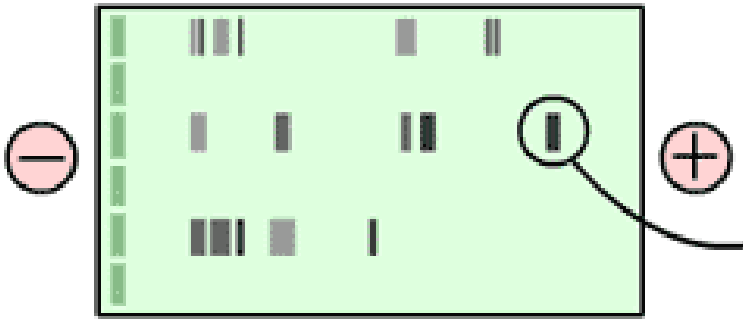


Gel Electrophoresis

DNA (or RNA) samples loaded into wells



2. DNA segments are loaded into wells in a porous gel. The gel floats in a buffer solution within a chamber between two electrodes.



3. When an electric current is passed through the chamber, DNA fragments move toward the positively-charged cathode.

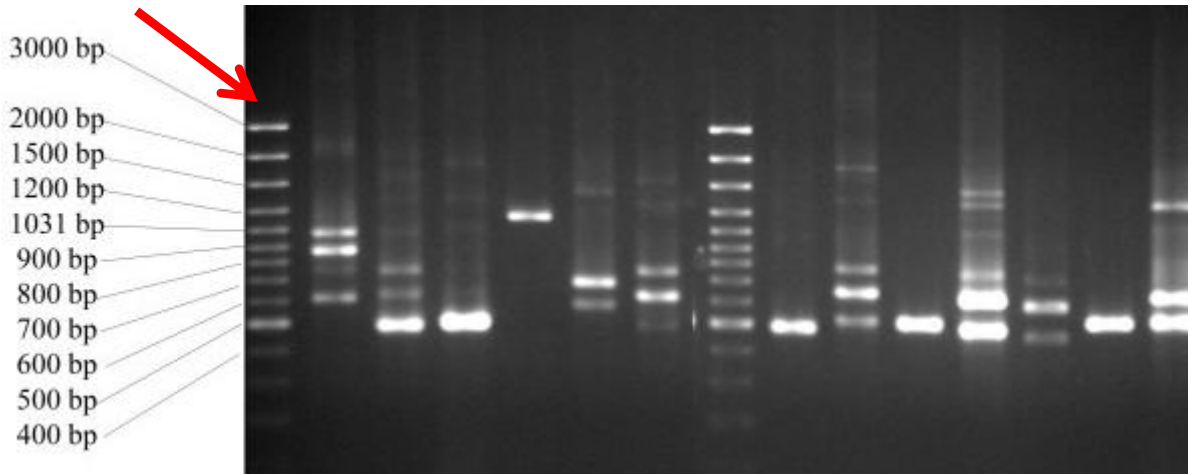
4. Smaller DNA segments move faster and farther than larger DNA segments.

How does gel electrophoresis work?

- DNA is forced by an electrical current through a firm gel
  - Phosphate group in DNA is negatively charged so it is moved towards a positive electrode by the current
  - Longer fragments have more nucleotides
    - So have a larger molecular weight
    - So are bigger in size
    - So aren't able to pass through the small holes in the gel and get hung up at the beginning of the gel
  - Shorter fragments are able to pass through and move farther along the gel
  - Fragments of intermediate length travel to about the middle of the gel

How does gel electrophoresis work?

- DNA fragments are then visualized in the gel with a special dye
- The number of nucleotides are then estimated by comparing it to a known sample of DNA fragments which is run through the gel at the same time → ladder

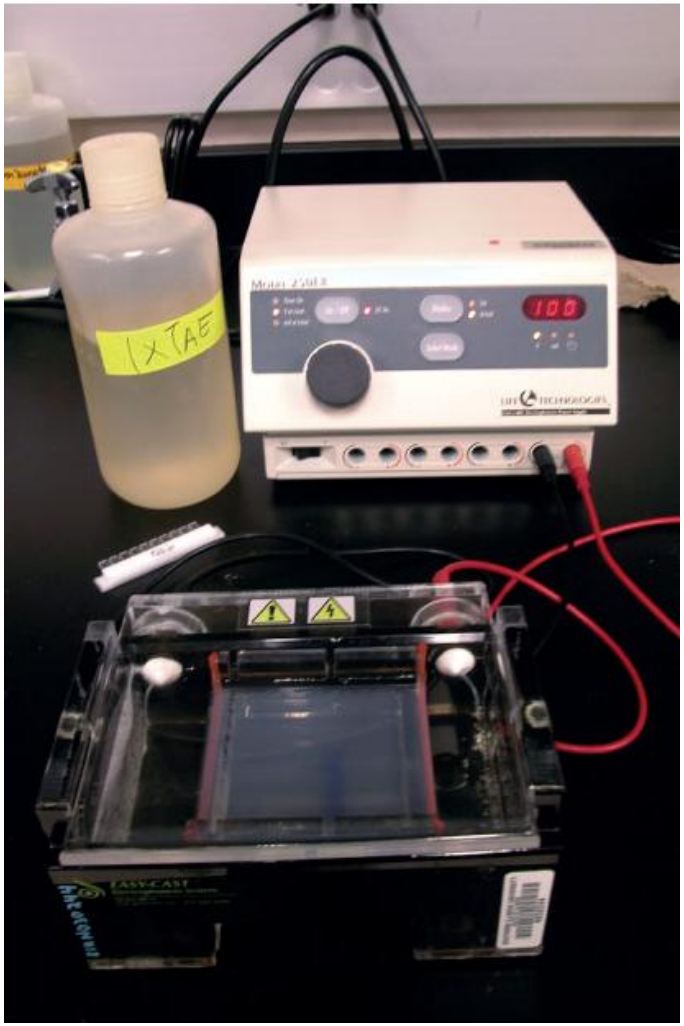


Reagents Needed for gel electrophoresis

- Sample of DNA fragments
- Known sample of DNA fragments
  - DNA ladder
- Gel
  - Agarose
- Dye to visualize the movement of the sample as it is traveling through the gel
  - Loading dye
- Dye to visualize DNA after it has traveled to its final spot in the gel
  - Ethidium bromide
- Buffer

Equipment Needed for gel electrophoresis work

- Box to hold the gel
- Comb to create small wells in the agarose gel to put the DNA sample in
- Positive and negative electrodes to create the electrical current
- Power supply
- Gel photo imaging system



What are the different types of PCRs?

Long range PCR

Long range PCR

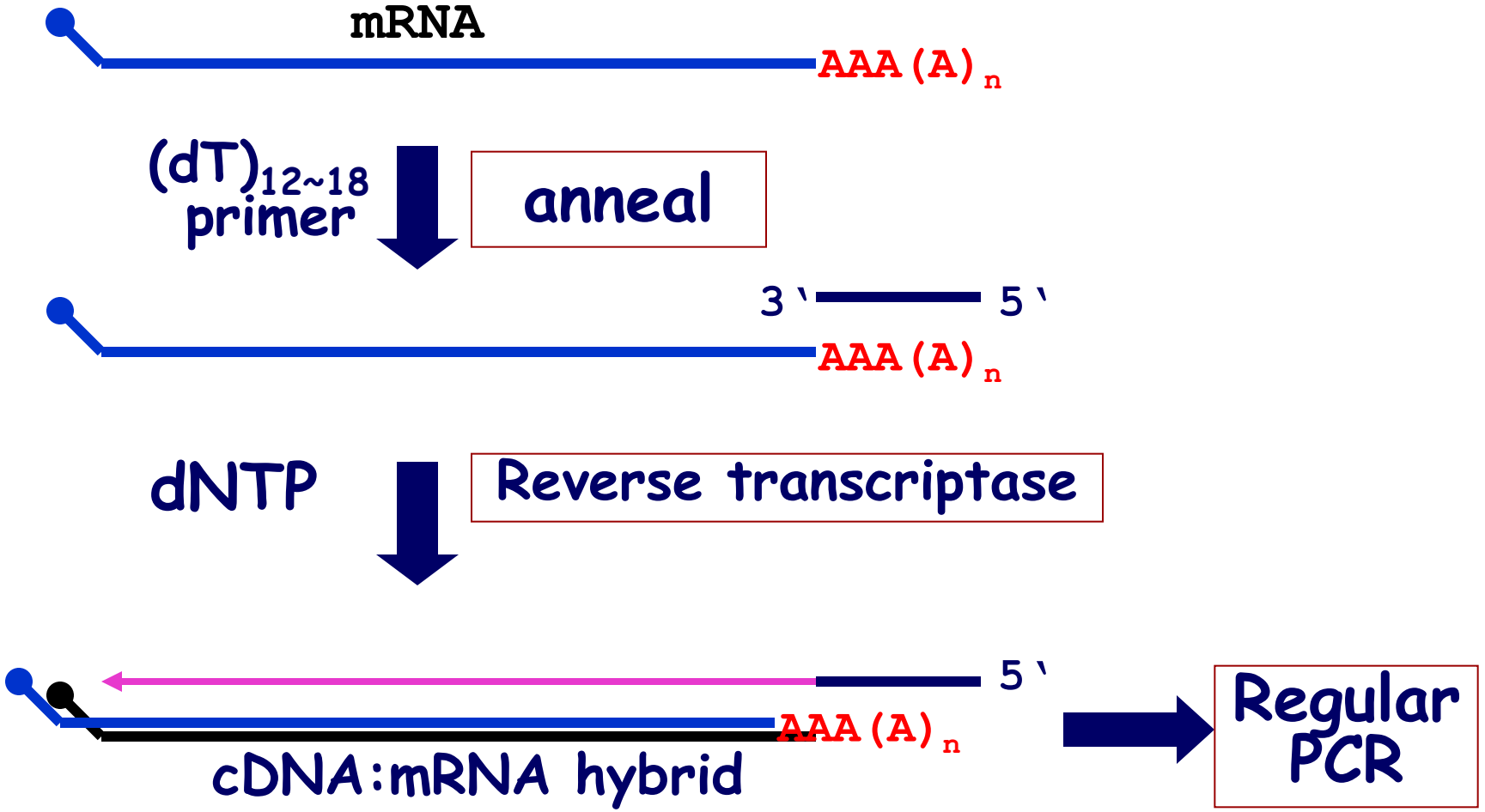
- PCR amplification of very long DNA templates (long range PCR)
- DNA proofreading activity allows for high fidelity amplification from a variety of templates
- High yields are possible with the robust enzyme-buffer system – includes an optimized buffer

RT-PCR

RT-PCR

- PCR of cDNA is used to detect specific transcripts in RNA sample.
- In this procedure, known as RT-PCR, reverse transcriptase is used to copy all of the mRNAs in an RNA sample into cDNA.
- Usually, oligo dT molecules, that anneal to the poly A tails of the mRNA, are used as primers.
- This single stranded cDNA can then be amplified by PCR using primers that anneal to a specific transcript sequence.

RT-PCR


mRNA
AAA(A)n
(dT)12~18 primer
anneal
3'
5'
AAA(A)n
dNTP
Reverse transcriptase
5'
AAA(A)n
cDNA:mRNA hybrid
Regular PCR

RT-PCR

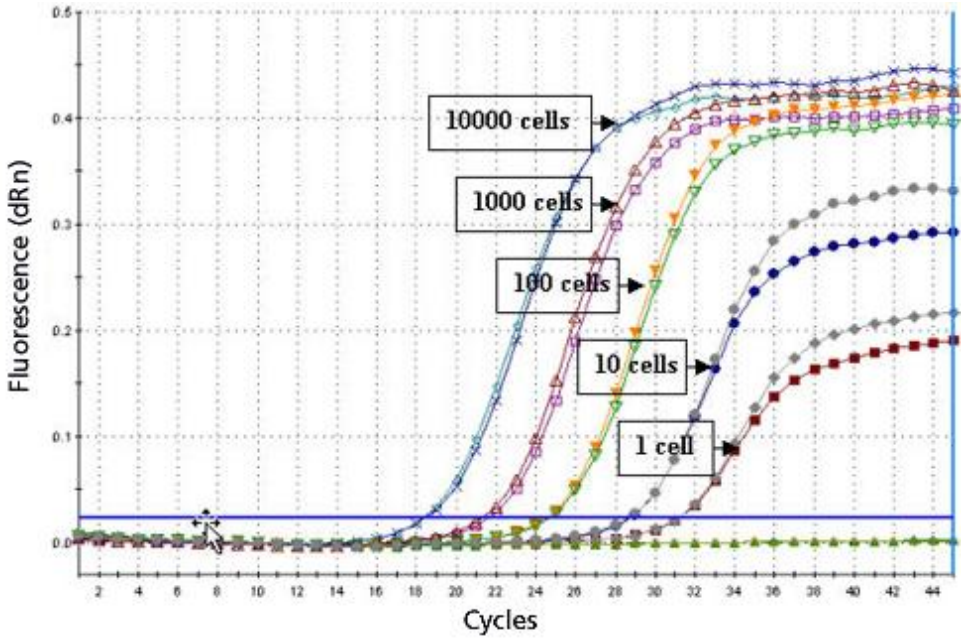
- The amplified DNA fragments that are produced can be analysed by agarose gel electrophoresis or fluorescent PCR or real time PCR.
- The amount of amplified fragment produced is proportional to the amount of target mRNA in the original RNA sample.
- RT-PCR is extremely sensitive and can be used to detect very rare mRNA species.

Real-time quantitative PCR:

Real-time quantitative PCR:

- Same as PCR, but measures the abundance of DNA as it is amplified.
- Useful for quantitatively measuring the levels of mRNA in a sample.
- Uses reverse transcriptase to generate cDNA for the template.
- Can also be used to quantitatively estimate fraction of DNA from various organisms in a heterogenous sample (e.g, can be used to measure abundance of different microbes in soil sample).
- Can be used to type SNPs if primer binding is stringent.
- Fluorescent dye, SYBR Green, is incorporated into PCR.
- SYBR Green fluoresces strongly when bound to DNA, but emits little fluorescence when not bound to DNA.
- SYBR Green fluorescence is proportional to the amount of DNA amplified, detected with a laser or other device.
- Experimental samples are compared to control sample with known concentration of cDNA.

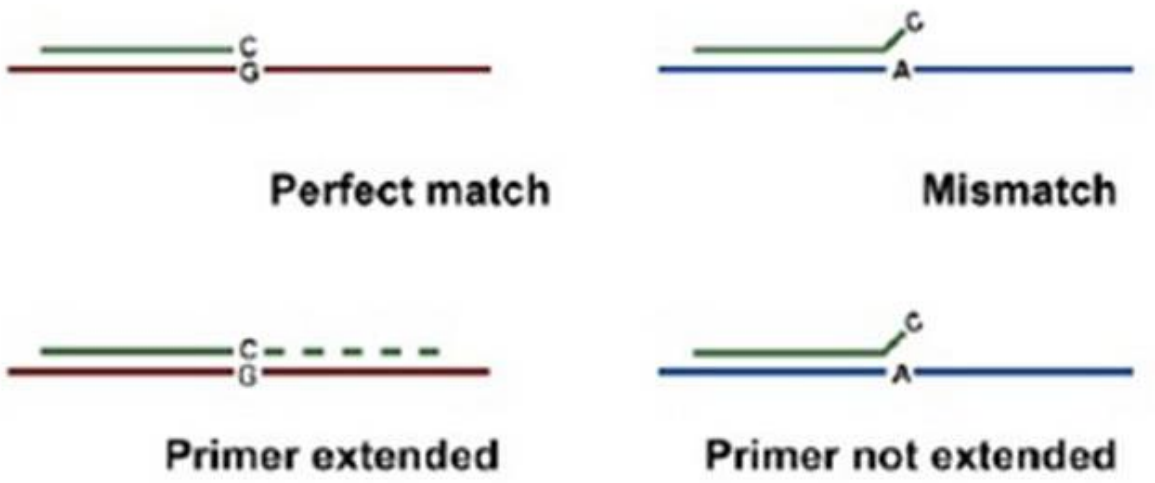
Real-time quantitative PCR amplification plot:



Allele-specific PCR

Allele-specific PCR

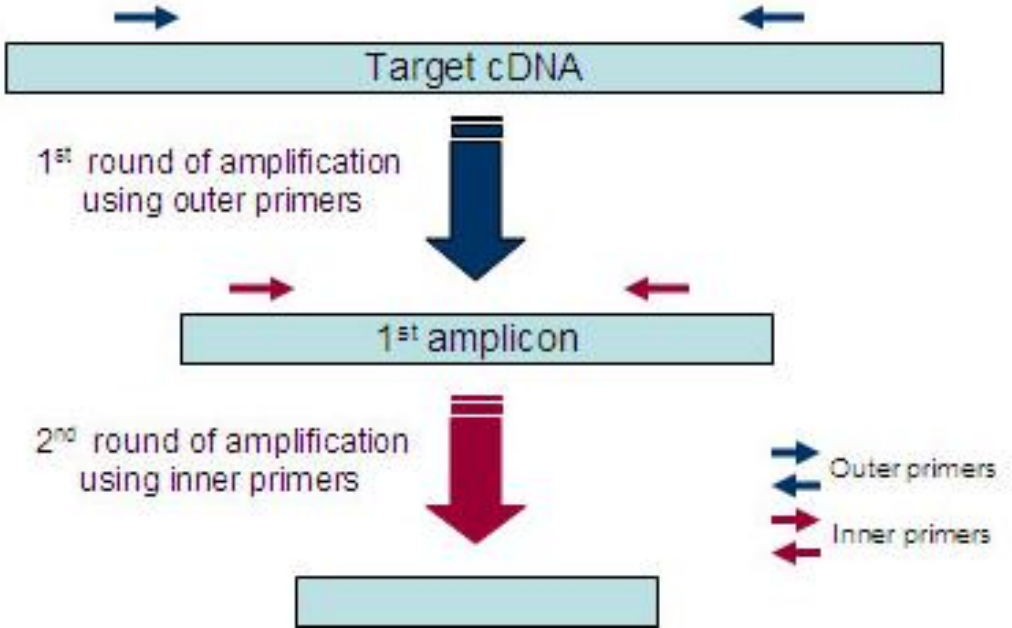
- Allele-specific PCR is used to identify or utilize single nucleotide polymorphisms (SNPs: single base differences in DNA).
- It requires prior knowledge of a DNA sequence, including differences between alleles and uses primers whose 3' ends encompass the SNP.
- PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.



Nested PCR

Nested PCR

- Nested PCR increases the specificity of DNA amplification by reducing background due to non-specific amplification of DNA.
- Two sets of primers are being used in two successive PCR.
  - In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments.
  - The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction.
- Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.



Methylation-specific PCR (MSP):

Methylation-specific PCR (MSP):

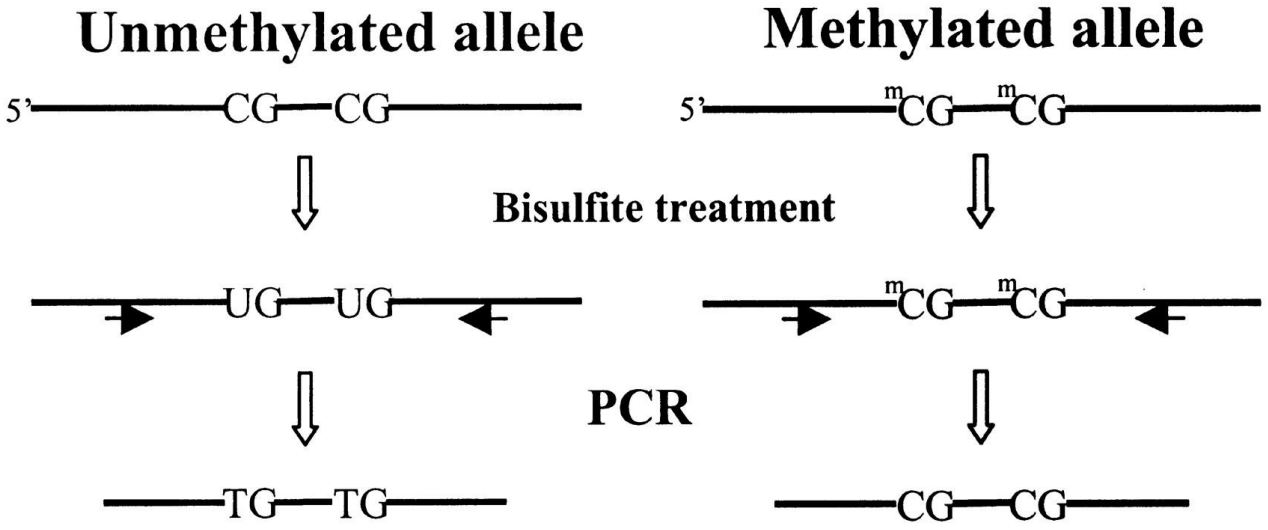
- MSP is used to detect methylation of CpG islands in genomic DNA.

1- DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine.

2- Two PCRs are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences.

At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA

One set recognizes DNA with uracil or thymine to amplify unmethylated DNA.



Microsattelites

Use of Microsatellites

- Repetitive DNA sequences

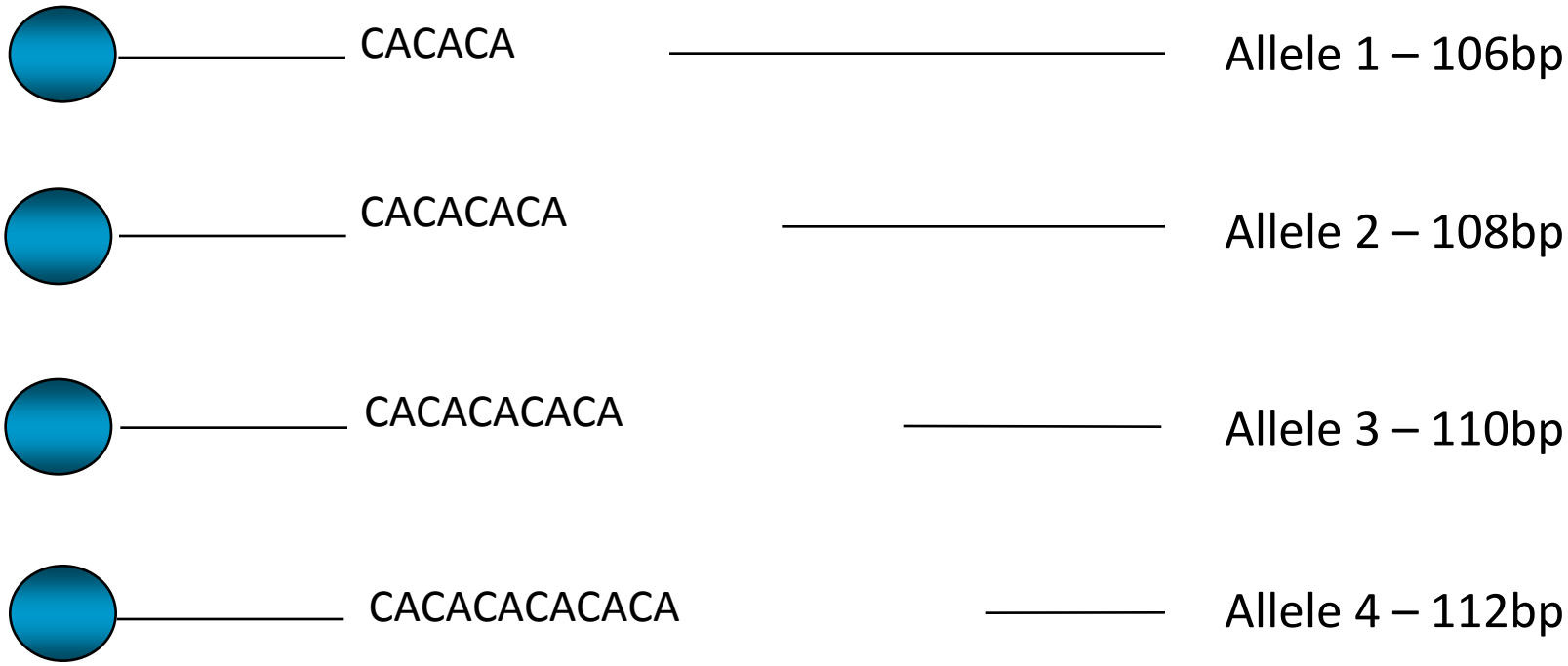
CACTCAGGAAGGGACCCTTAGACCTCAGCCCCA**CACACACACACACAC**CTCCCGCAGGAGCAGCA
TGGGATTAGGACCTATGGGATATAATGCAGCACTTTGGGAG

- Used for linkage analysis

- DNA fingerprinting

Microsatellite fragment sizing

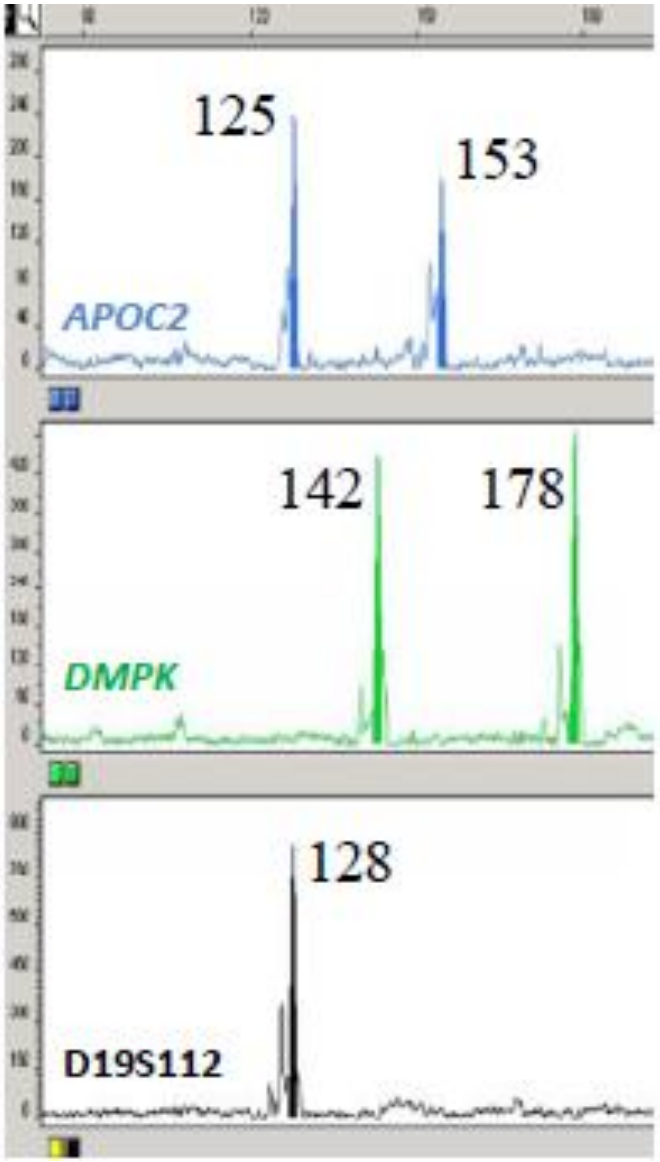
- Alleles distinguished by number of repeat units
  - Change in PCR product length



Fluorescent PCR

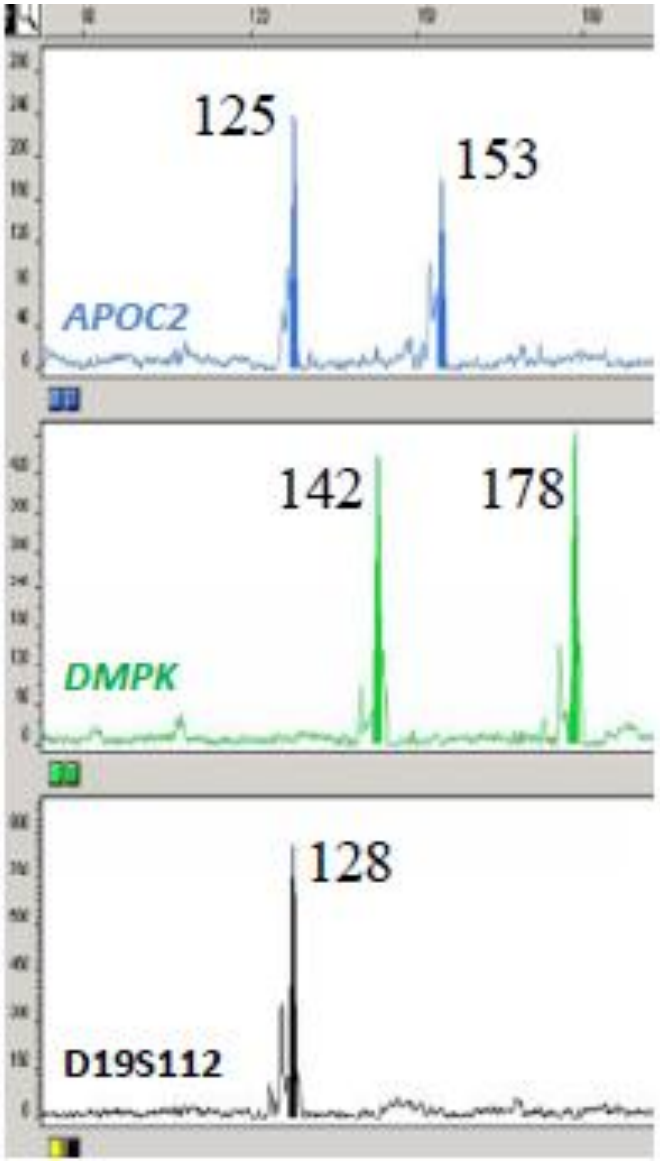
Fluorescent PCR

- Primers labelled at 5’terminus with fluorescent molecule



Flourescent PCR

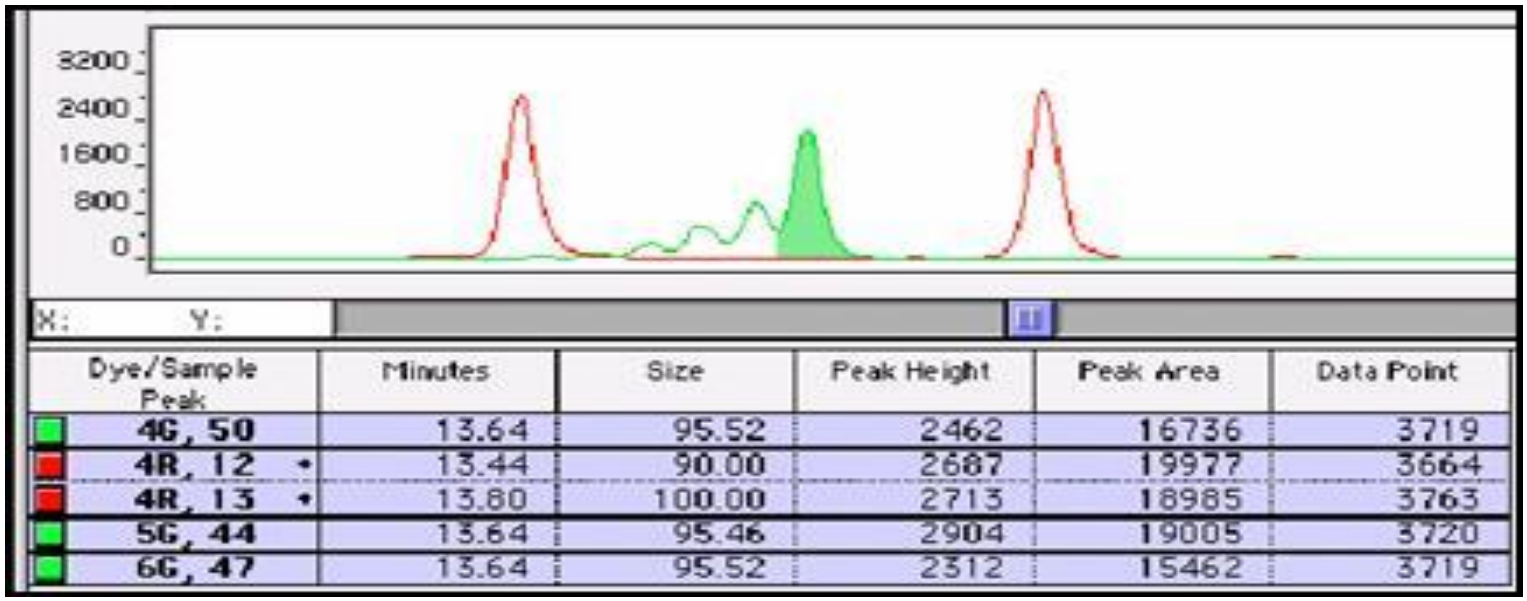
- PCR Products detected by laser analysis system
- Sizing of amplicon to single nucleotide accuracy
- Can use more than one colour of fluorescence → multiplex PCR
- Can differentiate same sized but different products
- Compatible with mutation analysis techniques



Detection of fluorescent PCR products by genetic analyser

Detection of fluorescent PCR products by genetic analyser

Amount



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
4G, 50	13.64	95.52	2462	16736	3719
4R, 12 •	13.44	90.00	2687	19977	3664
4R, 13 •	13.80	100.00	2713	18985	3763
5G, 44	13.64	95.46	2904	19005	3720
6G, 47	13.64	95.52	2312	15462	3719

Time / Length

Short time/Short fragments

Long time/Long fragments

Sequencing

Sequencing

- ✓ DNA sequencing = determining the nucleotide sequence of DNA

TTGTTATCCGCTCACAATTCCACACAAC

- ✓ Dideoxy sequencing developed by Frederick Sanger in the 1970s.

1980: Walter Gilbert (Biol. Labs) & Frederick Sanger (MRC Labs)

Sequencing

1. DNA template is denatured to single strands.

2. Single DNA primer (3' end near sequence of interest) is annealed to template DNA and extended with DNA polymerase.

3. Reactions contain:

    1. DNA template
    2. Primer annealed to template DNA
    3. DNA polymerase
    4. dNTPs (dATP, dTTP, dCTP, and dGTP)

4. Next, a different labeled dideoxynucleotide (ddATP, ddTTP, ddCTP, or ddGTP) is added to each of the four reaction tubes at 1/100th the concentration of normal dNTPs.

5. ddNTPs possess a 3'-H instead of 3'-OH, compete in the reaction with normal dNTPS, and produce no phosphodiester bond.

Sequencing

7. Whenever the labeled ddNTPs are incorporated in the chain, DNA synthesis terminates.

8. Dideoxy DNA sequencing also called dye terminator sequencing.

9. Each of the four reaction mixtures produces a population of DNA molecules with DNA chains terminating at all possible positions.

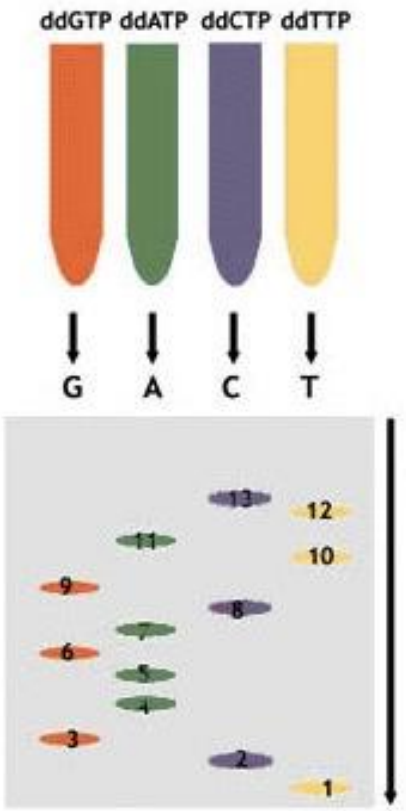
10. Extension products in each of the four reaction mixtures also end with a different labeled ddNTP (depending on the base).

11. Next, each reaction mixture is electrophoresed on a polyacrylamide gel or analysed by genetic analyser.

Sequencing

Polyacrylamide gels

- Pattern of bands in each of the four lanes is visualized on X-ray film.
- Location of “bands” in each of the four lanes indicate the size of the fragment terminating with a respective labeled ddNTP.
- DNA sequence is deduced from the pattern of bands in the 4 lanes.



Automated DNA Sequencing

1. Automated DNA sequencing uses ddNTPs labeled with fluorescent dyes.

2. Combine 4 dyes fluorescing at different wavelengths in one reaction tube and electrophores in one lane on a capillary containing polyacrylamide.

3. Capillary is thinner then gel → higher voltage → even faster.

4. UV laser detects dyes and reads the sequence.

5. Sequence data is displayed as colored peaks (chromatograms) that correspond to the position of each nucleotide in the sequence.

6. Throughput is high, up to 1200 bp per reaction and 96 reactions every 3 hours with capillary sequencers.

7. Most automated DNA sequencers can load robotically and operate around the clock for weeks with minimal labor.

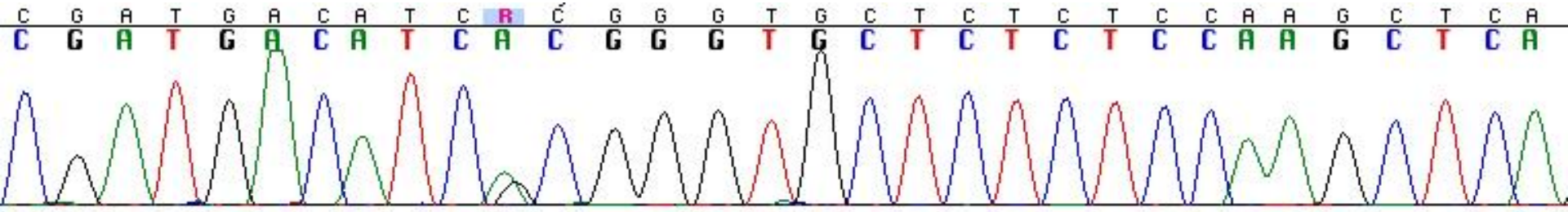
Automated DNA Sequencing

Applied Biosystems PRISM 3700
(Capillary, 96 capillaries)

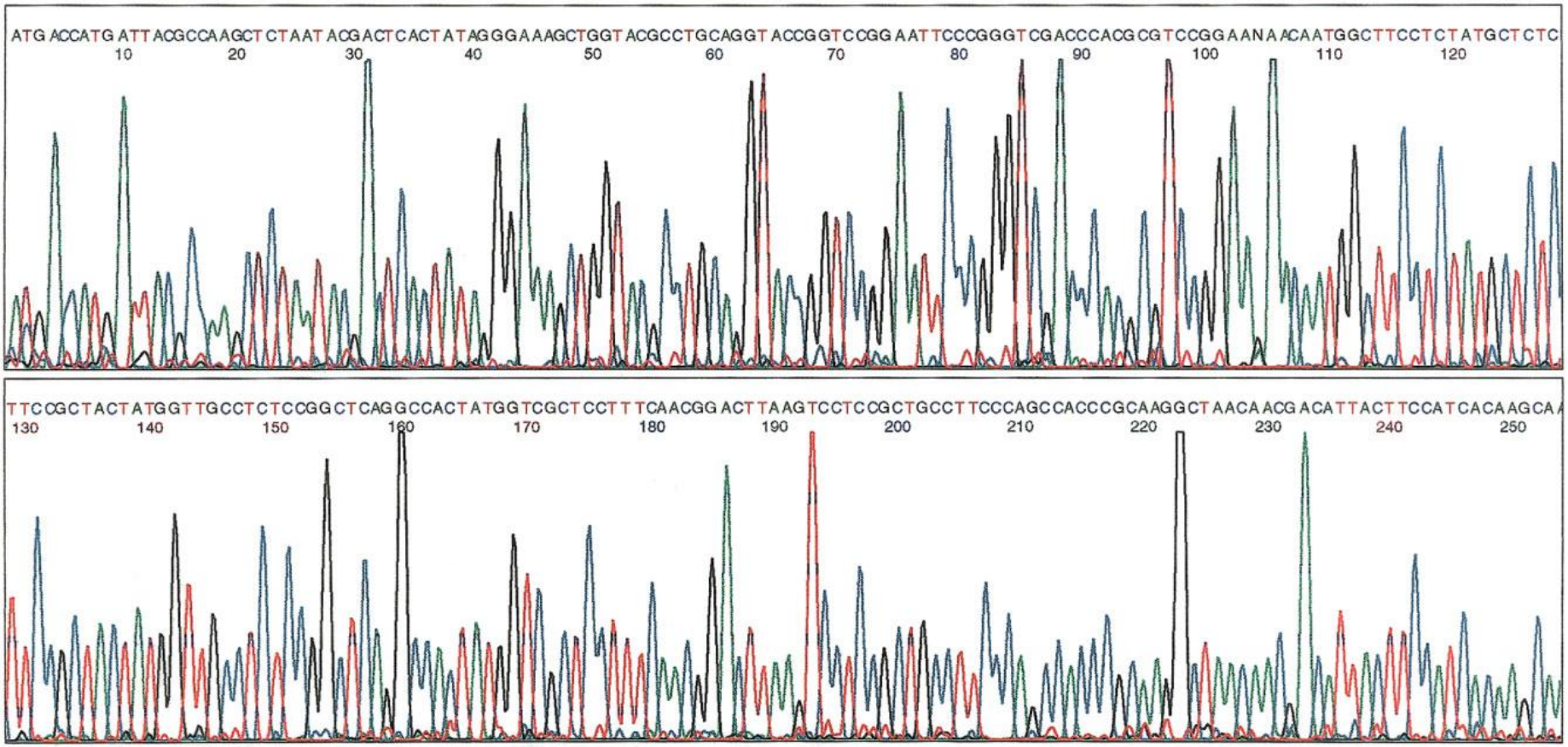
Applied Biosystems PRISM 3100
(Capillary, 16 capillaries)

Automated DNA Sequencing

Trace files (dye signals) are analyzed and bases create chromatograms.



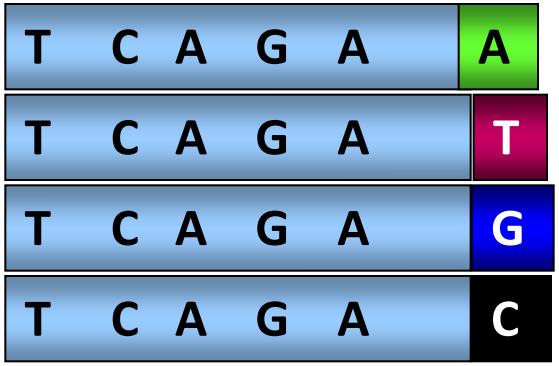
c)

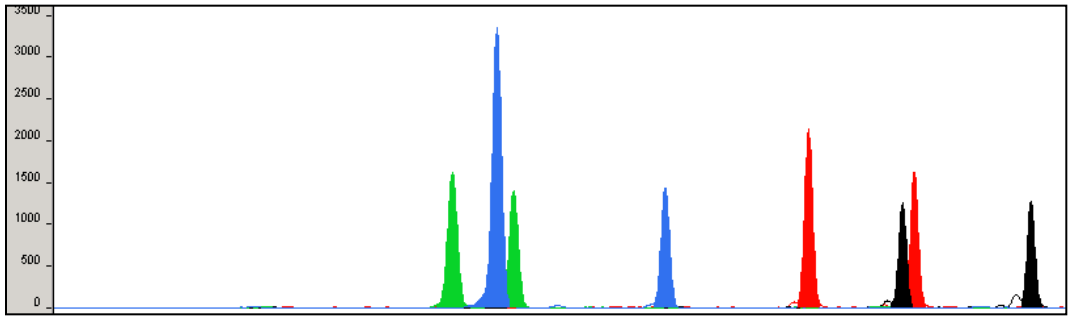


Mini-sequencing

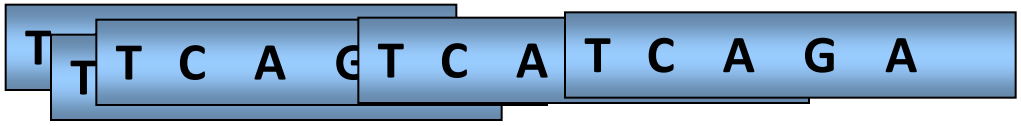
- Mini-sequencing is a fast and simple method to detect any known point mutation or allelic variation of DNA.
- Similar to sequencing
- Only uses ddNTPs
- Only one nucleotide added to primer
- Colour of ddNTP reveals the nucleotide at this site
- Useful for diseases caused by many different mutations
- Design one set of primers to encompass multiple mutation sites

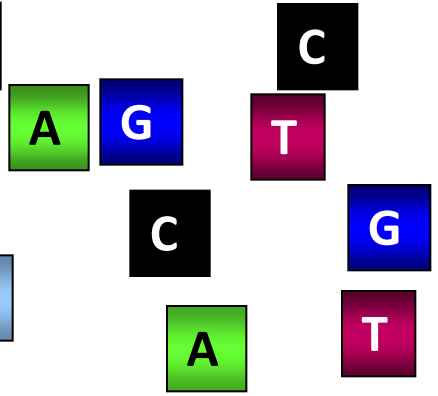
Mini sequencing (SnaPShot)





3’ - A A G T C T T G A T C -5’





PCR has become a very powerful tool in molecular biology

- One can start with a single sperm cell or stand of hair and amplify the DNA sufficiently to allow for DNA analysis.
- One can amplify fragments of interest in an organism's DNA by choosing the right primers.
- One can use the selectivity of the primers to identify the likelihood of an individual carrying a particular allele of a gene.

PCR and Disease

- Primers can be created that will only bind and amplify certain alleles of genes or mutations of genes
    - This is the basis of diagnostic tests and genetic counseling
    - PCR is used for diagnosis of genetic diseases.
- Some diseases that can be diagnosed with the help of PCR:
    - Huntington's disease
    - Cystic fibrosis
    - Human immunodeficiency virus

PCR and Forensic Science

- It is often of interest in forensic science to identify individuals genetically.
  - In these cases, one is interested in looking at variable regions of the genome as opposed to highly-conserved genes.

- PCR is used to amplify highly variable regions of the human genome. These regions contain runs of short, repeated sequences (STRs) .
  - Primers are chosen that will amplify these repeated areas and the genomic fragments generated give us a unique "genetic fingerprint" that can be used to identify an individual.

DNA Fingerprinting
Paternity testing