REAL-TIME PCR

Course: Molecular Biology (BIOL 333)
Instructor: Dr. M. A. Srour

Reference:
Bruns DE, Ashwood ER, Burtis CA. Fundamentals of Molecular Diagnostics. St. Louis, Missouri: Saunders Elsevier 2007. Chap 5

Real-time PCR

- In rt-PCR, data are collected during nucleic acid amplification rather than at a single end point. >> It allows measurement of the kinetics of the reaction in the early phases of PCR
- The normal PCR is not fully exponential, because it plateaus at around 10^8 copies of an amplicon, thus for quantitative work, PCRs should be maintained within 20 cycles
- In rt-PCR the exponential phase is monitored during early phase of rxn using fluorescently labeled molecules. While traditional methods use agarose gel for detection of PCR amplification at the final phase or end-point of PCR rxn.

Real-time PCR

- During exponential phase, the amount of PCR amplicons present in the rxn tube is proportional to the starting material
- Limitations of end-point PCR
  - Poor precision
  - Size-based discrimination only
  - Not quantitative
  - Low sensitivity
  - Non-automated
  - Poor resolution of about 10-fold
  - Results are not expressed as numbers

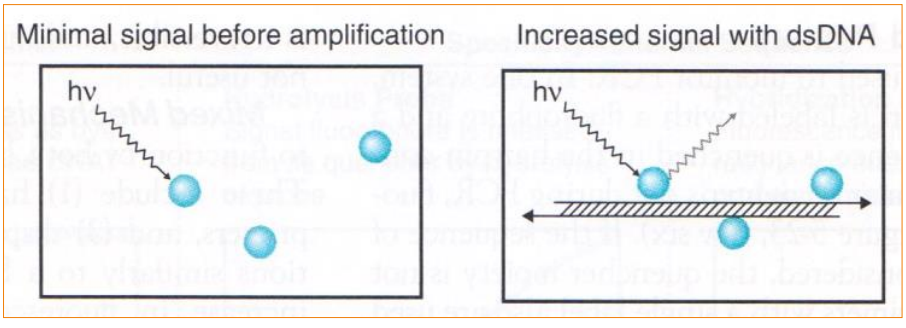
- Rt-PCR
  - Can detect 2-fold change in template
  - Can be applied for quantification of DNA or RNA (RT-PCR)
  - High sensitivity and specificity

Dyes and probe formats for rt-PCR

- Double-stranded DNA binding dyes
  - Dye: SYBR Green I, a fluorescent dye
  - Dye shows a significant increase in fluorescence when bound to dsDNA

Dyes and probe formats for rt-PCR

Double-stranded DNA binding dye



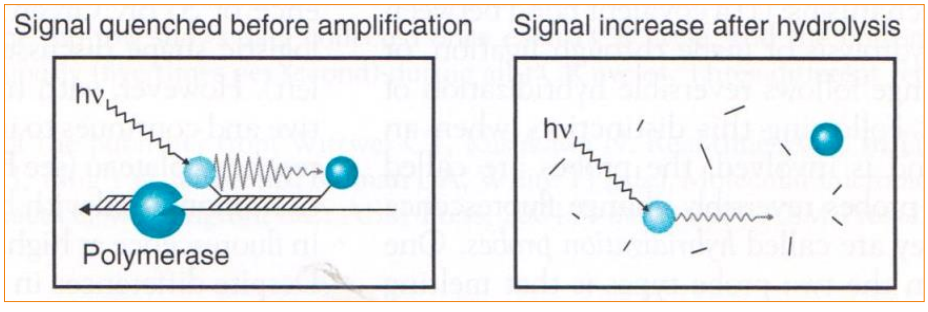
Animation: https://www.youtube.com/watch?v=ob3teCrpgxY

Dyes and probe formats for rt-PCR

- Probe-specific detection /hydrolysis probes
  - The use of fluorescent probes (TaqMan) probes provides additional level of specificity to the process
  - The probe is designed to anneal to a specific sequence of template between the F and R primers
  - When the enzymes reaches the annealed probe, the 5" exonuclease activity of the enzymes cleaves the probe
  - Probe has a Reporter & Quencher, the R is suppressed by Q > when separated the R starts to fluoresce

Dyes and probe formats for rt-PCR

Exonuclease hydrolysis of probe

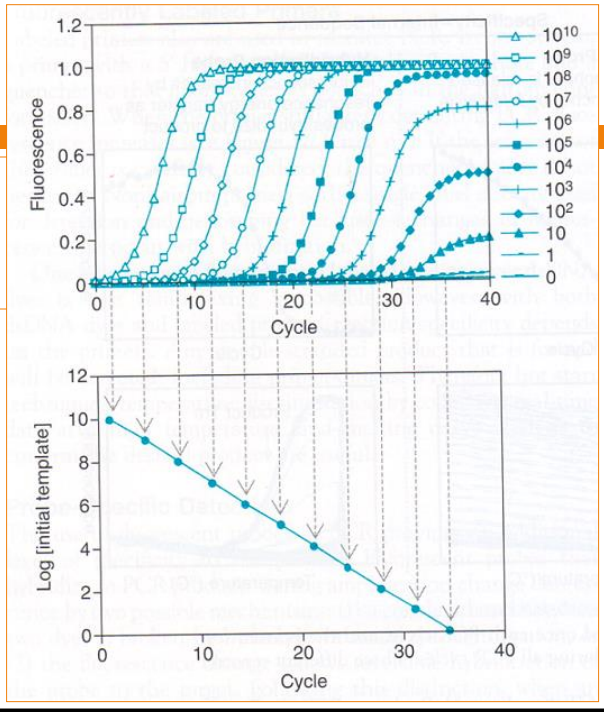


hv: excitation light, R: Reporter dye; Q: quencher molecule

Detection & quantification in rt-PCR

- a fluorescent signal that increases during PCR and follows one of the expected curve shapes suggest the specific target is present & was amplified
- Quantification: theoretically there is a quantitative relationship between the amount of starting target sample and amount of PCR product at any given cycle number. The data is measured at the exponential phase of the PCR rxn. The data are plotted in log format and the "copy number" of target amplicon is measured from a standard curve

Quantification by rt-PCR



Applications of rt-PCR

- Quantification of viral load, e.g., HIV, HBV, HCV
- Genotyping
- Quantification of mRNA in gene expression studies
- Pathogen detection

DNA microarray

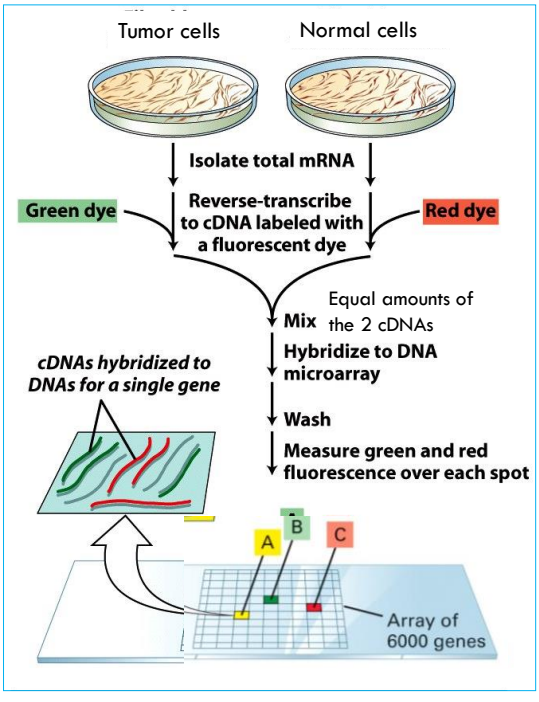
DNA Microarray (Gene Chips)

- Analyze 1000's of genes simultaneously
- Method
  - Different DNA 'probes' (at least 20 nts in length) are fixed (covalently linked) onto solid surface (glass) in array
  - Fluorescent labeled target cDNA (mRNA) incubated with chip
- A typical array might contain ~6000 spots of DNA in a 2X2 cm grid
- Uses
  - Identification of sequence polymorphisms & mutations
  - Quantification of gene expression

DNA Microarray:

Analysis of differences in gene expression in tumor cells compared to normal cells

C: if a spot is Red, expression of that gene increases in Normal cells
B: if a spot is Green, expression of that gene increases in Tumor cells
A: if a spot is Yellow, expression of that gene is the same in both Normal & Tumor cells



DNA Microarray Analysis:

a micrograph of a small segment of an actual DNA microarray.

Yellow spot : G=R, no change in gene expression.

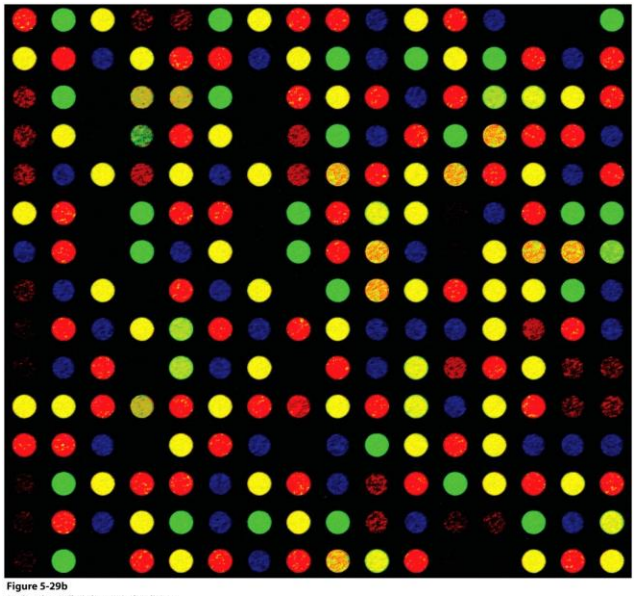


Figure 5-29b
Molecular Cell Biology, Sixth Edition

CLINICAL APPLICATIONS OF PCR

Course: Molecular Biology

Instructor: Dr. M A Srour

Textbook/ References:

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

Bruns DE, Ashwood ER, Burtis CA. Fundamentals of molecular diagnostics. St. Louis, Missouri: Saunders Elsevier. Chap 5/ p.46-79; Chap 7/p9197.

Clinical applications of PCR

- Molecular techniques and PCR in particular are of importance in clinical diagnostics in **most disciplines of laboratory medicine** and pathology and their applications will continue to grow in essentially all areas of testing

What lab tests are suitable for molecular testing?

- The type of tests chosen for molecular testing depends on:
  - The test's diagnostic accuracy
  - Its clinical utility
  - The local demand for the test
  - The skills and interests of the personnel
  - The likelihood that the test being financially viable
  - The ability of the lab to deliver acceptable turnaround time

What type of molecular techniques are used for molecular diagnosis?

- PCR
- DNA sequencing
- Hybridization techniques
  - Southern blot
  - Dot blot
  - Northern blot
- Real time PCR
- DNA microarray (mostly for research and not yet used for routine testing)

Molecular diagnostic lab: centralization or decentralization?

- Should each lab discipline that use molecular techniques have its own molecular space or a centralized molecular lab a more suitable model to deliver molecular testing services?
- The answer depend on several local factors like work load, availability of expertise and costs.
- As a compromise some large hospitals have a lab for molecular genetic and oncology while all tests for microbial identification and characterization in the existing microbiology lab

Types of PCR used in clinical diagnostics

- RT-PCR
- Nested PCR
- Multiplex PCR
- ARMS PCR
- RFLP PCR
- Real time PCR

Polymorphisms versus mutations

Individual genomes show extensive variations

- Polymorphism can be detected at the
  - Phenotypic level when sequence affects gene function (mutation)
  - At restriction fragment level when it affects a restriction site,
  - At the amplicon size level by PCR
  - At the sequence level by direct DNA analysis
- **Polymorphism:** clinically harmless DNA variation that does not affect the phenotype. Often occur in intervening sequences
- **Mutation:** an infrequent but potentially harmful, genome variation that is associated with specific human disease

Human genome & its sequence variations

- 99.9 % identity
- 1 difference every 1250 bases between randomly selected haploid genomes These variations include both polymorphism and mutations.
- SNPs are identified every 200-300 bp, 97% are within noncoding DNA and 3% within exons

Ref: Bruns, Ashwood & Burtis, 2007, Chap 2 p.18

Human genome & its sequence variations

- Disease causing variants
  - 70 % SNPs
  - 49% missense (aa substitution)
  - 11% nonsense (termination)
  - 9% Splicing
  - <1% regulatory
  - 23% small insertions &/or deletions
  - 7% gross lesions (large insertions &/or deletions, repeats, rearrangements, complex alterations)

Ref: Bruns, Ashwood & Burtis, 2007, Chap 2 p.18

Restriction Fragment Length Polymorphism (RFLP)

- RFLP: a genetic variant that can be examined by cleaving the DNA into fragments with a restriction enzyme
- RFLP can be used for
  - genetic mapping
  - to detect human genetic defects and DNA fingerprinting

Restriction Fragment Length Polymorphism (RFLP)

- Two types of DNA variation commonly result in in RFLPs
  1. Single base changes in DNA: about 90 % of human genome variation comes from single nucleotide polymorphisms (SNP, pronounced snips)
     - Defined by their SNPs, every human being is unique
     - >1 million SNPs are identified
  2. Tandem repeats or variable number tandem repeats (VNTR): short sequences of DNA at scattered locations in the genome repeated in tandem

RFLPs

- Example
- SNPs in Hb S

ATG CAC CTG ACT CCT GTG GAG – HB S

ATG CAC CTG ACT CCT GAG GAG – HB A

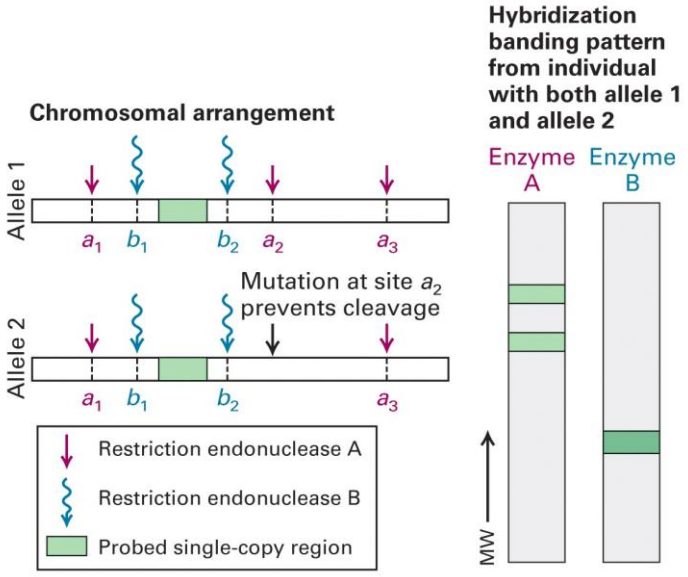
- VNTR (variable number tandem repeat)

--- GC GC GC GC GC ---- subject 1 (5 repeats)

--- ----- GC GC GC -------- subject 2 (3 repeats)

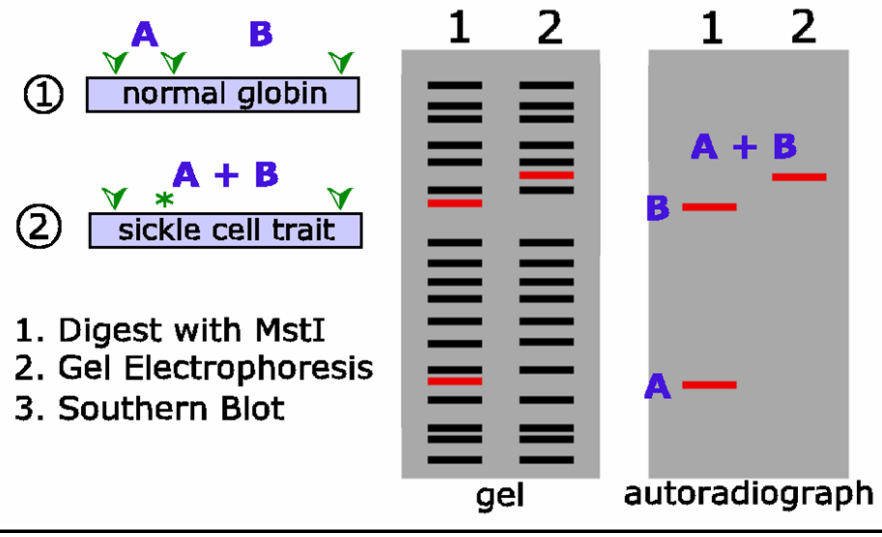
Diagnosis of Genetic Diseases by RFLP

(*R*estriction *F*ragment *L*ength *P*olymorphism)



Diagnosis of Genetic Diseases by RFLP

(*R*estriction *F*ragment *L*ength *P*olymorphism)



DNA Fingerprinting, used to identify individuals in paternity cases & criminal investigations:

A single PCR rxn using several sets of primers flanking the minisatellite repeat

(a) C has Minisatellite inherited from M & F1

(b) The Minisatellite of the specimen match that from suspect 1

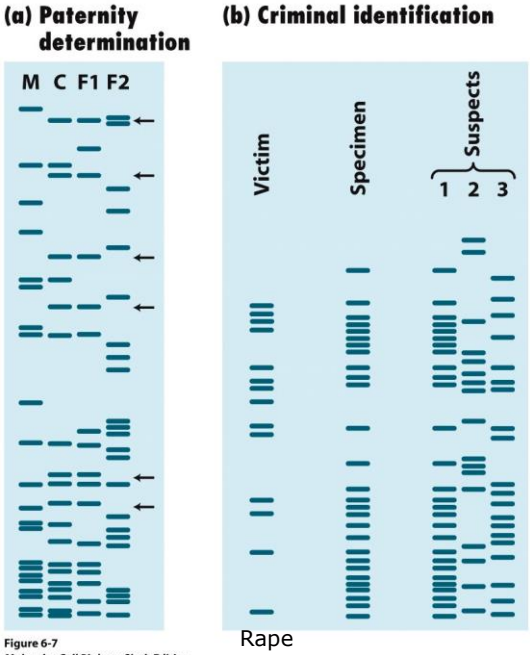


Figure 6-7
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Rape victim/ control

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