Arab School of Pathology Precongress Workshop

Introduction to Molecular Diagnostics

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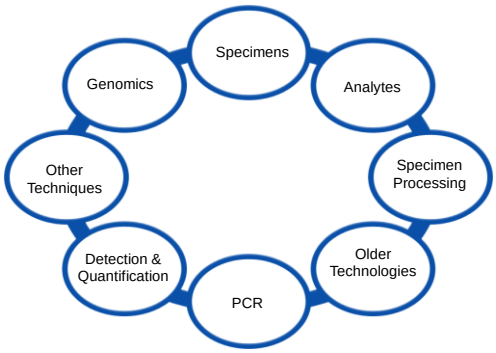
EMORY

Co-sponsored by AMP

Topic Summary



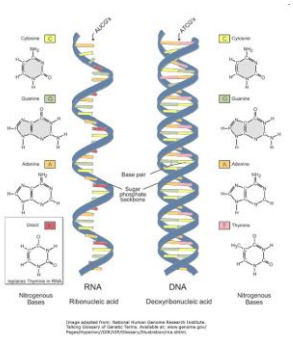
Molecular Diagnostics

Using DNA and/or RNA to make a diagnosis or provide information for clinical decision making

Sources of DNA and RNA

- DNA
  - Microorganisms
  - Malignancies
  - Genetic testing
- RNA
  - Microorganisms
  - Malignancies

Analytes



How are RNA and DNA Different?

- Ribose vs deoxyribose (RNA has 2' hydroxyl)
- RNA is less stable
- U instead of T
- More often single stranded
- Catalytic properties

Specimens

- Fluids
  - Serum/Plasma
  - CSF
  - Urine
  - Sputum
  - Others

Specimens

- Cells
  - Blood/Bone Marrow
  - Culture
- Tissue
  - Fresh
  - Frozen
  - Formalin fixed

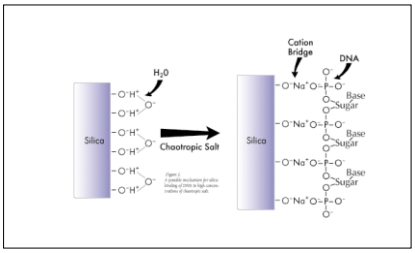
Difficult Samples

- Urine – urea is inhibitory
- Sputum/stool/wounds – bacteria may degrade nucleic acids
- CSF – little or no nucleic acid normally
- Eye – Polysaccharides may inhibit analysis
- Formalin fixed tissue – cross linked proteins make retrieval of DNA very difficult

Principles of Isolation

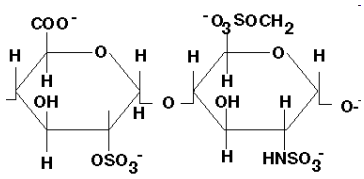
- DNA and RNA often bound by protein
- Components of primary sample may inhibit enzymes
- Enzymes in sample may degrade DNA or RNA
- Quantitatively purify nucleic acids

Extraction of Nucleic Acids



<http://www.bio101.com/newsletter/august98/3.html>

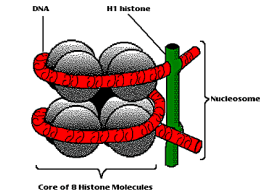
Heparin



Repeat unit of heparin

From: http://www.facs.org/jacs/graphics/sumpiofig1.gif

DNA Associates with Histones



Nucleosome
http://www.accessexcellence.com/AB/GG/nucleosome.gif

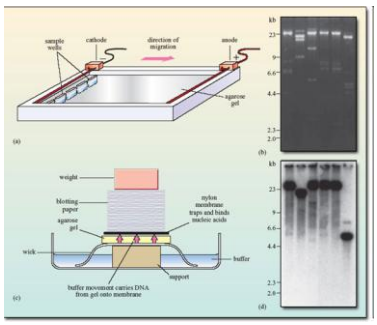
Special Considerations

- Formalin fixed tissue needs long protease digestion due to cross-linking of histones
- RNA may need to be stabilized if sample is to be stored prior to extraction
- Some specimens may require additional effort to isolate nucleic acids

Automation

- Many systems based on silica chemistry
  - Most use paramagnetic particles coated with silica
  - Some use silica filters or columns
- Iron oxide is new chemistry and selective for RNA
  - Naturally paramagnetic
  - Only used in one system

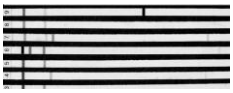
Southern blot

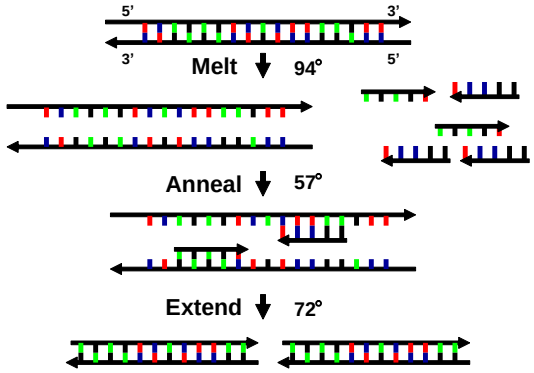


From: http://openlearn.open.ac.uk/file.php/2645/S377_1_007i.jpg

Probe Capture

- Oligonucleotide probes bound to immobile substrate capture specific nucleic acid
- Secondary probe added which is linked to horseradish peroxidase
- Probe binds and excess washed away
- Detection by color change
- Similar to ELISA, may be quantitative





Polymerase Chain Reaction

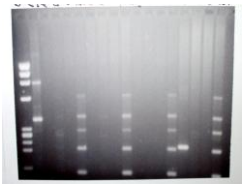
Amplification of selected DNA using a thermostable polymerase.

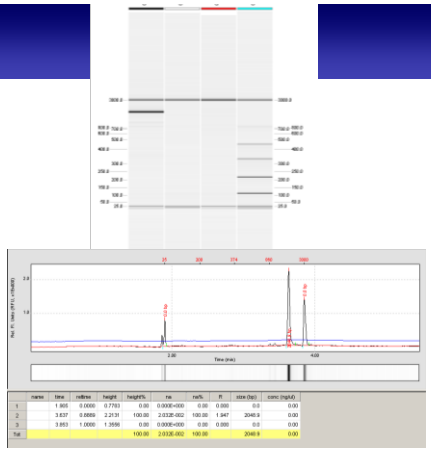
Mullis KB et al. "Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction." Cold Spring Harbor Symp. Quant. Biol. vol. 51 pp. 263-73 (1986).

Cycle #	Copies of Target
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024
.....	
16	65536
17	131072
18	262144
19	524288
20	1048576

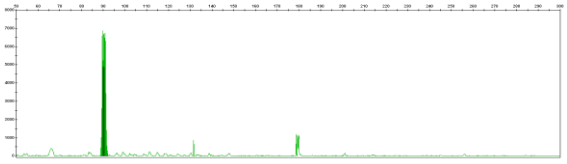
Detection of PCR Products

- Can be as simple as measuring DNA content after reaction
- Next simplest is gel electrophoresis with ethidium bromide



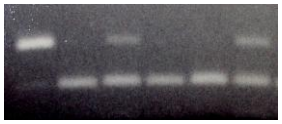


Capillary Electrophoresis



Restriction Digest with Gel electrophoresis

- Detect gain or loss of restriction enzyme recognition site
- Requires post PCR processing



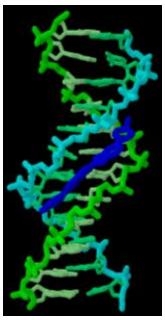
Real-time (Q) PCR

- Detection of PCR product during the PCR process
- Typically, optics added to thermocycler to detect fluorescence each cycle
- No need to open tube after PCR – closed system
- May use double stranded DNA binding dye or probe

Real-time PCR Product Detection

- Double Strand DNA Binding Dyes
  - SYBR Green
  - Power SYBR
- Probe Based Detection
  - 5'-nuclease detection
  - Molecular beacons
  - FRET Probes
  - Scorpion Probes

SYBR Green I

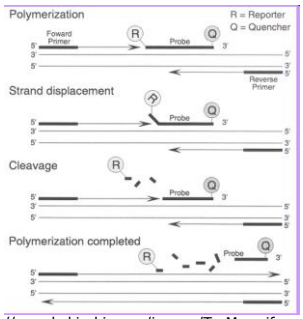


A minor groove binding dye.

No probe = low reagent cost.

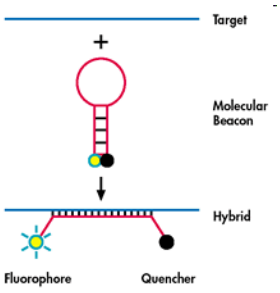
Cannot multiplex.

5' Nuclease Q-PCR (TaqMan)



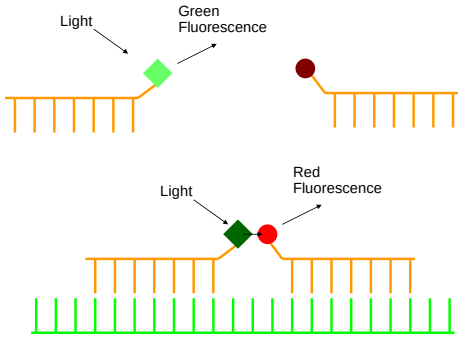
From: http://www.hgbiochip.com/images/TaqMan.gif

Molecular Beacons

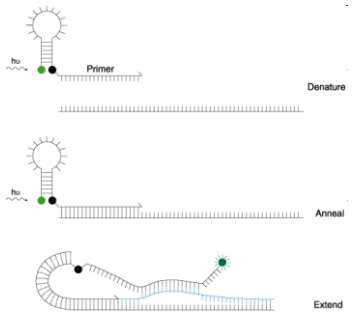


From: http://www.ebiotrade.com/buyf/productsf/qiagen/fig_molecular_beacons.gif

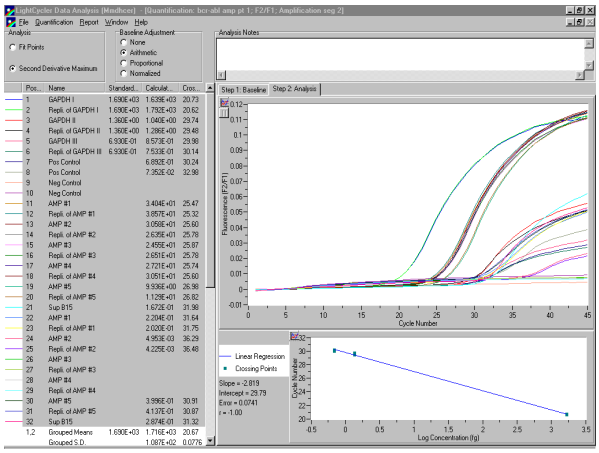
Fluorescent Resonant Energy Transfer

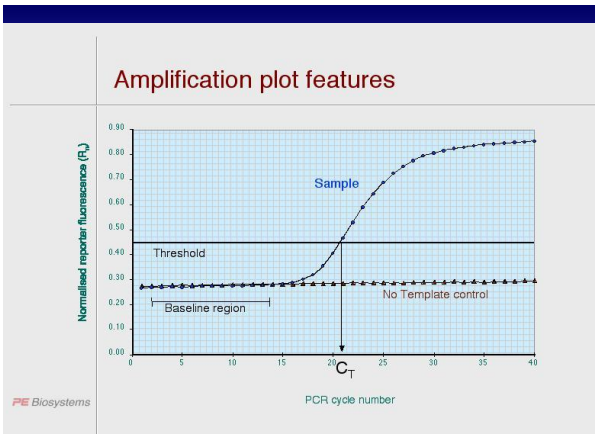


Scorpion Probe (ARMS)



From: http://probes.invitrogen.com/handbook/images/g001360.gif



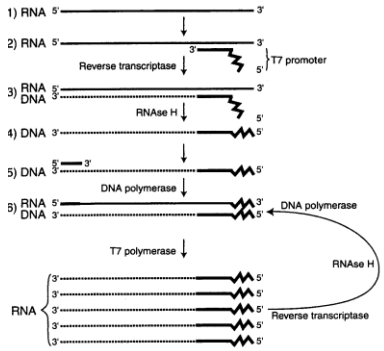


PCR Math

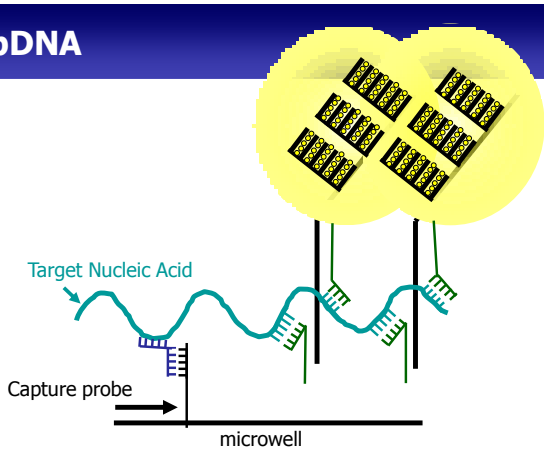
$$A_n = A_0(1+e)^n$$

Practical consequence is that if sample A takes one more cycle to be detected than sample B, sample A has half as much starting template as sample B.

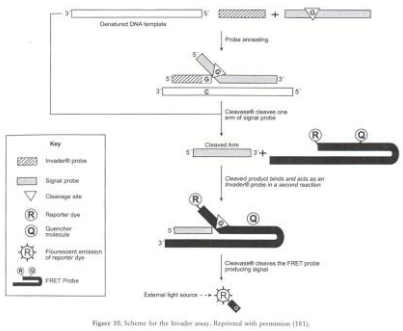
Transcription Mediated Amplification



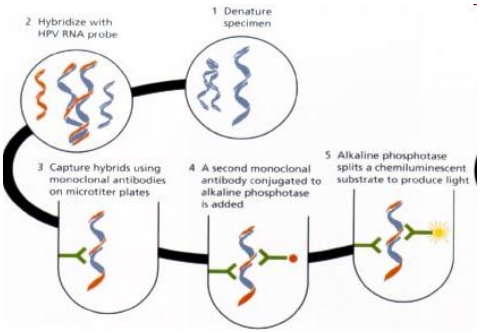
bDNA



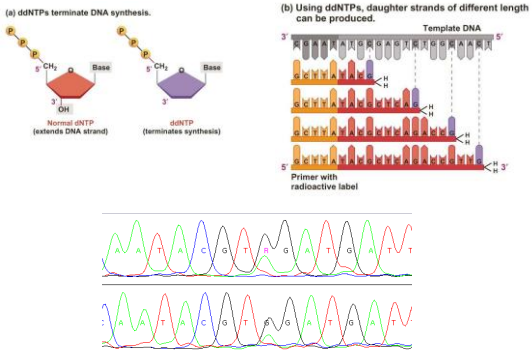
Serial Invasive Amplification



Hybrid Capture

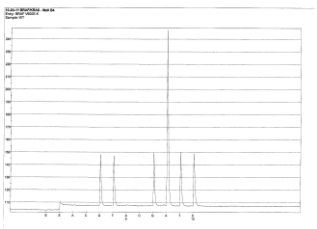


Sanger Sequencing



Pyrosequencing

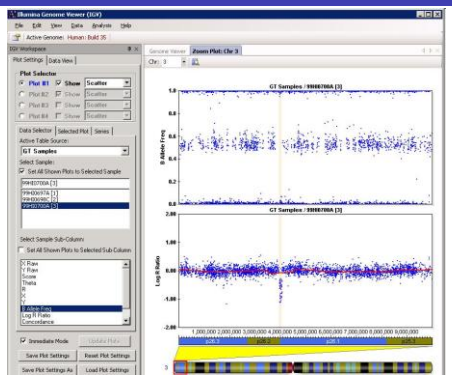
- Pipette nucleotide
- If added, pyrophosphate produced
- Pyrophosphate can participate with luciferase to produce light
- If nucleotide not added, degraded by apyrase



Genomics

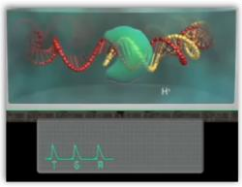
- Whole genome sequencing easily within reach
- Whole exome sequencing
- Cost/benefit
- Human Genome Project ~$3B USD
- Now possible to achieve less than $5K/genome

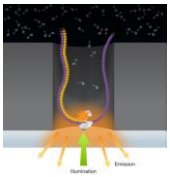
Arrays



Genomics

- Massively parallel sequencing
- Generally sequencing individual DNA strands (not population sequencing)
- Whole genomes/exomes in very little time
- Short vs long read lengths





Genomic Challenges

- Too much information
- Confidence in the data?
- Archiving the data
- Tumor heterogeneity?
- Reporting
- Unintended Consequences

Summary

- Molecular Diagnostics includes DNA/RNA analysis
- Sample and preanalytic variables are critical
- PCR is the cornerstone technique
- Detection and quantification
- Genomics will change the practice of pathology

Thank you!

Dr. Rami Mahfouz
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Dr. Corinne Fantz