TECHNIQUES OF MOLECULAR BIOLOGY:

POLYMERASE CHAIN REACTION

Instruct: Dr. M. A. Srour

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.html
- Virtual labs for PCR & gel electrophoresis: http://learn.genetics.utah.edu/content/labs/

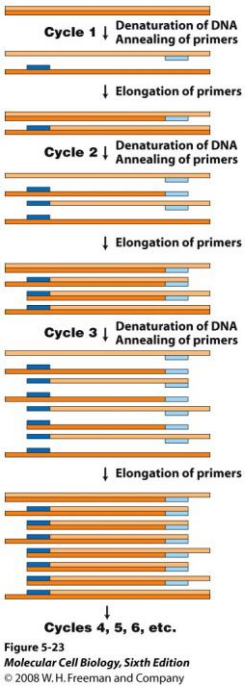


Figure 5-23
Molecular Cell Biology, Sixth Edition

PCR involves Geometric Amplification of template

2^0

↓ 1st cycle

2^1

↓ 2nd cycle

2^2

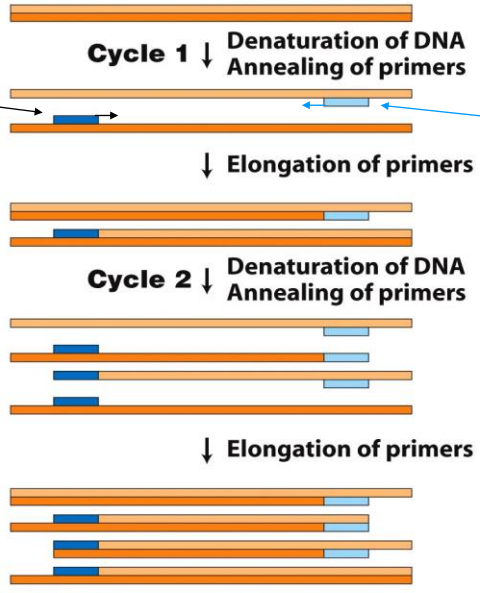
↓ nth cycle

↓

2^n

PCR amplification

Sense/
Forward/
Upward
Primer



Antisense/
Reverse/
Downward
Primer

Figure 5-23 part 1
Molecular Cell Biology, Sixth Edition

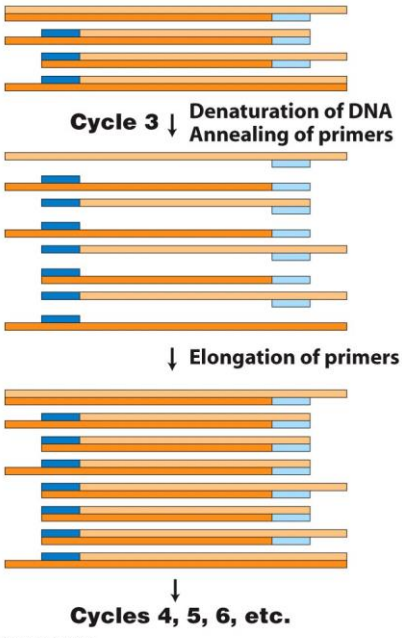
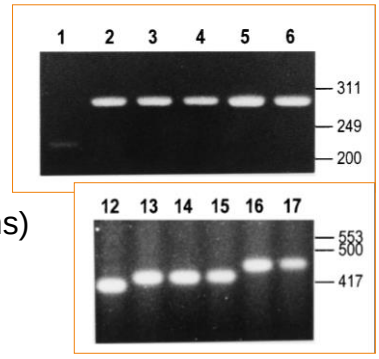


Figure 5-23 part 2
Molecular Cell Biology, Sixth Edition

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

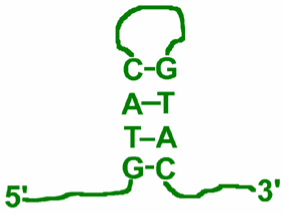
- Melting Temperature (Tm)
  - Temperature at which strands separate
  - Can estimate T_m with formulas: **
- Synthetic Oligonucleotides:
  $T_m = 2(A + T) + 4(G + C)$
- Effective $T_m = 81.5 + 16.6\log[Na^+] + 0.41(\%GC) - 0.65(600/N) - 1.4(\%mismatch)$*

Stringency and Melting Temperature

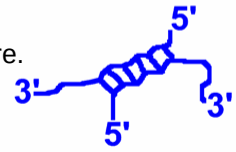
- Stringency of a hybridization reaction: refers to the tolerance (or lack thereof) for mistakes in base pairing
- High stringency: low salt conc, high formamide, high temperature > require exact base pairing
- Low stringency: high salt conc, low formamide, low temperature>> more base pair mismatches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

Design of Oligonucleotide Primers

- Criteria for selection of an oligo
  - Uniqueness (18-28 bases)
  - $T_m > 55^o$
  - 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 inhibiters are usually used tin cDNA synthesis rxn
- The cDNA is further amplified using a normal PCR rxn using gene-specific primers

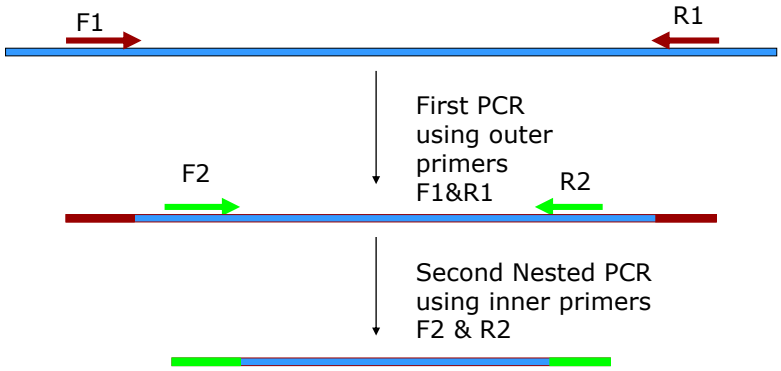
Types of PCR: RT-PCR

- 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

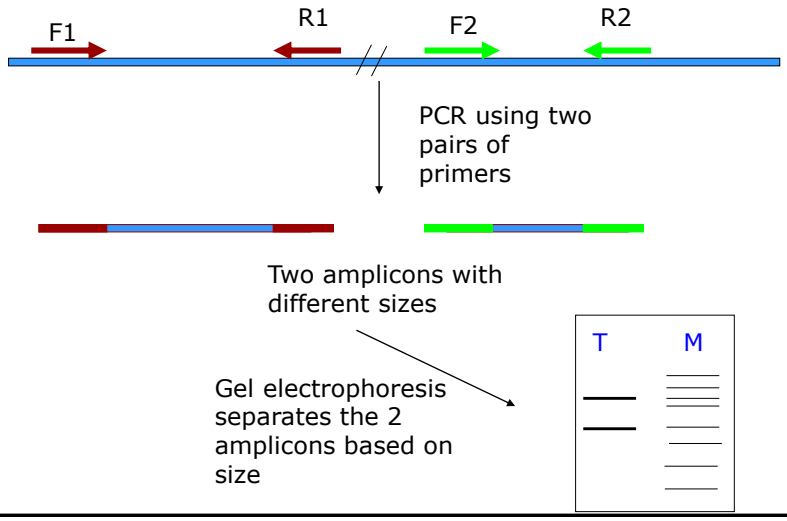
Types of PCR: Nested PCR



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)

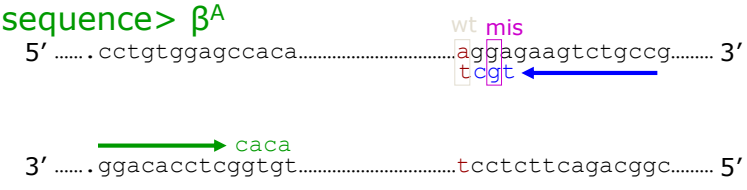
- ARMS is ideally suited for detection of point mutation and small insertion/deletions
- It involves two primers> wt allele & mutant allele >> two PCRs, one for each allele
- Multiplex ARMS
- Advantages of ARMS
  - Quick, inexpensive
  - Not suited for detection of unknown mutations

ARMS primers

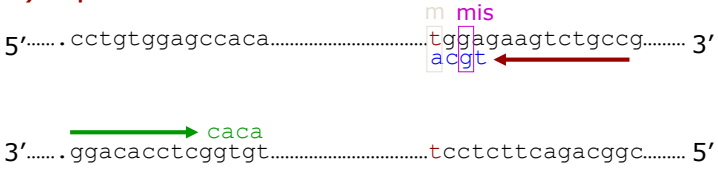
- It is useful to increase the length of primers to about 30 nts.
- Primers usually include a mismatch close to the 3'end at position -2 to -3, to improve specificity (but may decrease yield)
- A second mismatch at nt -5 is sometimes included
- The most discriminatory mismatch involves A:G/G:A

ARMS PCR

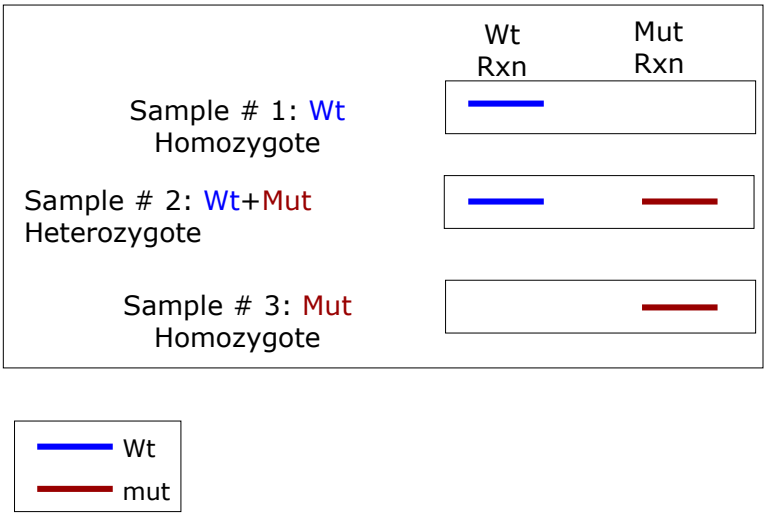
Wild type β-globin gene sequence> β^A



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



ARMS results: 2 rxns per sample

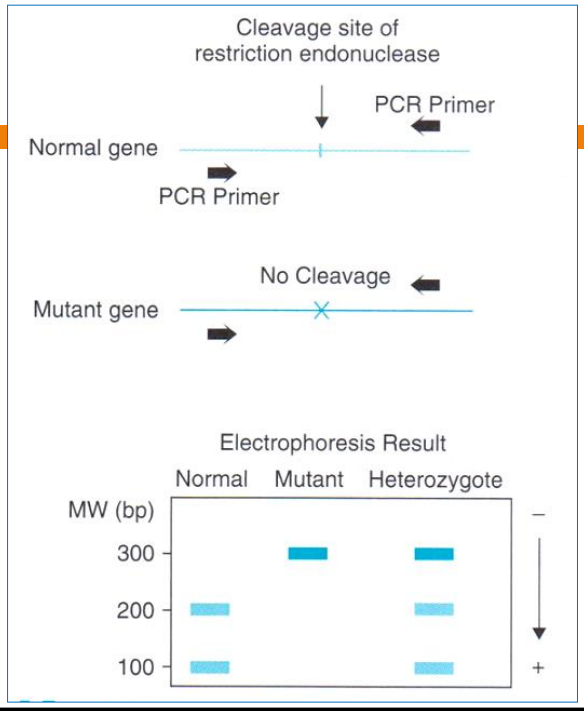


Types of PCR: RFLP-PCR

- 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:

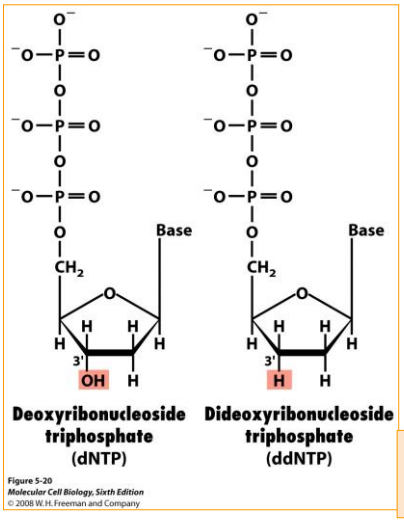
RFLP-PCR



DNA sequencing

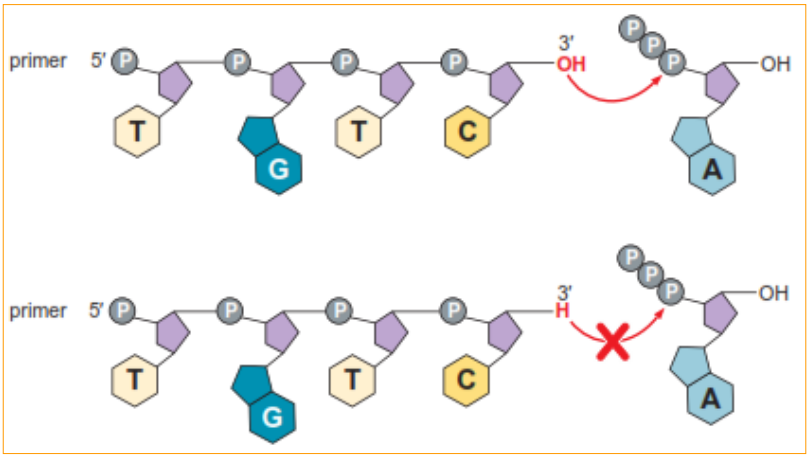
DNA sequencing: https://dnalc.cshl.edu/view/15479-Sanger-method-of-DNA-sequencing-3D-animation-with-narration.html

DNA sequencing by **Sanger method** / Dideoxy chain termination method:



Structure of dNTP & ddNTP

Dideoxy chain termination method in presence of ddNTPs



DNA sequencing by Dideoxy chain termination method

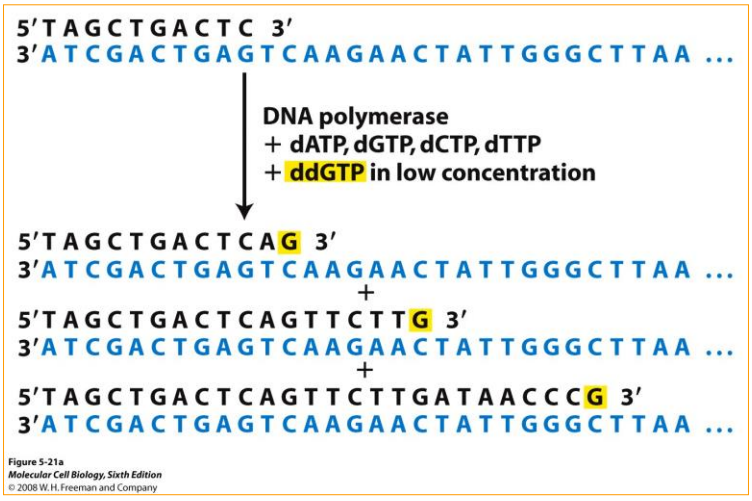


Figure 5-21a
Molecular Cell Biology, Sixth Edition

DNA sequencing by Dideoxy chain termination method

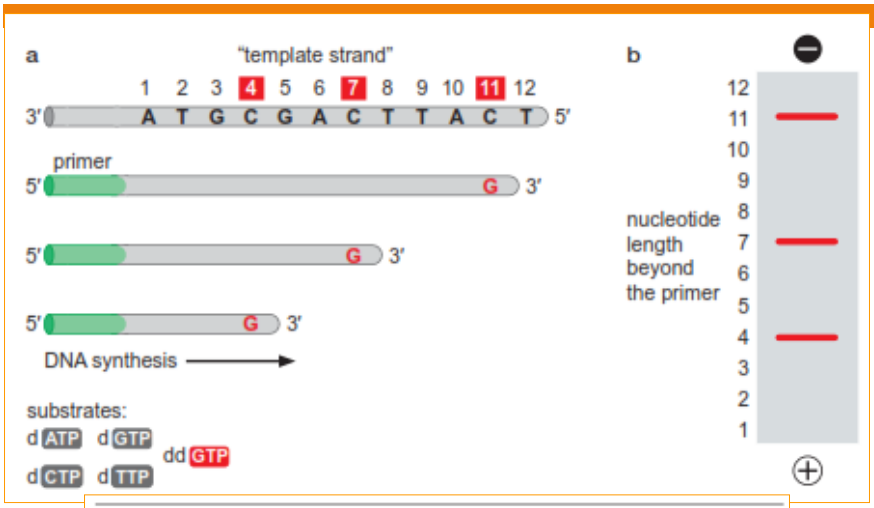
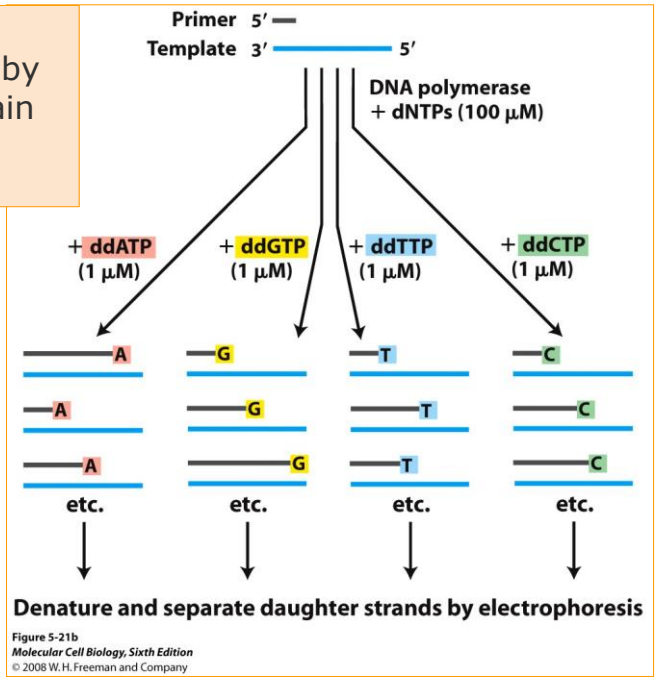
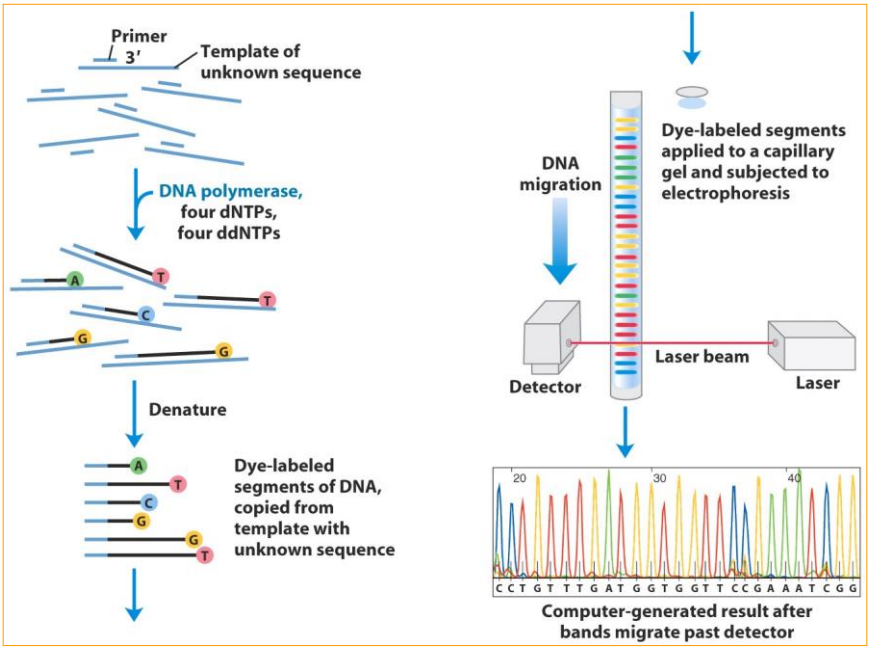


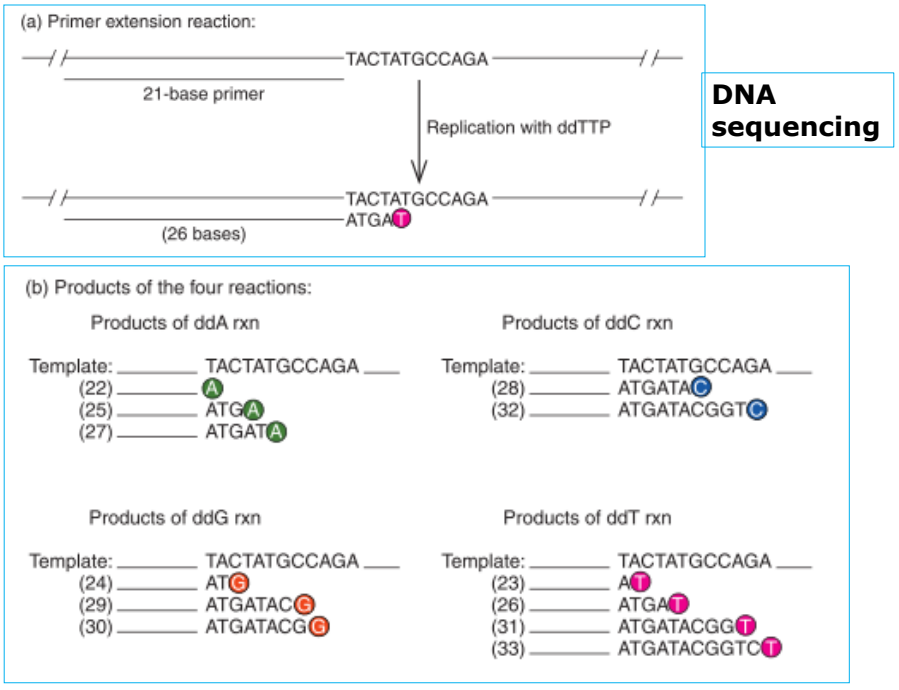
FIGURE 7-15 **DNA sequencing by the chain-termination method.** As described in the text, chains of different length are synthesized in the presence of dideoxynucleotides. The length of the

DNA sequencing by Dideoxy chain termination method

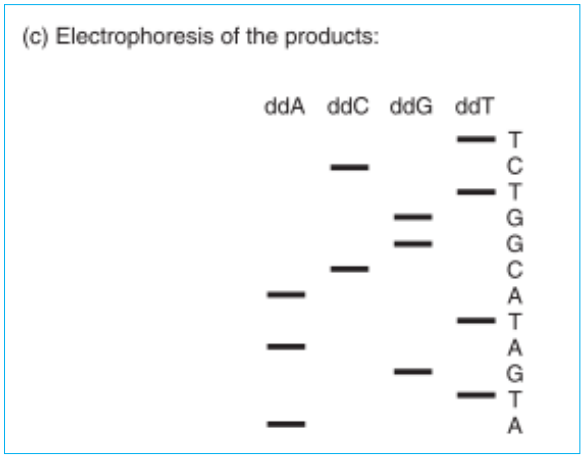




DNA sequencing



DNA sequencing



DNA sequence readout: by Dideoxy chain termination method

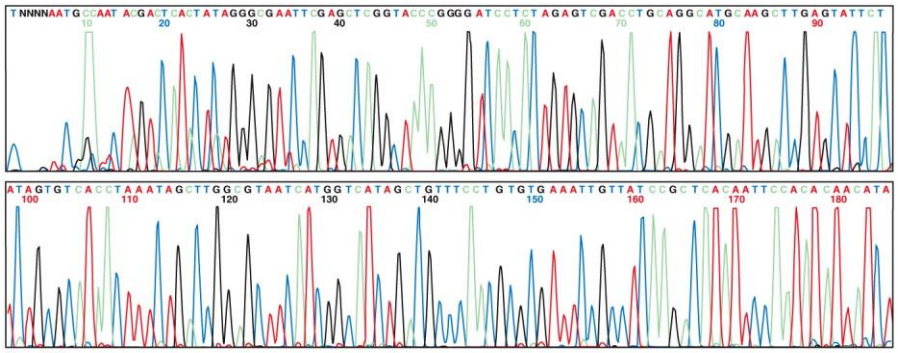
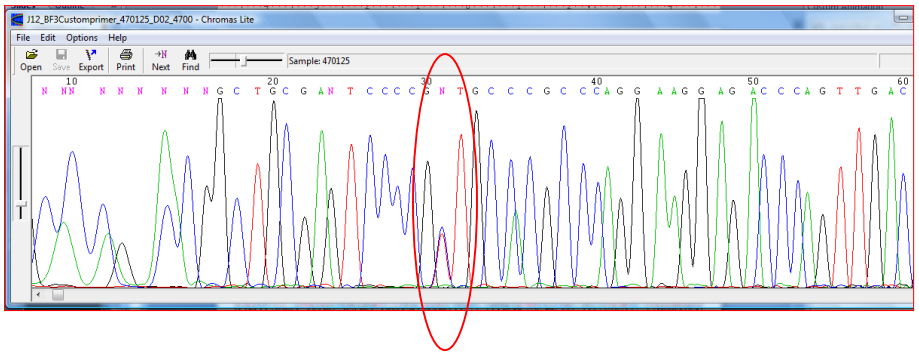


Figure 5-21c
Molecular Cell Biology, Sixth Edition

DNA sequence readout: chromatogram

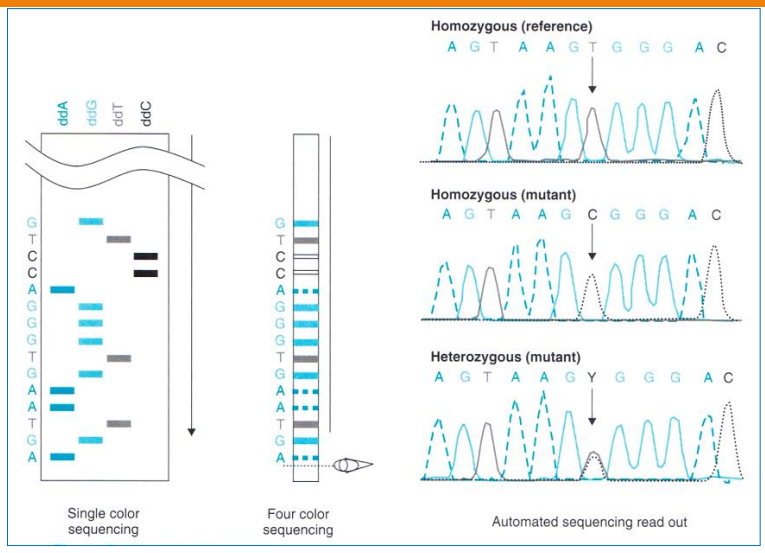


Applications of DNA sequencing

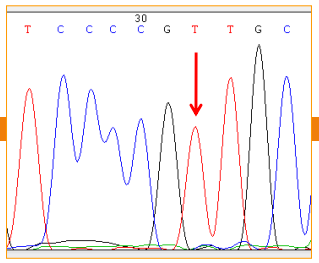
- Study gene sequence & structure
- Allows genome sequencing, e.g. Human genome project completed sequencing human genome in 2003
- Detection of mutations (known & unknown)

- Sequencing is now being done using "DNA sequencing machines" or "Sequenators"

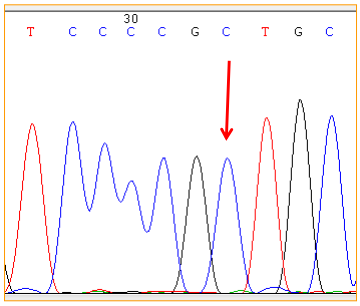
Detection of point mutations using DNA sequencing



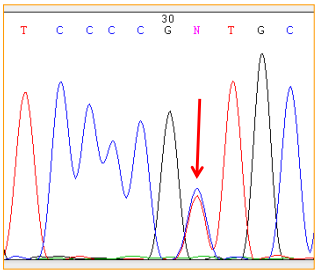
Detection of point mutations using DNA sequencing



Homozygous (Mutant)
5′-TCCCC G **T** T G C-3′



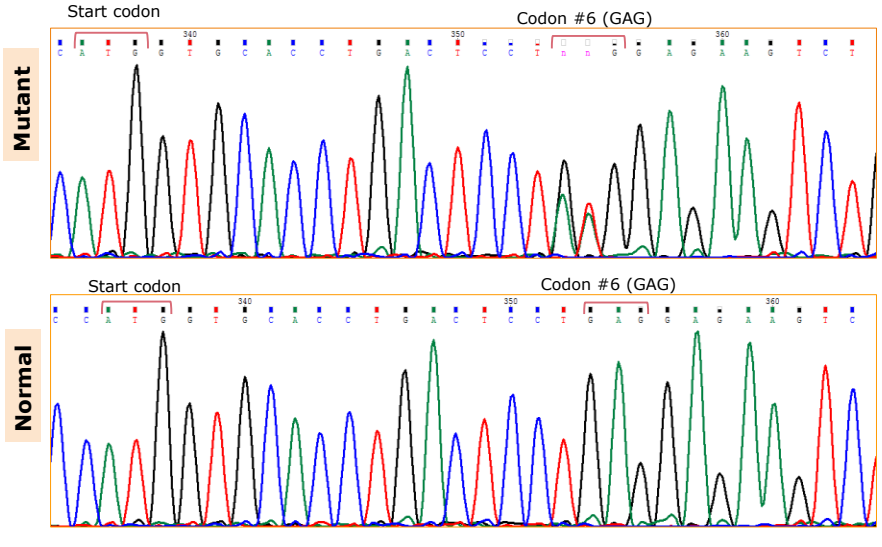
Homozygous
(Reference, Normal)
5′-TCCCCG **C** T G C-3′



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 phosphoamidines

Chemical synthesis of DNA oligos (cont'd)

- In chemical synthesis, the DNA chain grows by addition to the 5' end of the molecule (3' → 5'), while in vivo polymerization is 5' → 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 >

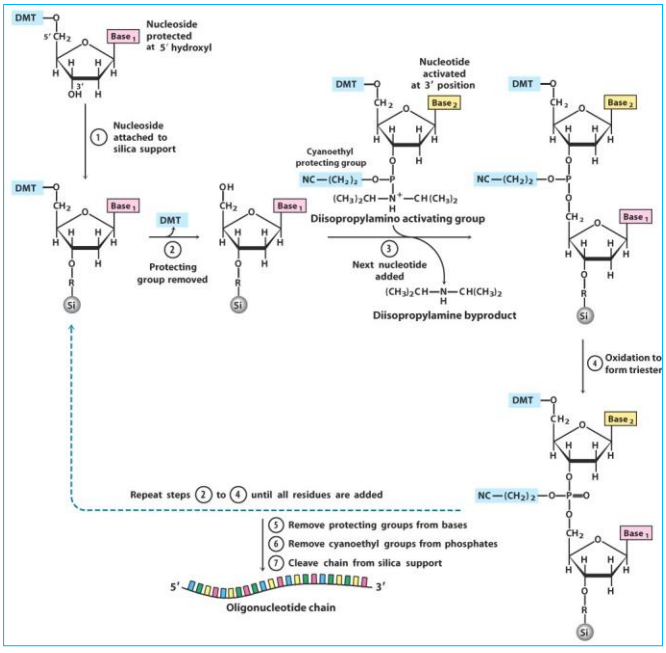
DMT: 4′,4′-dimethoxytrityl

Activation >

MeO: diisopropylamino group

Protection >

NIP3: Cyanoethyl group



Blocking >

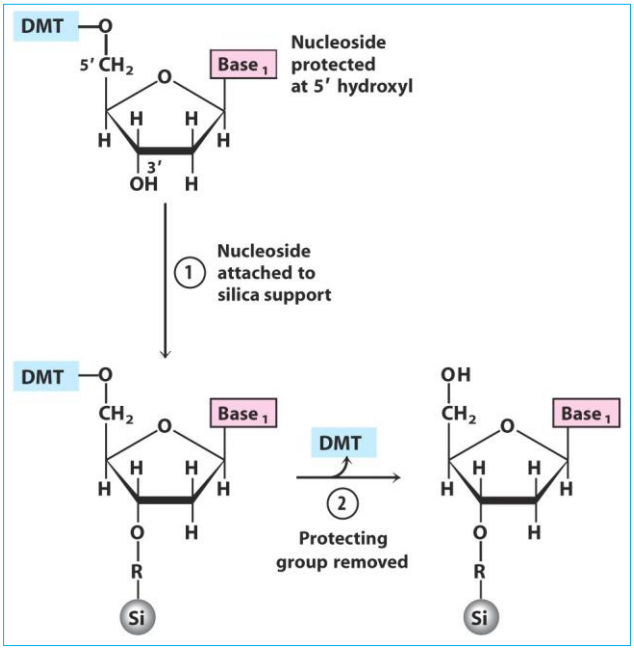
DMT: 4′,4′-dimethoxytrityl

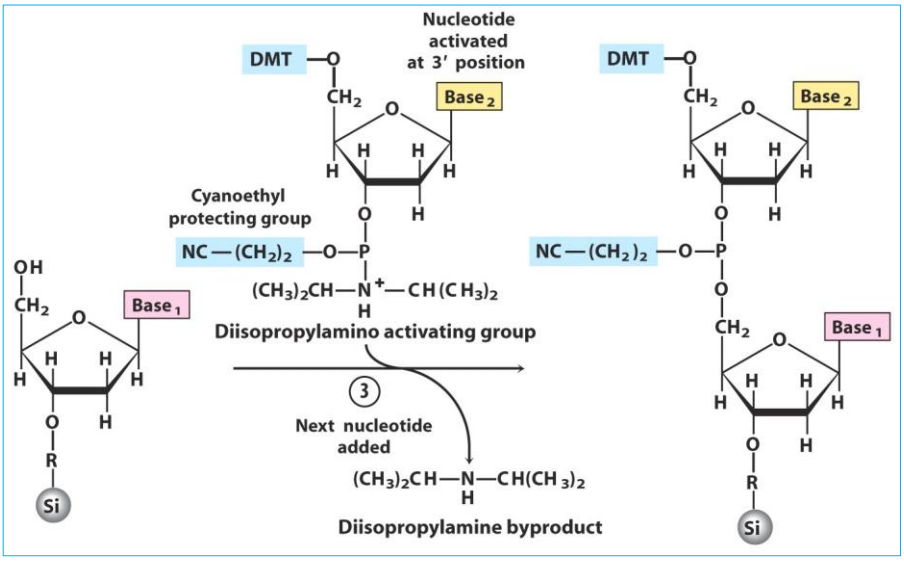
Activation >

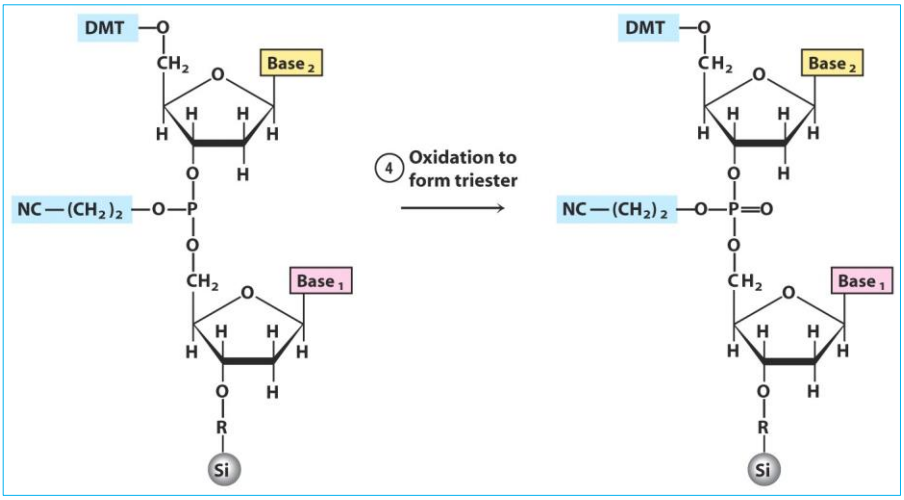
MeO: diisopropylamino group

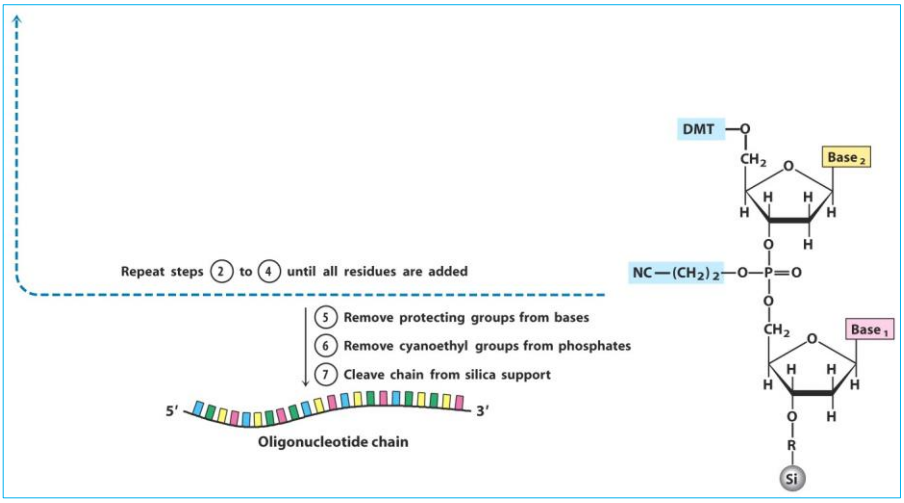
Protection >

NIP3: Cyanoethyl group




Nucleotide activated at 3′ position
DMT
Base 2
Cyanoethyl protecting group
NC—(CH2)2—O—P
(CH3)2CH—N+—CH(CH3)2
H
Diisopropylamino activating group
3
Next nucleotide added
(CH3)2CH—N—CH(CH3)2
H
Diisopropylamine byproduct
OH
Base 1
Si


DMT
Base 2
NC—(CH2)2—O—P
Base 1
Si
4
Oxidation to form triester
NC—(CH2)2—O—P=O



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