

Pathogen Detection using DNA Amplification Technologies

Lab 14

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- How do we identify plant pathogens?
- Traditional methods - morphological features, physiology, serology


1) **Morphology** - used to identify fungal and nematode pathogens

PROBLEM: genetically distinct fungi can look very similar, morphological characters can be unstable

2) **Physiology** - used to identify bacteria and fungi

PROBLEM: genetically distinct pathogens can have similar physiological attributes, tests can be finicky

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3) **serology** - used to identify fungi, bacteria and viruses

PROBLEM: some pathogens may not be antigenic, antigens are not specific, tests are finicky

- alternative - use organism's DNA as characters for identification
- more variation in DNA than in proteins
- 1970's - development of molecular biology - tools to move DNA between organisms, clone and sequence DNA
- 1983 - development of polymerase chain reaction (PCR) - allows amplification of DNA directly

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Polymerase Chain Reaction

In vitro amplification of nucleic acid (DNA) by using a DNA polymerase

=what happens in a living cell is now possible in a test tube

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Polymerase Chain Reaction (PCR)

- developed in 1983 by Kary Mullis - awarded Nobel prize in Chemistry for the discovery in 1993
- utilizes a thermostable DNA polymerase from the thermophilic bacterium *Thermophilus aquaticus*
- *Taq* polymerase assembles a new DNA strand from nucleotide building blocks and DNA oligonucleotides (primers)
- thermal cycling used to exponentially increase amount of target DNA from sample

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Dr. Kary Mullis Nobel Prize in Chemistry 1993

Born Dec. 28, 1944, in Lenoir, N.C.
B.S. Chemistry, Georgia Tech
Ph.D. Biochemistry, UC Berkeley
Scientist, Cetus Corp., CA

National Inventors Hall of Fame
in 1998

Autobiography,
"Dancing Naked in the Mind Field,"
1988



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Components of PCR

- Template DNA
- Primers (upstream and downstream aka forward and reverse primers)
- DNA polymerase
- dNTPs (dATP, dCTP, dGTP, dTTP)
- Buffer for the enzyme
- Mg (co-factor for Taq DNA polymerase)
- Incubation at optimum temperature (for the enzyme)

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Taq DNA polymerase

- *Thermus Aquaticus*: a bacterium that lives in extremely hot environments
- Its DNA polymerase is heat-stable: remains active even at 80 C!
- This enzyme was purified and used in PCR
- The gene was cloned and is now produced in *E. coli* (no longer needs to be purified from *T. aquaticus*)

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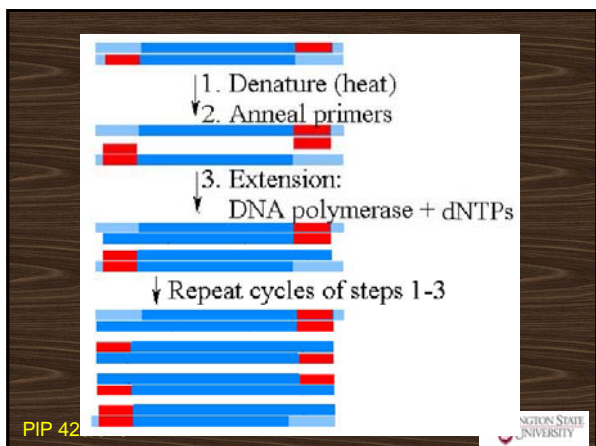


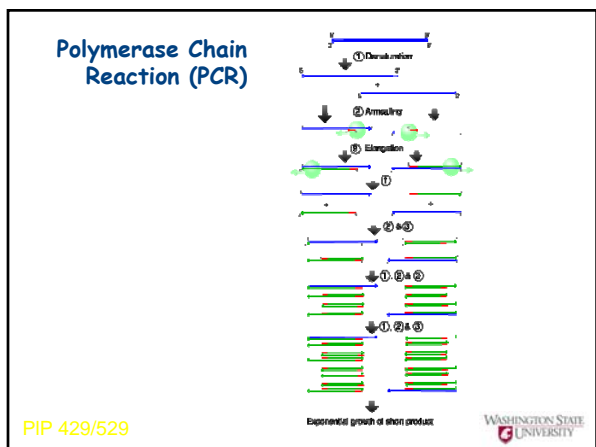
Polymerase Chain Reaction (PCR) - cont'd

- STEP 1** - denaturation or melting of target DNA strand - 20-30 seconds at 94-98C
 - STEP 2** - annealing of primers to single-stranded template - 20-40 seconds at 50-65C
 - STEP 3** - extension or elongation of DNA strand - 10-60 seconds at 72C - time depends on target size
- use a thermocycler to alternate rapidly between the three different temperatures

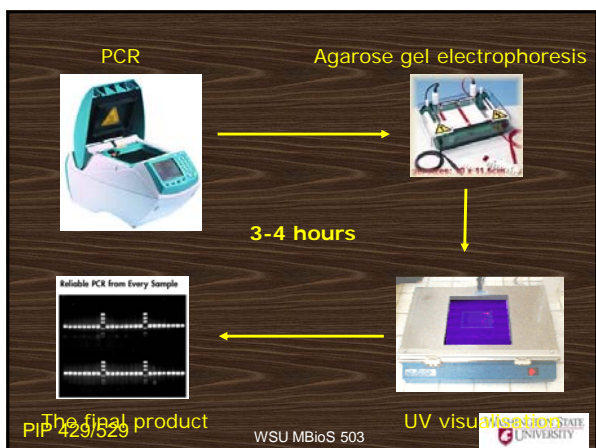
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PCR Animation:
<http://www.maxanim.com/genetics/PCR/PCR.htm>


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Advantages of PCR

- Highly sensitive (can detect picogram)
- Process is automated: very rapid, it takes 2 hrs or less for the test
- Versatile: can be used for detecting RNA or DNA
- Very useful where other methods are not effective

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Reverse Transcriptase PCR

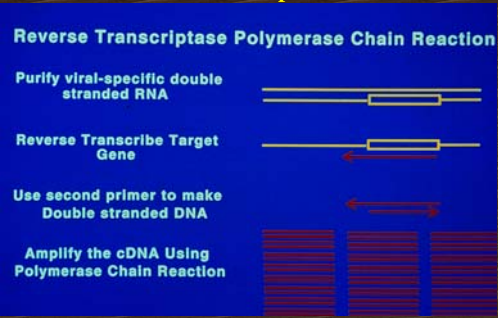
Reverse Transcriptase Polymerase Chain Reaction

Purify viral-specific double stranded RNA


Reverse Transcribe Target Gene

Use second primer to make Double stranded DNA

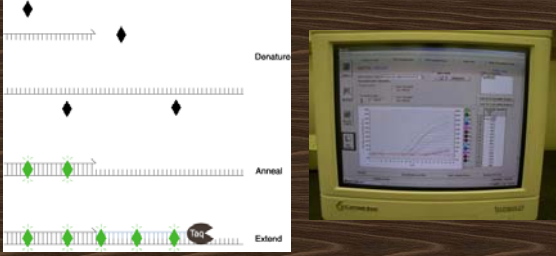
Amplify the cDNA Using Polymerase Chain Reaction



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Real-time PCR: SYBR Green



Denature

Anneal

Extend

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COX1 primer design

Cytochrome oxidase

- oxidizing enzyme
- found in mitochondria and important in cell respiration
- enzyme has multiple subunits - 13 subunits in mammals
- we are focusing on cytochrome oxidase subunit 1 (COX1)
- COX1 gene located in mitochondrial genome


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COX1 primer design

- partial *COX1* sequences available for *Penicillium expansum* and *P. digitatum*
- align *COX1* sequences to compare
- identify nucleotide sites that differ between the two species
- try to design primers to these regions
- use these primers in PCR

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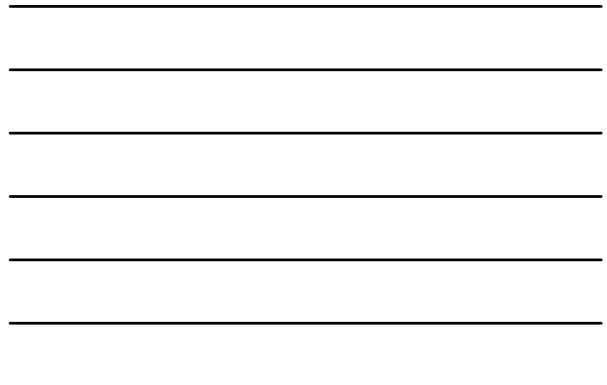



CLUSTAL X (1.83.1) multiple sequence alignment

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EP180260expansum      AATTCGAAAGATATGGACTTTAATCTTAATGTTTCATATTTCTGGTTAGTTGG 60
EP180235digitatum    TATTCGAAAGATATGGACTTTAATCTTAATGTTTCATATTTCTGGTTAGTTGG 60
EP180260expansum      AACAGGTTTTCAAGTTTAAATAGATTAGAGTTATCAGGTCAGGTTTCAATATATATC 120
EP180235digitatum    AACAGGTTTTCAAGTTTAAATAGATTAGAGTTATCAGGTCAGGTTTCAATATATATC 120
EP180260expansum      AGATAATCAATATATAAATAGTATAATACAGACACATCTATCTGTATGTTTTCT 180
EP180235digitatum    AGATAATCAATATATAAATAGTATAATACAGACACATCTATCTGTATGTTTTCT 180
EP180260expansum      GOTTATGCTCTGATTAAATGAGGTTTTGGTAATTTCTTATACCAATT  AGTTGGGG 240
EP180235digitatum    GOTTATGCTCTGATTAAATGAGGTTTTGGTAATTTCTTATACCAATT  AGTTGGGG 240
EP180260expansum      TCAGATATGGCAATTTCTAGATTAAATATATAAGTTTTGGATTA  TTAGTCTGATTT 300
EP180235digitatum    TCAGATATGGCAATTTCTAGATTAAATATATAAGTTTTGGATTA  TTAGTCTGATTT 300
EP180260expansum      ATTTTATTATATCTCAGCTACTATAGAAAATGGAGCTG  GTCAGGTTGAATATA 360
EP180235digitatum    ATTTTATTATATCTCAGCTACTATAGAAAATGGAGCTG  GTCAGGTTGAATATA 360
EP180260expansum      TCACCAATTATCAGGAATACAATCTCAGATGGACC  TAGTGTAGATTAGCTATTTGG 420
EP180235digitatum    TCACCAATTATCAGGAATACAATCTCAGATGGACC  TAGTGTAGATTAGCTATTTGG 420
EP180260expansum      TTACATTTAAGTGTATAGTATATGTTAGTGTATGATTTTATACACTACTATCT 480
EP180235digitatum    TTACATTTAAGTGTATAGTATATGTTAGTGTATGATTTTATACACTACTATCT 480
EP180260expansum      AAATATGAGAAGTCTGGTATACGTTTACACAAATAGCTTTATTTGGATGAGCTGTTAT 540
EP180235digitatum    AAATATGAGAAGTCTGGTATACGTTTACACAAATAGCTTTATTTGGATGAGCTGTTAT 540
EP180260expansum      TATTA 545
EP180235digitatum    TATTA 545
    
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M	Primer Set 1			Primer Set 2		
	PE	PD	AA	PE	PD	AA
500	—	—	—	—	—	—
400	—	—	—	—	—	—
300	—	—	—	—	—	—
200	—	—	—	—	—	—
100	—	—	—	—	—	—

Expected banding patterns in agarose gel loaded with PCR products from fungal species-specific PCR. M = DNA size marker, PE = *Penicillium digitatum*, PE = *P. expansum*, AA = *Alternaria alternata*

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Always remember:

- PCR is a highly sensitive technique (contamination with unwanted DNA)
- Run NEGATIVE controls (H₂O)
- Include a positive control if possible

--Wear gloves to protect yourselves

We add ethidium bromide into the gel mix in order to visualize to bands.

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Today's Exercises:

- Load horizontal electrophoresis gels with PCR products generated previously (Johnson 312)
- Set up PCR reactions

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