BME 215
Applied Gene Technology

Molecular Based Methods of Diagnosis
Molecular Based Methods of Diagnosis

DNA based molecular methods
Why use a molecular test to diagnose an infectious disease?

- Need an accurate and timely diagnosis
  - Important for initiating the proper treatment
  - Important for preventing the spread of a contagious disease
Leading uses for nucleic acid based tests

- Nonculturable agents
  - Human papilloma virus
  - Hepatitis B virus

- Fastidious, slow-growing agents
  - Mycobacterium tuberculosis
  - Legionella pneumophila

- Highly infectious agents that are dangerous to culture
  - Francisella tularensis
  - Brucella species
  - Coccidioides immitis
Leading uses for nucleic acid based tests

- In situ detection of infectious agents
  - Helicobacter pylori
  - Toxoplasma gondii
- Agents present in low numbers
  - HIV in antibody negative patients
  - CMV in transplanted organs
- Organisms present in small volume specimens
  - Intra-ocular fluid
  - Forensic samples
Leading uses for nucleic acid based tests

- Differentiation of antigenically similar agents
  - May be important for detecting specific virus genotypes associated with human cancers (Papilloma viruses)

- Antiviral drug susceptibility testing
  - May be important in helping to decide anti-viral therapy to use in HIV infections
Leading uses for nucleic acid based tests

- Molecular epidemiology
  - To identify point sources for hospital and community-based outbreaks
  - To predict virulence
- Culture confirmation
What are the different types of nucleic acid molecular techniques that are used?

- Direct probe testing – better for identification than for detection because it is not as sensitive as amplification methods.
- Amplification methods – used to improve the sensitivity of the nucleic acid testing technique.
  - Target amplification
  - Probe amplification
  - Signal amplification
  - Combinations of the above
Direct probe testing

- Hybridization – to come together through complementary base-pairing.
  - Can be used in identification.
  - In colony hybridization the colony is treated to release the nucleic acid which is then denatured to single strands.
    - Labeled single-stranded DNA (a probe) unique to the organism you are testing for is added and hybridization is allowed to occur.
    - Unbound probe is washed away and the presence of bound probe is determined by the presence of the label.
Direct probe testing

(a) Salmonella DNA fragment cloned in E. coli

(b) Cloned DNA fragments marked with fluorescent dye and separated into single strands, forming DNA probes

(c) Unknown bacteria collected on filter

Cells lysed, DNA released

DNA separated into single strands

DNA probes added to DNA from unknown bacteria

Salmonella DNA

DNA from other bacteria

(d) DNA probes hybridize with Salmonella DNA from sample; excess probe is washed off; fluorescence indicates presence of Salmonella
Target amplification

- Target amplification requires that the DNA to be tested for be amplified, i.e., the number of copies of the DNA is increased.

- To understand this we must first review the activity of the enzyme, DNA polymerase, that is used to amplify the DNA.
Polymerase template and primer requirements

- DNA polymerase cannot initiate synthesis on its own.
  - It needs a primer to prime or start the reaction.
  - The primer is a single stranded piece of DNA that is complementary to a unique region of the sequence to be amplified.
Polymerase template and primer requirements
DNA synthesis

- Synthesis can occur only in the 5’ to 3’ direction.
DNA synthesis

- Remember that DNA replication is semiconservative:
Target amplification – The PCR reaction

- Polymerase chain reaction – used to amplify something found in such small amounts that without PCR it would be undetectable.
  - Uses two primers, one that binds to one strand of a double-stranded DNA molecule, and the other which binds to the other strand of the DNA molecule,
  - all four nucleotides and
  - a thermostable DNA polymerase.
  - The primers must be unique to the DNA being amplified and they flank the region of the DNA to be amplified.
PCR

- The PCR reaction has three basic steps
  - Denature – when you denature DNA, you separate it into single strands (SS).
    - In the PCR reaction, this is accomplished by heating at 95°C for 15 seconds to 1 minute.
    - The SS DNA generated will serve as templates for DNA synthesis.
  - Anneal – to anneal is to come together through complementary base-pairing (hybridization).
    - During this stage in the PCR reaction the primers base-pair with their complementary sequences on the SS template DNA generated in the denaturation step of the reaction.
The primer concentration is in excess of the template concentration.

The excess primer concentration ensures that the chances of the primers base-pairing with their complementary sequences on the template DNA are higher than that of the complementary SS DNA templates base-pairing back together.

The annealing temperature used should ensure that annealing will occur only with DNA sequences that are completely complementary. WHY?

The annealing temperature depends upon the lengths and sequences of the primers. The longer the primers and the more Gs and Cs in the sequence, the higher the annealing temperature. WHY?

The annealing time is usually 15 seconds to 1 minute.

PCR
PCR

- Extension – during this stage of the PCR reaction, the DNA polymerase will use dNTPs to synthesize DNA complementary to the template DNA.
  - To do this DNA polymerase extends the primers that annealed in the annealing step of the reaction.
  - The temperature used is 72°C since this is the optimum reaction temperature for the thermostable polymerase that is used in PCR.
  - Why is a thermostable polymerase used?
  - The extension time is usually 15 seconds to 1 minute.

- The combination of denaturation, annealing, and extension constitute 1 cycle in a PCR reaction.
PCR

- Most PCR reaction use 25 to 30 of these cycles to amplify the target DNA up to a million times the starting concentration.
What are the advantages of using a molecular test?

- **High sensitivity**
  - Can theoretically detect the presence of a single organism

- **High specificity**
  - Can detect specific genotypes
  - Can determine drug resistance
  - Can predict virulence

- **Speed**
  - Quicker than traditional culturing for certain organisms
What are the advantages of using a molecular test?

- Simplicity
  - Some assays are now automated
What are the disadvantages of using a molecular test?

- Expensive
- So specific that must have good clinical data to support infection by that organism before testing is initiated.
- Will miss new organisms unless sequencing is done (not practical in a clinical setting).
- May be a problem with mixed cultures – would have to assay for all organisms causing the infection.
What are the disadvantages of using a molecular test?

- Too sensitive? Are the results clinically relevant?