

TECHNIQUES OF MOLECULAR BIOLOGY: POLYMERASE CHAIN REACTION

Instruct: Dr. M. A. Srouf

Course: Molecular Biology (BIOL 333)

Textbook:

Watson J, et al. (2014). Molecular Biology of the Gene, 7th ed. Chap 7

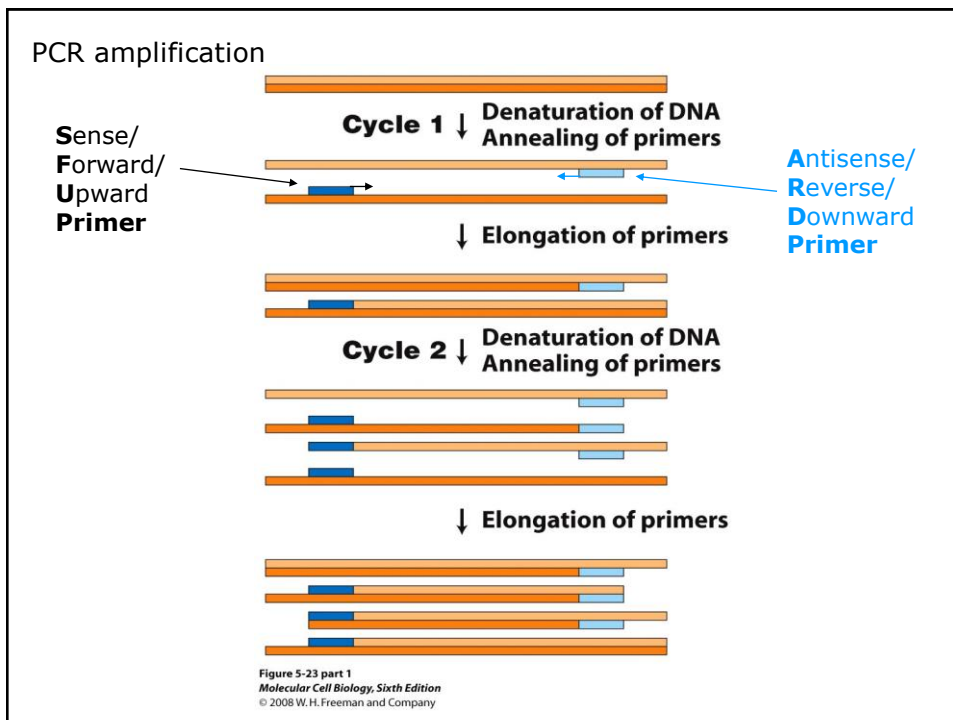
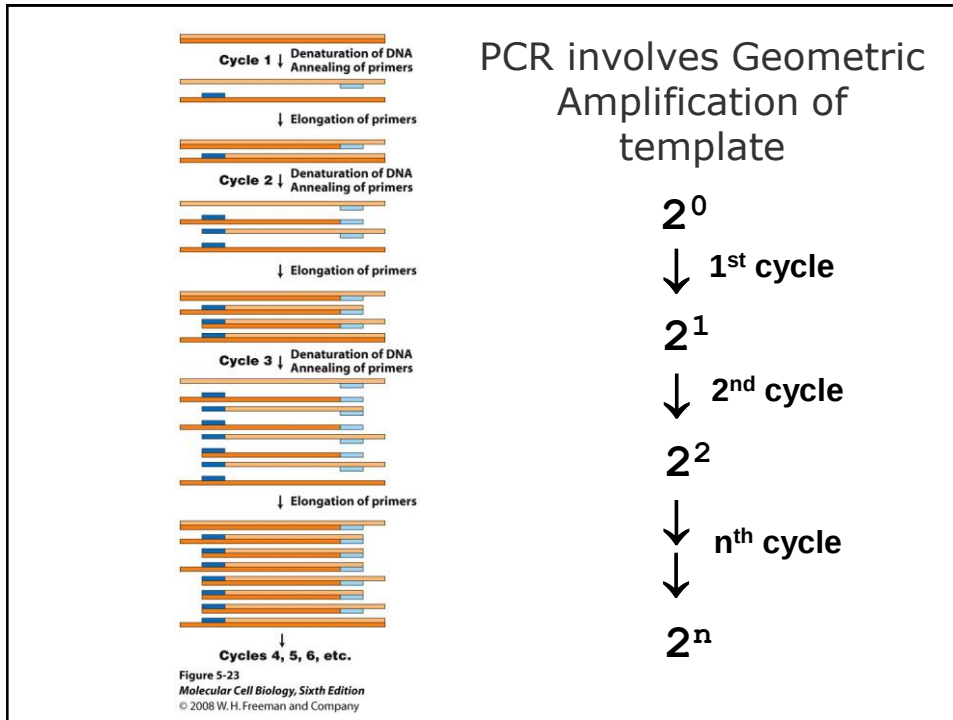
Polymerase Chain Reaction (PCR)

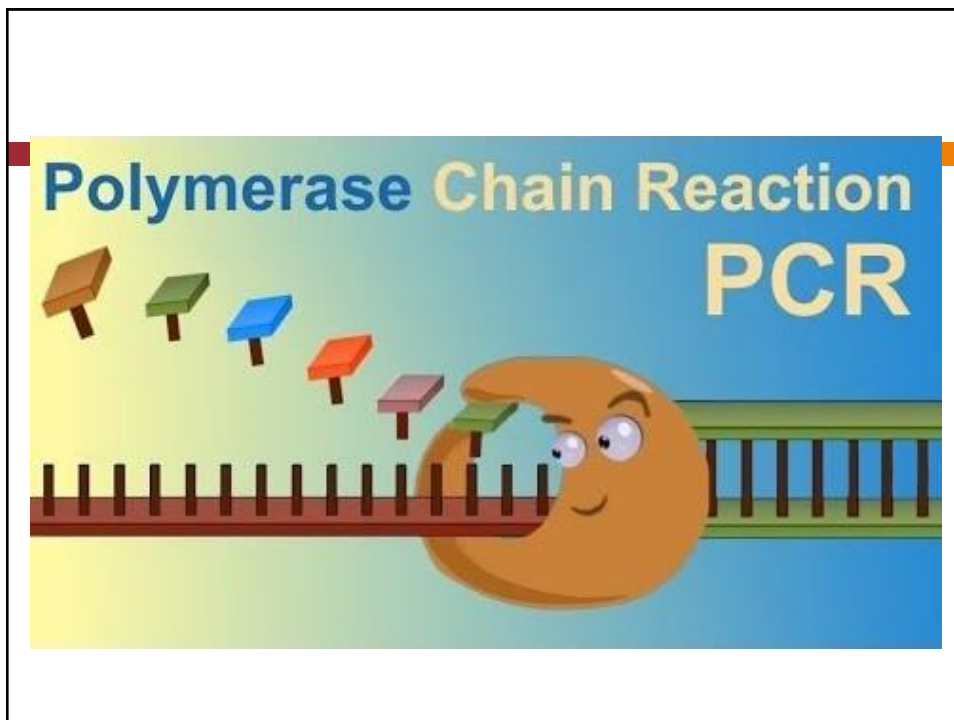
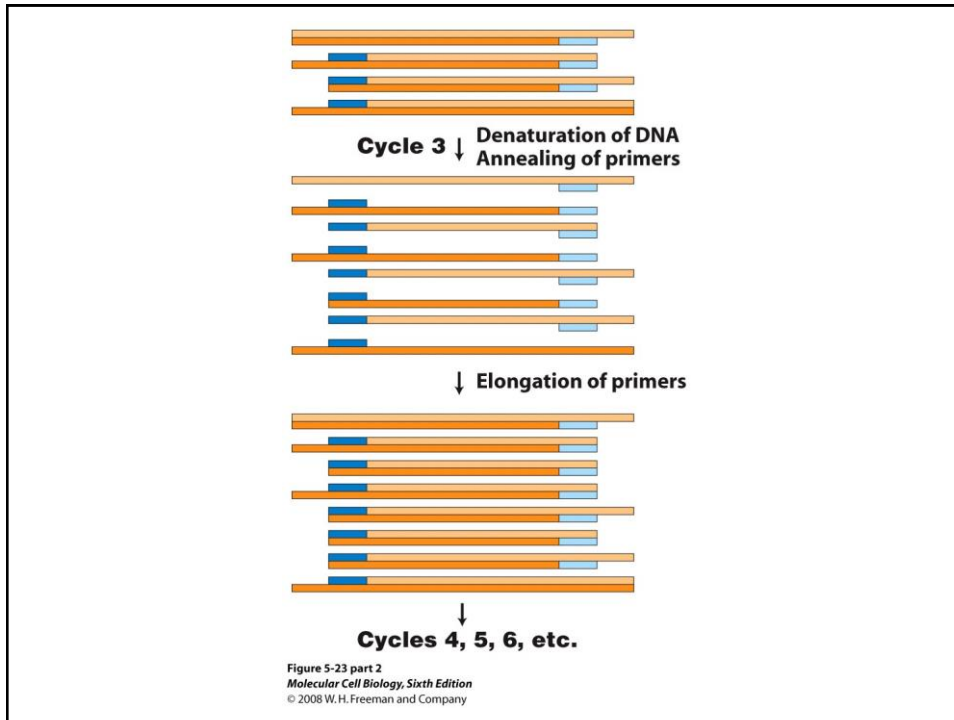
- ❑ PCR: *in vitro* amplification of a specific DNA region flanked by known sequences
- ❑ Specific DNA fragment(s) are enzymatically amplified
- ❑ 10^6 -fold amplification possible
- ❑ Can detect single molecule
- ❑ Tolerates impure DNA
- ❑ Assay time < day

PCR

- PCR Requirements:
 - Heat-stable DNA polymerase
 - Deoxynucleotides (dNTPs)
 - Target DNA
 - A pair of oligonucleotides (primers)
 - Thermocycler
- Taq Polymerase
- *Thermus aquaticus* DNA polymerase
- Thermophilic organism
- Enzymes resistant to high temperatures
- 72-74°C optimum

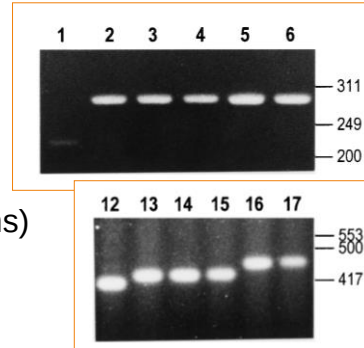
- DNA Learning center at Cold Spring Harbor Lab:
<https://dnalc.cshl.edu/resources/animations/pcr.htm>
!
- Virtual labs for PCR & gel electrophoresis:
<http://learn.genetics.utah.edu/content/labs/>





PCR Protocol

- Mix DNA, primers, dNTPs, Taq, buffer, Mg^{2+}
- Program thermocycler for times and temperatures
 - ▣ Denaturation
 - ▣ Annealing
 - ▣ Extension
- Thermal cycling: 30-35 cycles
- Analyze amplified DNA (amplicons) by agarose gel electrophoresis



PCR: Thermal cycling

Step	Temp	Time	Notes
Initial Denaturation	94-96°C	2-5 min	
30-35 cycles:			
Denaturation	94-96°C	0.5-2 min	Longer: ↑ denaturation, but ↓ enzyme & template
Annealing	45-65°C	0.5-2 min	Higher/shorter: ↑ specificity, but ↓ yield
Extension	72°C	~1 min/kb	Taq processivity = 150 nt/sec
Final extension	72°C	5 min	

Stringency and Melting Temperature

- Melting Temperature (T_m)
 - Temperature at which strands separate
 - Can estimate T_m with formulas: **
- Synthetic Oligonucleotides:

$$T_m = 2(A + T) + 4(G + C)$$
- Effective T_m = 81.5 + 16.6log[Na⁺] + 0.41(%GC) - 0.65(600/N) - 1.4(%mismatch)*

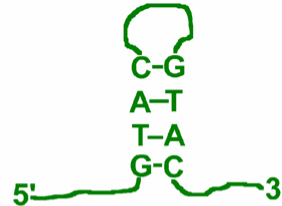
Stringency and Melting Temperature

- Stringency of a hybridization reaction: refers to the tolerance (or lack thereof) for mistakes in base pairing
- High stringency: low salt conc, high formamide, high temperature > require exact base pairing
- Low stringency: high salt conc, low formamide, low temperature >> more base pair mismatches can be tolerated

Design of Oligonucleotide Primers

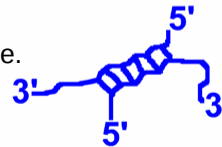
□ Criteria for selection of an oligo

- Uniqueness (18-28 bases)
- $T_m > 55^\circ$
- 50% GC composition
- 3'-GC 'caps'
- No internal complementarity
- No "primer dimers"



□ How to design an oligo?

- ▣ Manual
- ▣ Analyze sequence with computer software.
- ▣ Online software: NCBI/Primer BLAST; available at: www.ncbi.nlm.nih.gov



Types of PCR: RT-PCR

- Isolation of RNA template, requires careful handling of RNA templates
- Synthesis of cDNA using Oligo-dT, random hexamers or gene-specific primers, and reverse transcriptase at 37-42°C
- RT: Avian myeloblastosis virus (AMV) or Molony murine leukemia virus (MMLV) RT
- RNase inhibitors are usually used in cDNA synthesis rxn
- The cDNA is further amplified using a normal PCR rxn using gene-specific primers

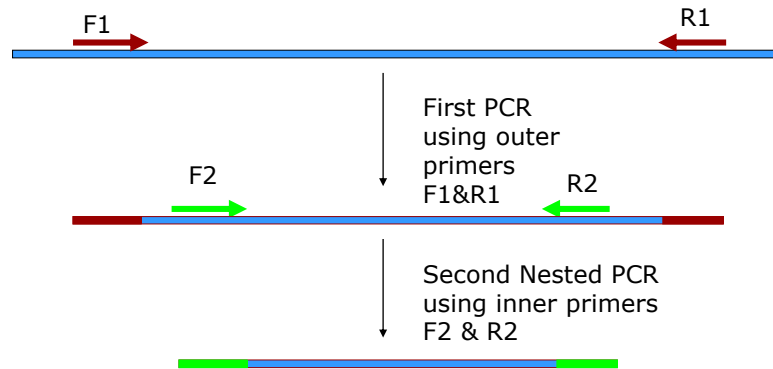
Types of PCR: RT-PCR

- Applications
 - ▣ Study of gene expression
 - ▣ Quantitation of mRNA and viral RNA levels
 - ▣ Detection of specific gene expression/ mRNA
 - ▣ Detection of RNA viruses

Types of PCR: Nested PCR

- Nested: 2 outer and 2 inner primers
- Why nested PCR?
 - ▣ Increase sensitivity and specificity

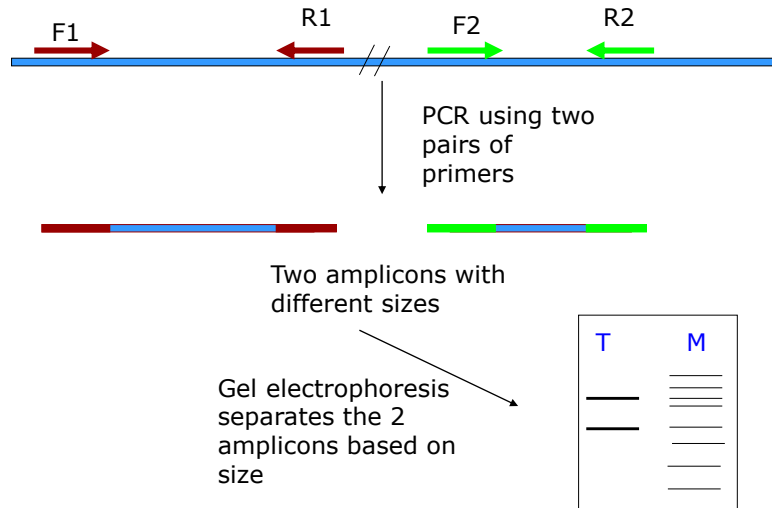
Types of PCR: Nested PCR



Types of PCR: Multiplex PCR

- It uses more than one pair of primers (multiplex)
- Allows co-amplification of more than one fragment/ target in one tube
- Can involve the target and internal control fragments
- Decreases the number of tubes per rxn per sample
- Requires careful optimization of rxn and design of primers

Types of PCR: Multiplex PCR



Types of PCR:

Amplification Refractory Mutation System (ARMS)

- ARMS is ideally suited for detection of point mutation and small insertion/deletions
- It involves two primers > wt allele & mutant allele >> two PCRs, one for each allele
- Multiplex ARMS
- Advantages of ARMS
 - ▣ Quick, inexpensive
 - ▣ Not suited for detection of unknown mutations

ARMS primers

- It is useful to increase the length of primers to about 30 nts.
- Primers usually include a mismatch close to the 3' end at position -2 to -3, to improve specificity (but may decrease yield)
- A second mismatch at nt -5 is sometimes included
- The most discriminatory mismatch involves A:G/G:A

ARMS PCR

Wild type β -globin gene sequence > β^A

5'cctgtggagccaca.....[aggagaagtctgccg..... 3'

wt mis
[tcgt] ←

3'→ caca.....tcctcttcagacggc..... 5'

Mutant β -globin gene sequence > Codon 6 (A→T) > β^S

5'cctgtggagccaca.....[tggaagtctgccg..... 3'

m mis
[acgt] ←

3'→ caca.....tcctcttcagacggc..... 5'

ARMS results: 2 rxns per sample

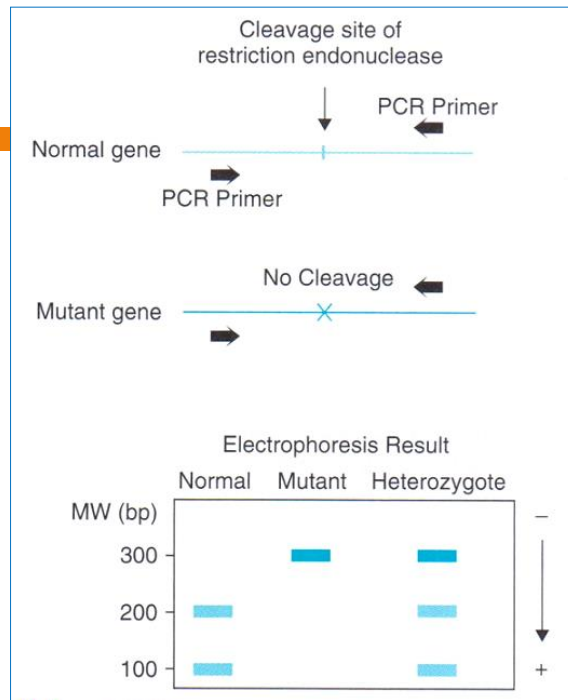
	Wt Rxn	Mut Rxn
Sample # 1: Wt Homozygote		
Sample # 2: Wt+Mut Heterozygote		
Sample # 3: Mut Homozygote		

	Wt
	mut

Types of PCR: RFLP-PCR

- RFLP: Restriction Fragment Length Polymorphism
- Allows detection of mutations that generate a new or delete an existing restriction site
- Amplicons flanking the target mutation are amplified by normal PCR and then digested using an appropriate Restriction enzyme (RE)
- RFLP-PCR increase the specificity of the first PCR

Types of PCR: RFLP-PCR



DNA sequencing

DNA sequencing:

<https://dnalc.cshl.edu/view/15479-Sanger-method-of-DNA-sequencing-3D-animation-with-narration.html>

DNA sequencing by **Sanger method**

/ Dideoxy chain termination method:

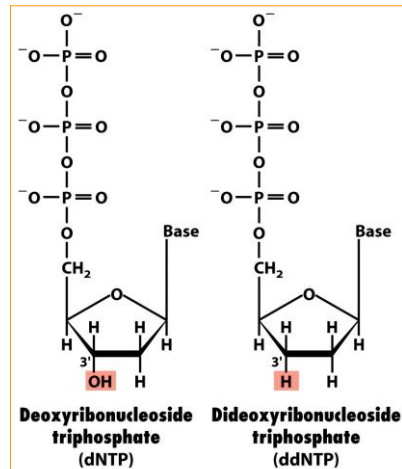
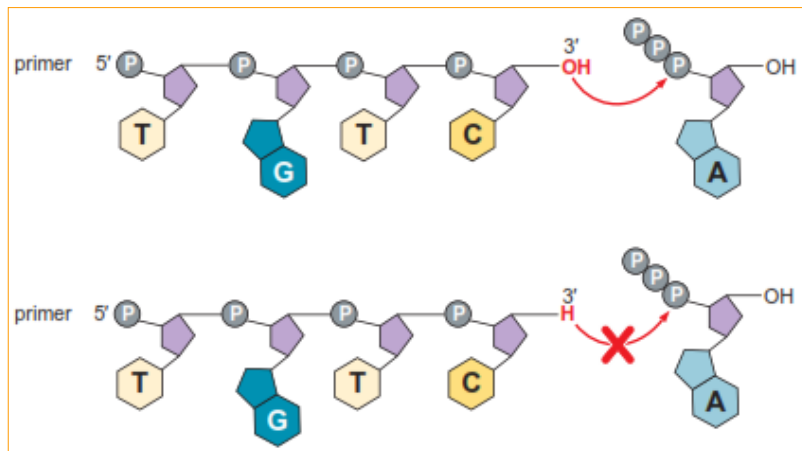


Figure 5-20
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Structure of
dNTP & ddNTP

Dideoxy chain termination method in presence of ddNTPs



DNA sequencing by Dideoxy chain termination method

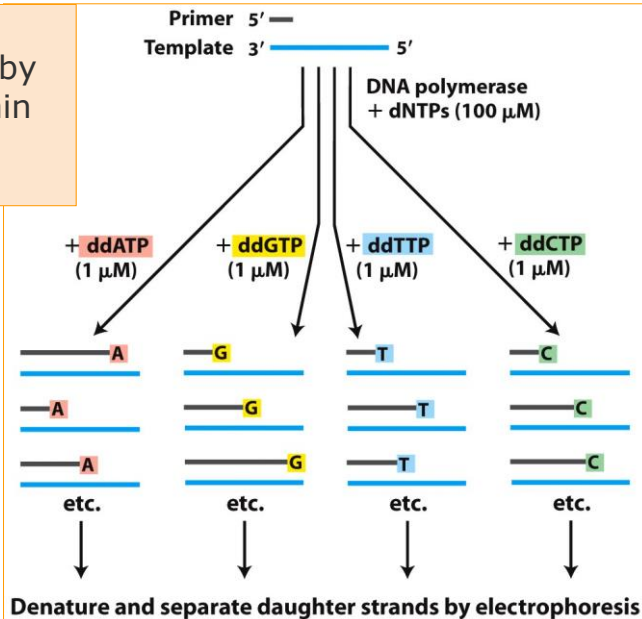
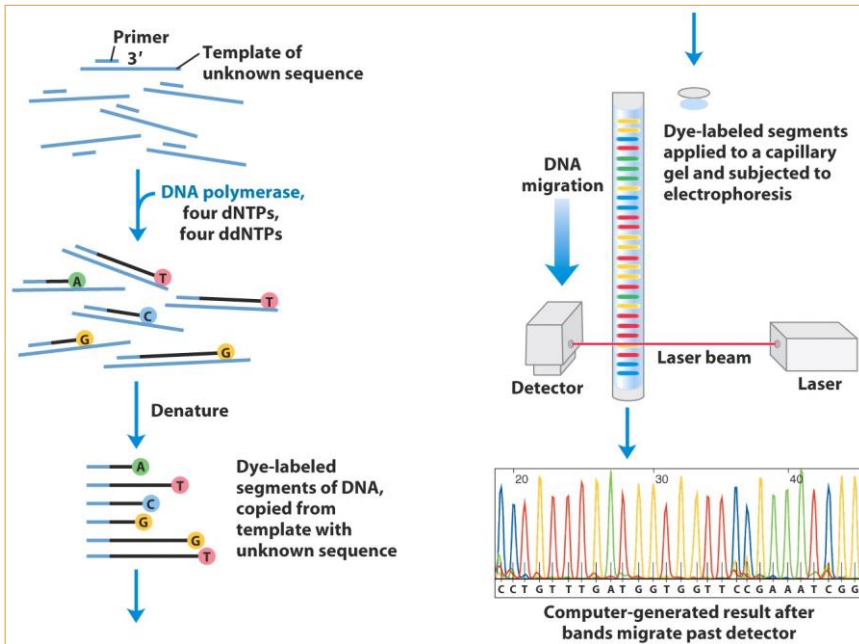
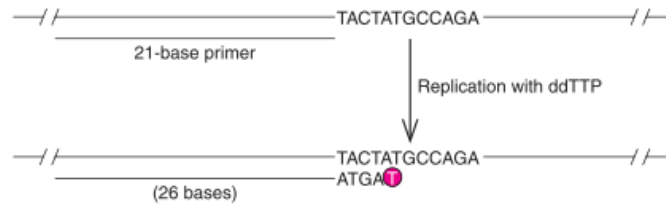


Figure 5-21b
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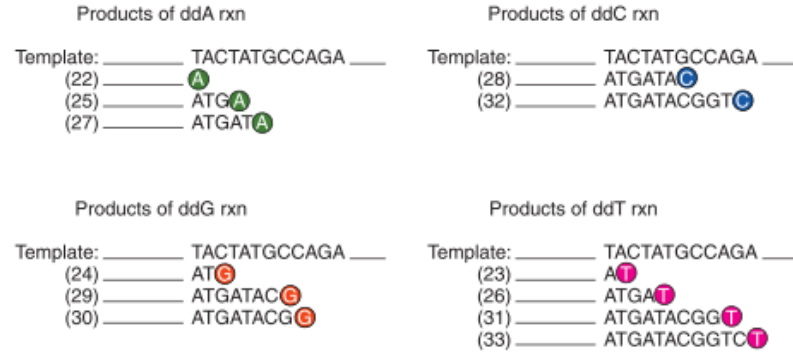


DNA sequencing

(a) Primer extension reaction:

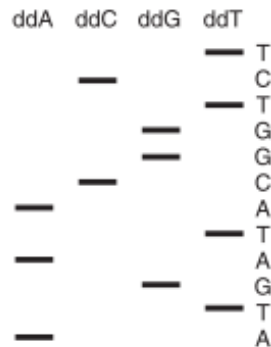


(b) Products of the four reactions:



DNA sequencing

(c) Electrophoresis of the products:



DNA sequence readout: by Dideoxy chain termination method

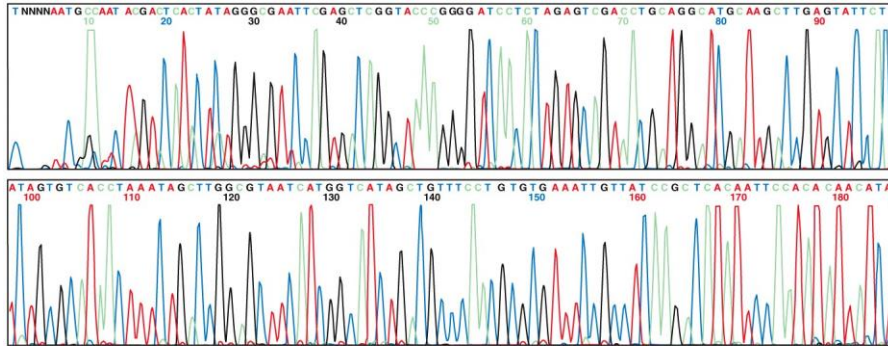
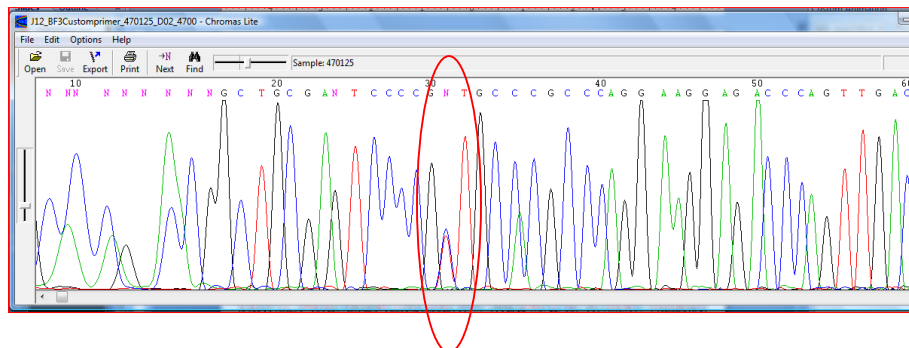


Figure 5-21c
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DNA sequence readout: chromatogram

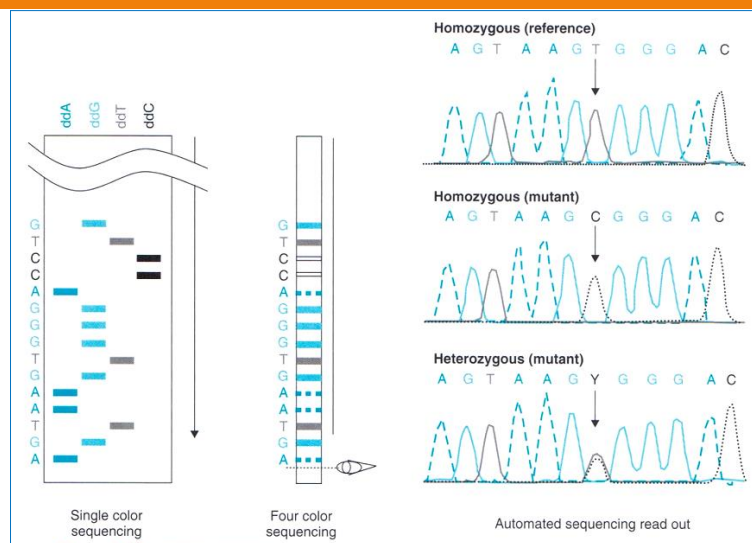


Applications of DNA sequencing

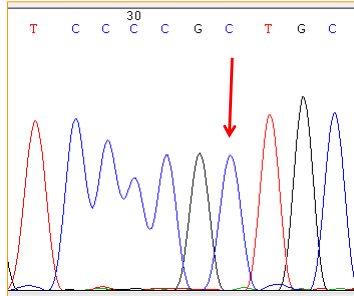
- Study gene sequence & structure
- Allows genome sequencing, e.g. Human genome project completed sequencing human genome in 2003
- Detection of mutations (known & unknown)

- Sequencing is now being done using “DNA sequencing machines” or “Sequencers”

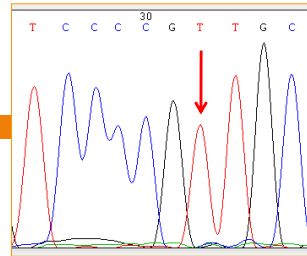
Detection of point mutations using DNA sequencing



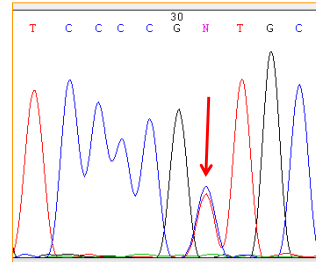
Detection of point mutations using DNA sequencing



Homozygous
(Reference, Normal)
5'-TCCCCG **C** T G C-3'



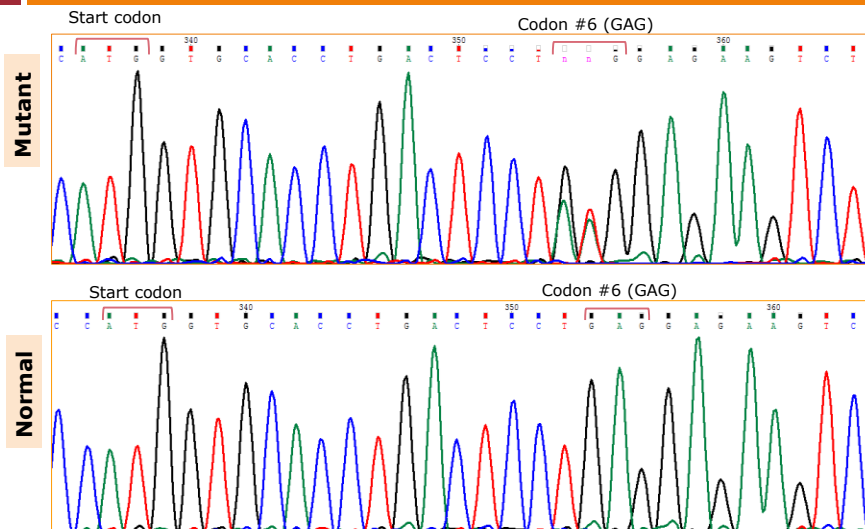
Homozygous (Mutant)
5'-TCCCCG **T** T G C-3'



Heterozygous (Mutant)
5'-TCCCCG (**C/T**) T G C-3'

Heterozygous for HbS & HbC

HbS: GAG>GTG, Glu>Val; **HbC:** GAG>AAG, Glu>Lys



Chemical synthesis of DNA

Chemical synthesis of DNA oligos

- It allows the chemical synthesis of short, custom designed segments of single-stranded DNA (ssDNA), known as oligonucleotides
- Synthesis is performed on solid supports using automated machines.
- Precursors used for nucleotide addition are chemically protected molecules called phosphoramidites

Chemical synthesis of DNA oligos (cont'd)

- In chemical synthesis, the DNA chain grows by addition to the 5' end of the molecule ($3' \rightarrow 5'$), while in vivo polymerization is $5' \rightarrow 3'$.
- Synthesis of ssDNA molecules 10-100 bases long is efficient and accurate
- Molecules >100 nucleotides long are difficult to synthesize in the quantity and with the accuracy desirable for most molecular analysis.

Chemical synthesis of DNA oligos

- Method:
- The first nt is attached to silica support via its 3'-OH & is protected at 5'-OH by an acid-labile Dimethoxytrityl (DMT) group
- The DMT is removed by washing with acid
- The next nt is activated with a Diisopropylamino group (MeO), which is oxidized with iodine to form phosphotriester linkage ($5'-3'$)
- The synthesis continues to the desired length
- The protecting groups are removed from P
- The oligo is separated from solid support & purified

Chemical synthesis of oligos

Blocking >

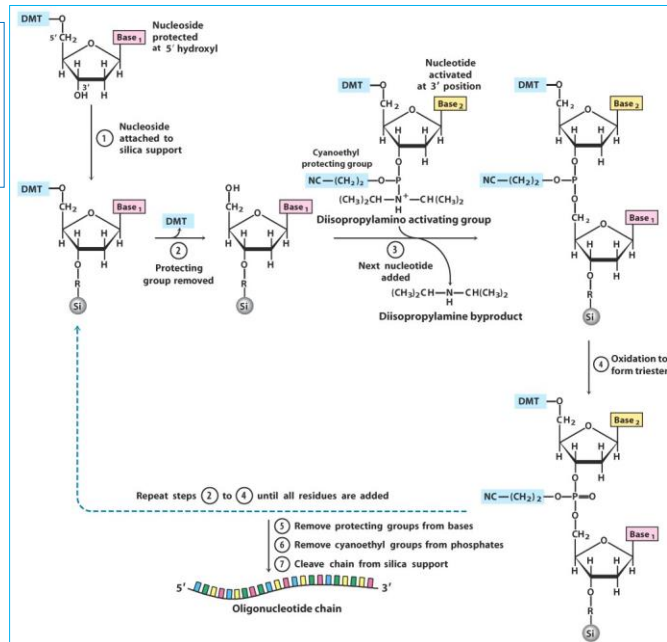
DMT: 4',4'-dimethoxytrityl

Activation >

MeO: diisopropylamino group

Protection >

NIP3: Cyanoethyl group



Blocking >

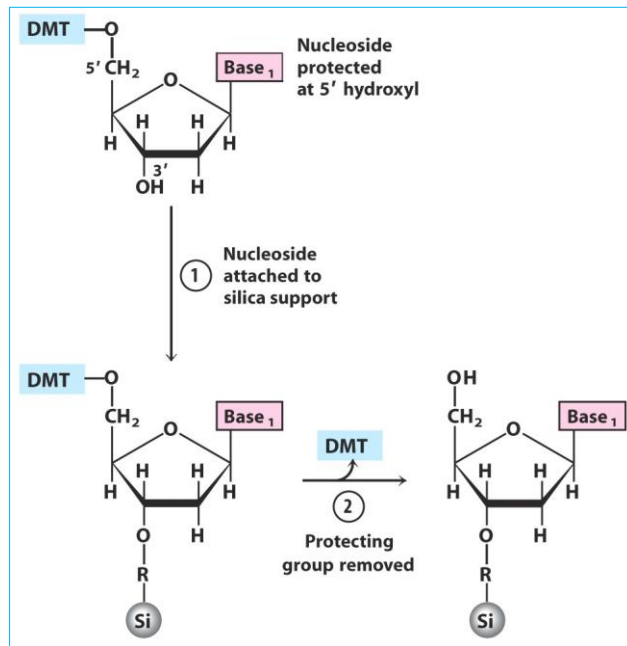
DMT: 4',4'-dimethoxytrityl

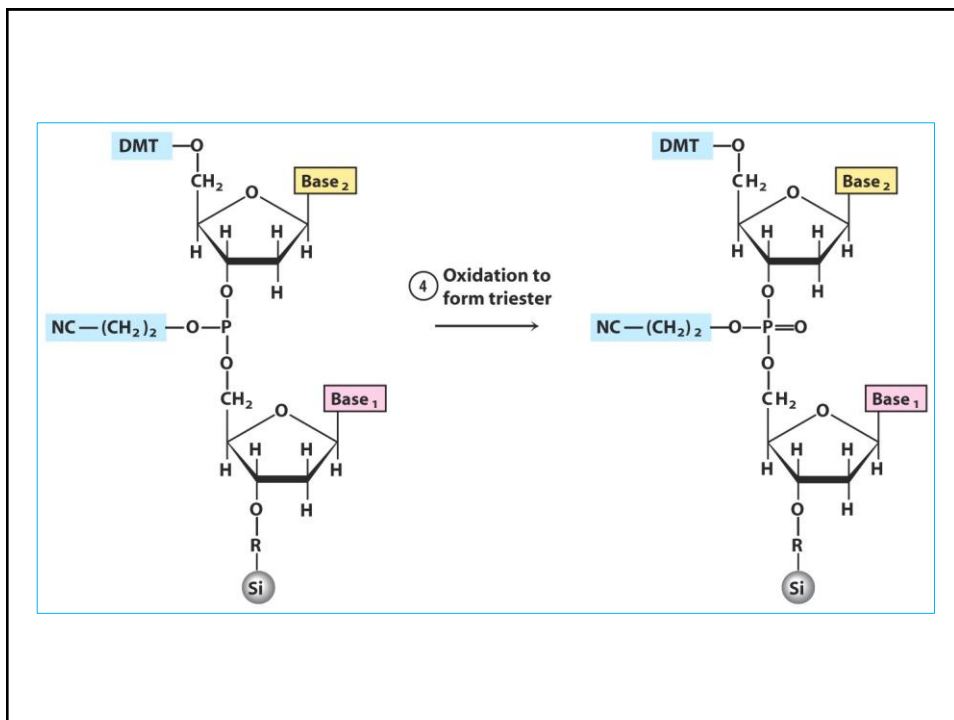
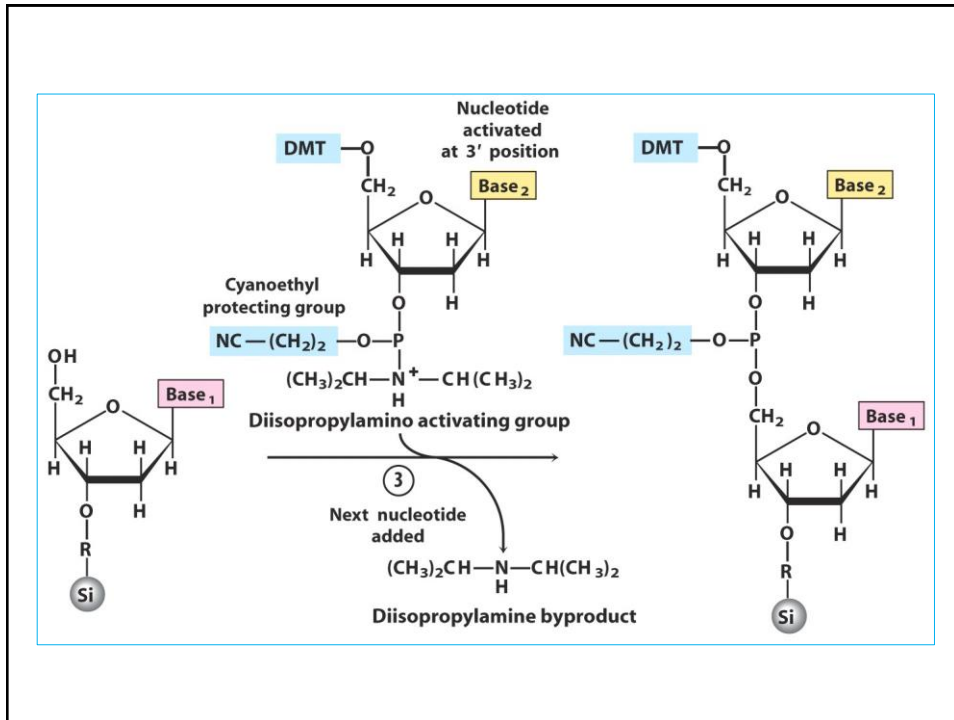
Activation >

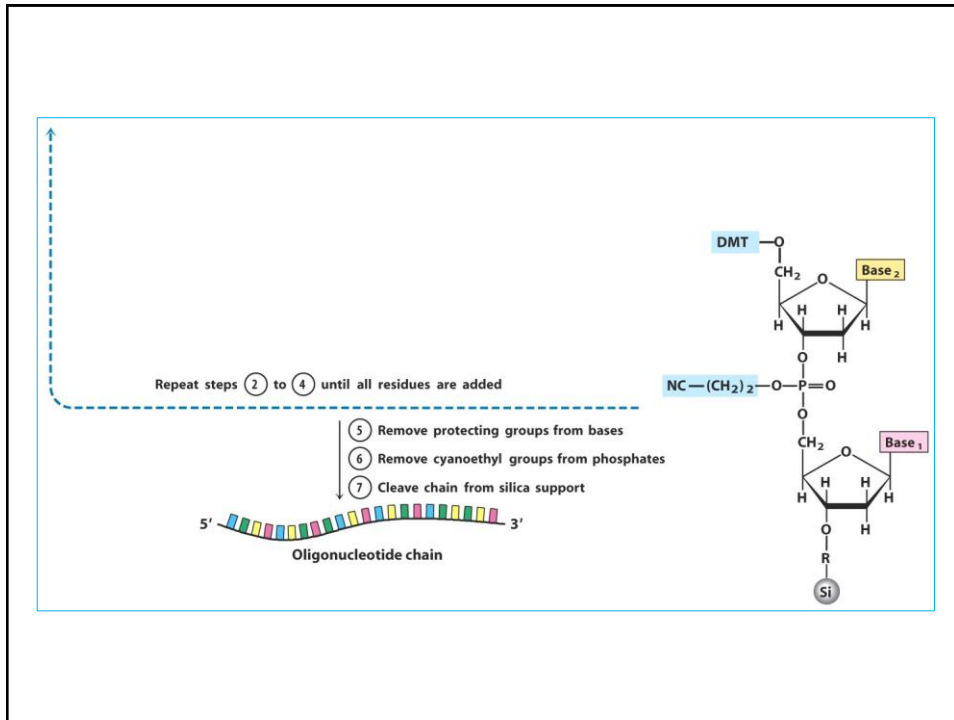
MeO: diisopropylamino group

Protection >

NIP3: Cyanoethyl group







Applications of chemically synthesized ssDNA molecules

- ❑ Custom designed oligonucleotides (oligos) or PCR primers: used for amplification of a specific DNA fragment using the PCR
- ❑ Custom designed oligos harboring a mismatch for a cloned DNA fragment>> a method called site-directed mutagenesis
- ❑ Custom designed oligos: used to introduce a restriction site in a DNA fragment to facilitate cloning or to create various recombinant DNAs
- ❑ Probes