TECHNIQUES IN MOLECULAR BIOLOGY

Course: Molecular Biology (02022312)

Instructor: Dr. M A Srour

Textbook:
Watson J, Baker TA, Bell SP, Gann A, Levine M, Losick R (2008). Molecular Biology of the Gene, 6th ed. Chap 21/ pp739-82

Lodish H, et al. (2008). Molecular cell biology, 6th edition. W.H. Freeman and Company: New York. Chapter 5

Lec # 5 Wed 15.02.2012

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

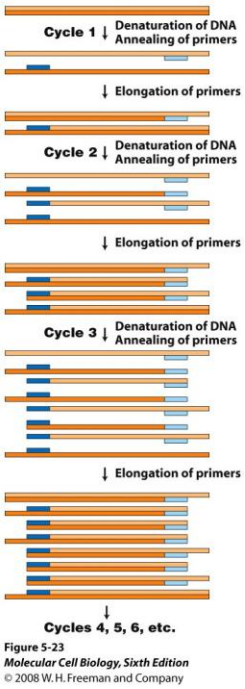


Figure 5-23
Molecular Cell Biology, Sixth Edition

PCR involves Geometric Amplification of template

2^0

↓ 1st cycle

2^1

↓ 2nd cycle

2^2

↓ nth cycle

↓

2^n

PCR amplification

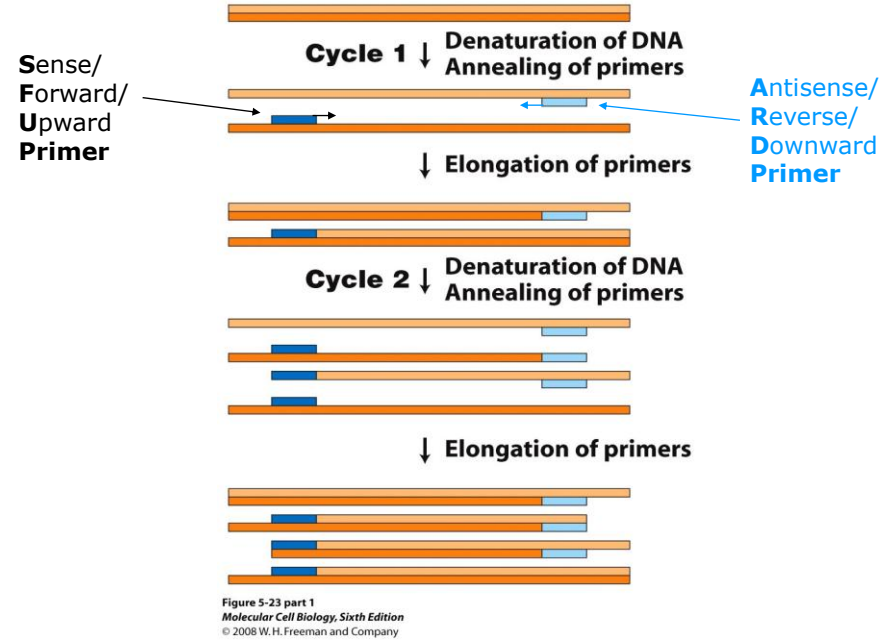


Figure 5-23 part 1
Molecular Cell Biology, Sixth Edition

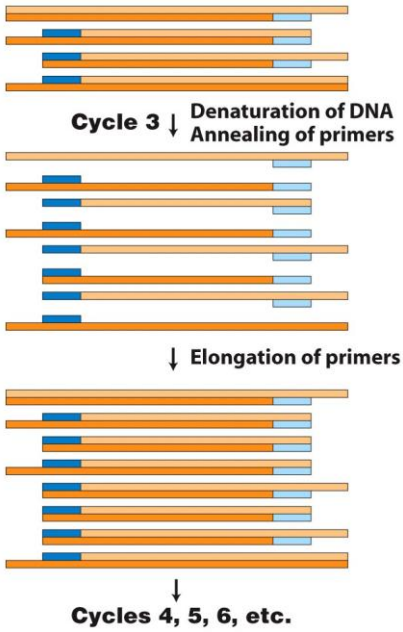
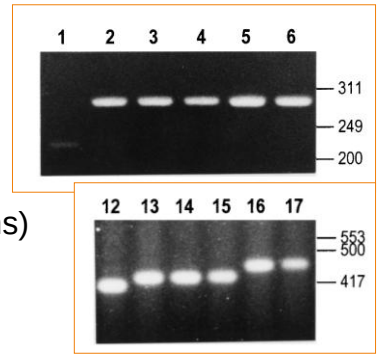


Figure 5-23 part 2
Molecular Cell Biology, Sixth Edition

PCR Protocol

- Mix DNA, primers, dNTPs, Taq, buffer, Mg^{2+}
- Program thermocycler for times and temps
  - 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

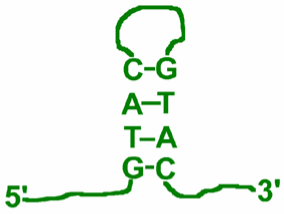
- Melting Temperature (Tm)
  - Temperature at which strands separate
  - Can estimate T_m with formulas: **
- Synthetic Oligonucleotides:
  $T_m = 2(A + T) + 4(G + C)$
- Effective $T_m = 81.5 + 16.6\log[Na^+] + 0.41(\%GC) - 0.65(600/N) - 1.4(\%mismatch)$*

Stringency and Melting Temperature

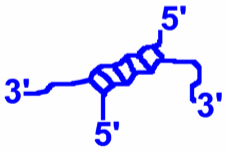
- Stringency of a hybridization reaction: refers to the tolerance (or lack thereof) for mistakes in base pairing
- High stringency: low salt conc, high formamide, high temperature > require exact base pairing
- Low stringency: high salt conc, low formamide, low temperature>> more base pair mismatches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

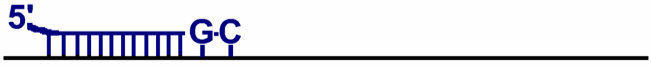
Design of Oligonucleotide Primers

- 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?
  - Analyze sequence with computer software
  - Manual





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

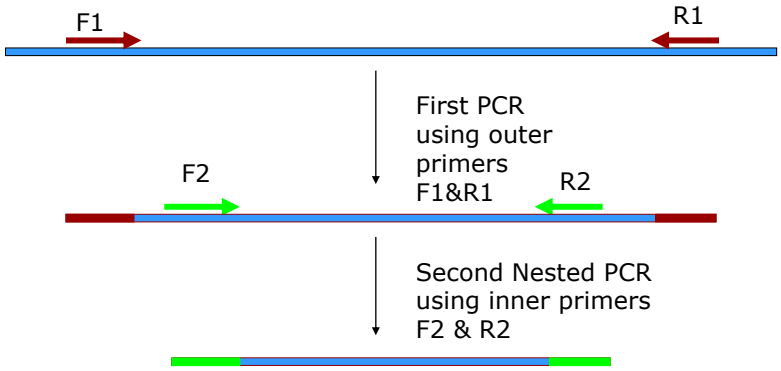
Types of PCR: RT-PCR

- 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

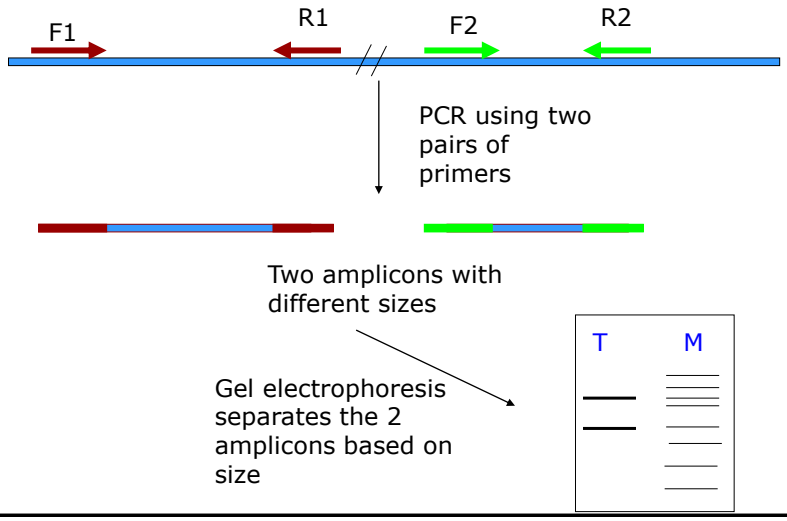
Types of PCR: Nested PCR



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)

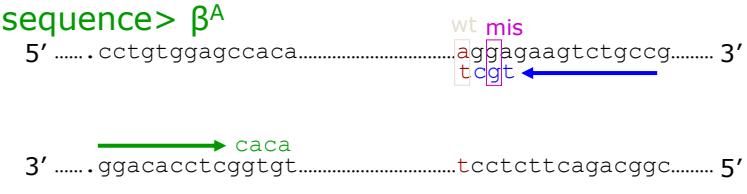
- ARMS is ideally suited for detection of point mutation and small insertion/deletions
- It involves two primers> wt allele & mutant allele >> two PCRs, one for each allele
- Multiplex ARMS
- Advantages of ARMS
  - Quick, inexpensive
  - Not suited for detection of unknown mutations

ARMS primers

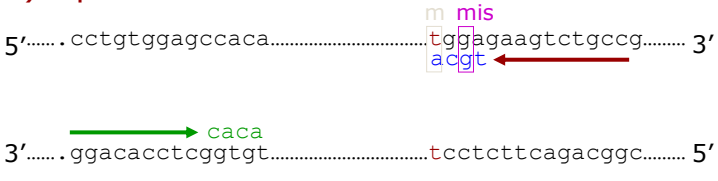
- It is useful to increase the length of primers to about 30 nts.
- Primers usually include a mismatch close to the 3'end at position -2 to -3, to improve specificity (but may decrease yield)
- A second mismatch at nt -5 is sometimes included
- The most discriminatory mismatch involves A:G/G:A

ARMS PCR

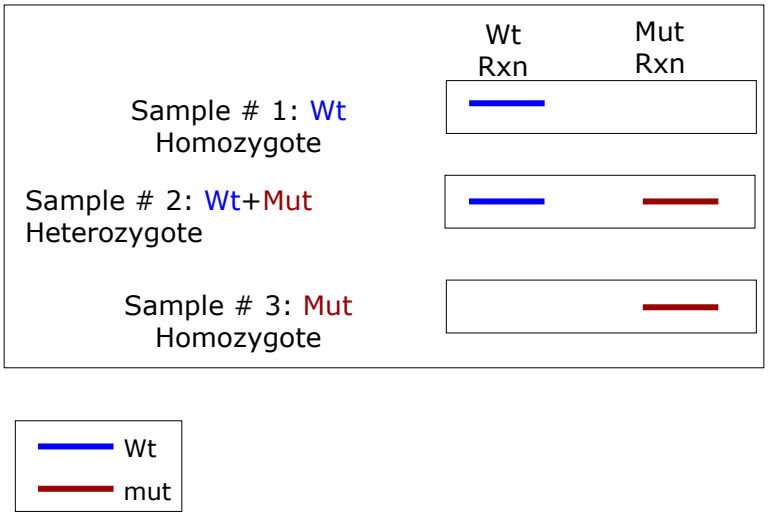
Wild type β-globin gene sequence> β^A



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