MOD1 – DNA ENGINEERING

Engelward, Fall 2009

Day 1

About this Module

Goals for this Module

Brief background: Homologous recombination is not just for meiosis!

Overview of the Experiment

Today's Experiment: **Design Primers and Perform PCR**

Brief Review of DNA Replication

Chemistry of nucleotide addition (5' vs 3' end)

PCR - Cycling

How you can use PCR to add a restriction site to your PCR product

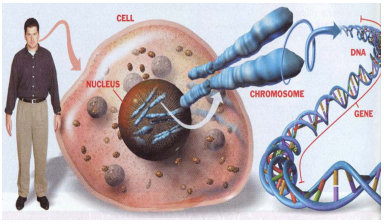
Conceptual Objectives

- Binding Conditions (primers)
- Enzymes and Reaction Conditions
- Concepts in purification
- Engineering Gene Expression Vectors
- Transfection and Transformation
- Restriction Mapping
- Internal standards (concentration)
- Cell Culture (mammalian cells)
- Cell Growth Kinetics
- Differentiation
- Flow Cytometry

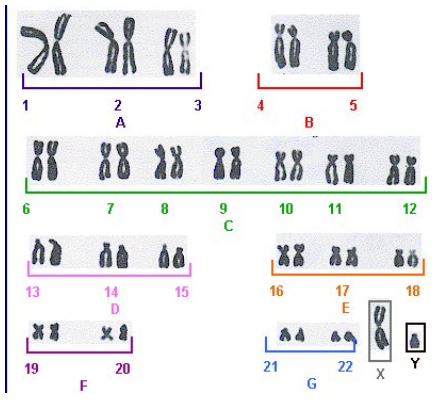
Module Objectives

- Experimental Design
  - -Quantitative Measurements
  - -Controls
  - -Experimental Variability
- Data Interpretation and Presentation Skills
  - -Statistics
  - -Data Interpretation
  - -Written & Oral Communication
- Basic Laboratory Skills
  - -Record Keeping
  - -Sources of Error
  - -Basic Laboratory Equipment
  - -Manipulation of enzymes, DNA and mammalian cells

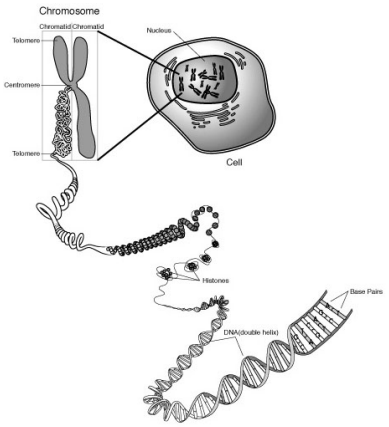
Homologous Recombination
& Why it is important to you

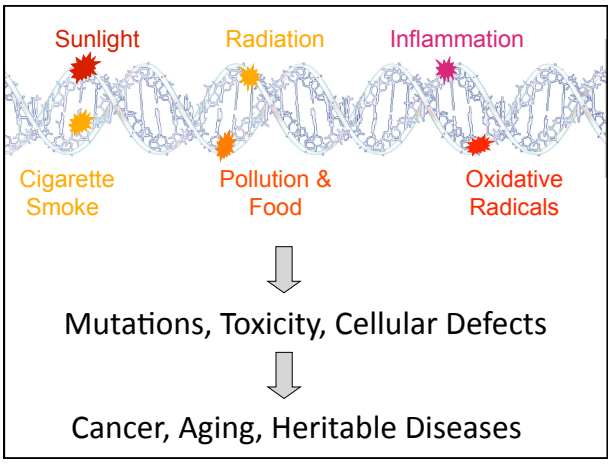


"*TIME*" Nov., 1999



http://homepages.uel.ac.uk/V.K.Sieber/human.htm





Homologous Recombination Repairs DNA

Werner Syndrome (WS)

* develop normally in early age
* premature aging starting at puberty
* short stature
* leg ulceration
* soft-tissue calcification
* average life span = 47
* cancer and cardiovascular diseases are primary cause of death

Defects in HR Promote Aging, Cancer, & Diseases

Homologous Recombination Repairs DNA

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Defects in HR Promote Aging, Cancer, & Diseases

Homologous Recombination Repairs DNA

Bloom Syndrome (BS)

* sun-sensitive skin
* dwarfism
* immune deficiencies
* male infertility
* female subfertile
* cancer as primary cause of death before age of 30

Werner Syndrome (WS)

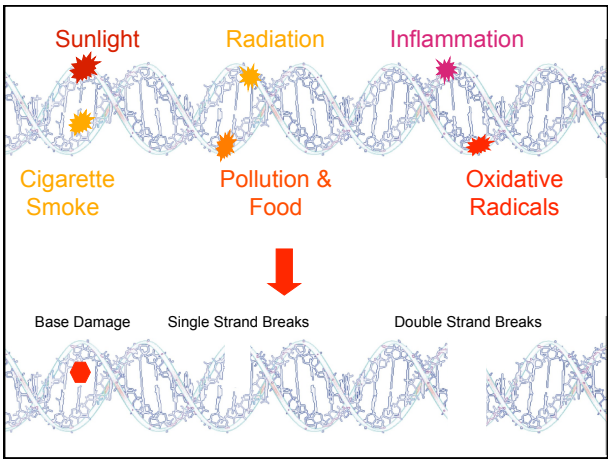
* develop normally in early age
* premature aging starting at puberty
* short stature
* leg ulceration
* soft-tissue calcification
* average life span = 47
* cancer and cardiovascular diseases are primary cause of death

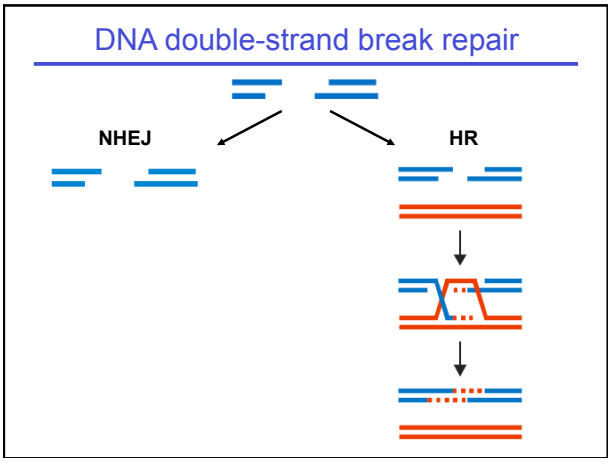
Rothmund-Thomson Syndrome (RTS)

* sun-sensitive
* hyper-pigmentation of skin
* short stature
* bone abnormality
* cancer predisposition, especially osteosarcoma

Defects in HR Promote Aging, Cancer, & Diseases

Life Cannot Exist without Homologous Recombination





Homologous Recombination Protects Cells from the Lethal Effects of DNA Damage

Your Experiment:

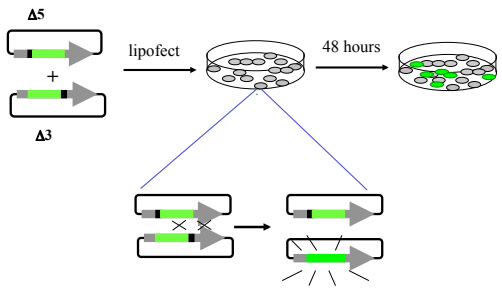
Create a plasmid that will be part of a homologous recombination assay.

Measure the frequency of cells in which homologous recombination between two plasmids gives rise to a fluorescent cell.

Test conditions that might affect the frequency of green cells!

Components of an Expression Cassette

A Plasmid-Based Assay for Homologous Recombination in Mammalian Cells



Roadmap: Blueprint of Plasmid Construction Plan

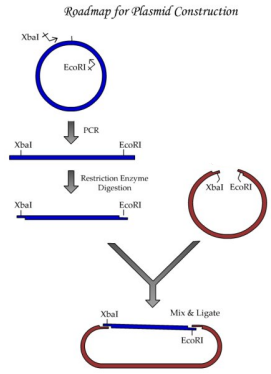
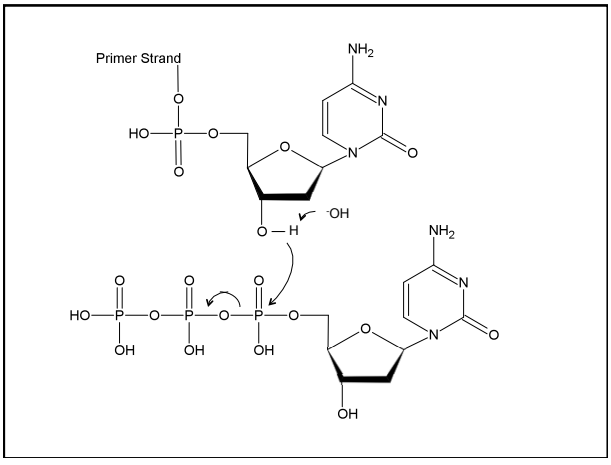


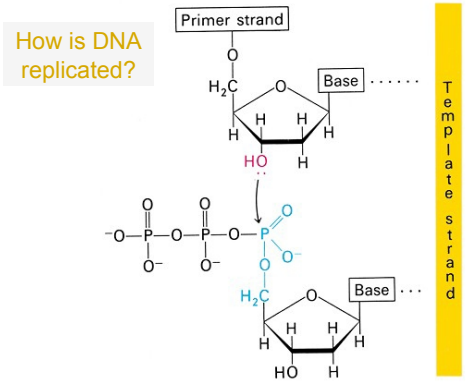
Figure by Justin Lo

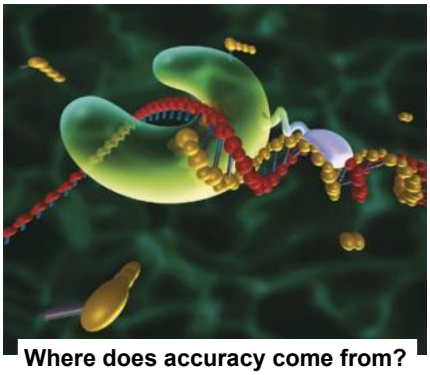
PCR

DNA Polymerases



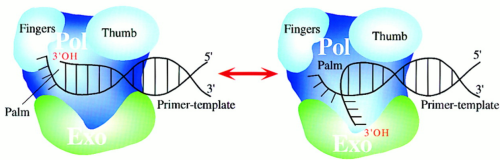
How is DNA replicated?





Where does accuracy come from?

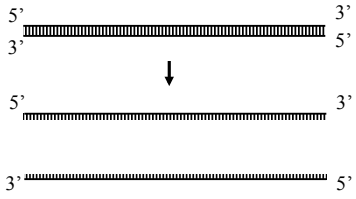
Image from BioPro



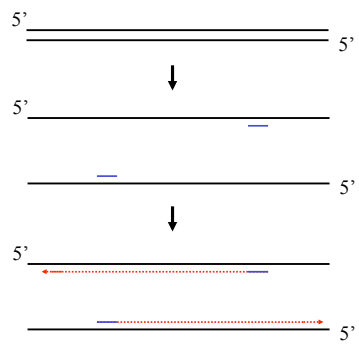
Proofreading prevents extension from a mismatch

Baker and Bell, Cell 1998

PCR



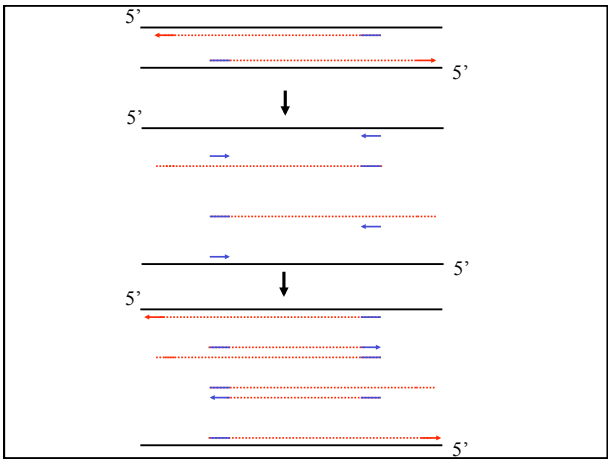
PCR

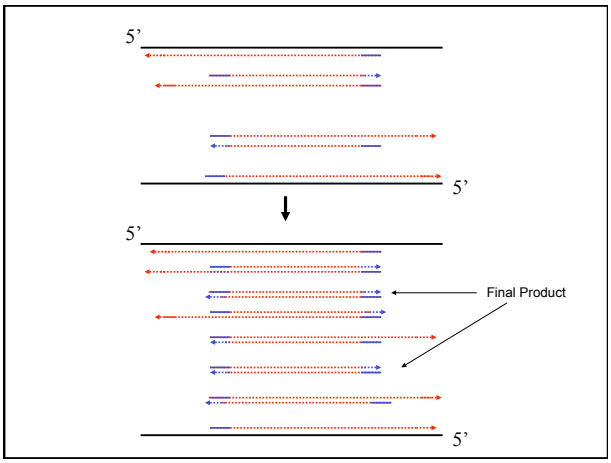


MELT 94°C

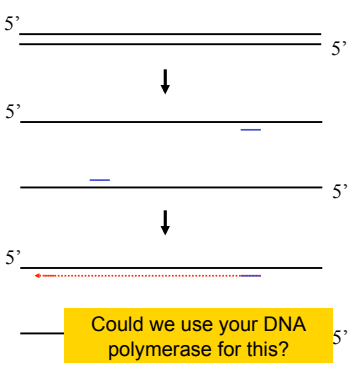
ANNEAL 55°C

EXTEND 72°C





PCR



MELT
94°C

ANNEAL
55°C

EXTEND
72°C

Could we use your DNA polymerase for this?

Error Rates

Taq

2.1 x 10^{-4} errors/bp
(Keohavang and Thilly, 1989)

Pfu

1.6 x 10^{-6} errors/base
(Lundberg et al., 1991)

What are the components of a PCR reaction?

Polymerase

Template

Primers

dNTPs

Mg++

Correct buffer conditions

Primer Design

1. 17-28 bases
3. 50-60% (GC)
4. Melting Temps should be ~65-80^{o}C
5. 3'-ends of primers should not be complementary to each other (why?)
9. Hairpins should be avoided (why?)
11. Check for 'accidental' annealing elsewhere in your target.

Why is it recommended that primers be about 50% GCs?

What would happen if there was a mismatch at the 5' end of the primer? ...the 3' end of the primer?

What would happen if the annealing temperature was too low? To high?

What are sticky ends and why are they useful?

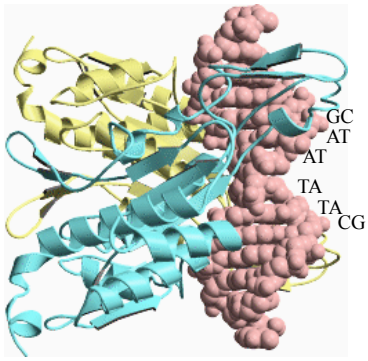
Restriction Enzymes

5' - G A A T T C - 3'
3' - C T T A A G - 5'

EcoRI

5' - G
3' - C T T A A

A A T T C - 3'
G - 5'



5' - G A A T T C - 3'
3' - C T T A A G - 5'

EcoRI

Image from: Rosenberg, J. M. Curr. Opin. Struct. Biol. 1: 104-110 (1991)

Roadmap: Blueprint of Plasmid Construction Plan

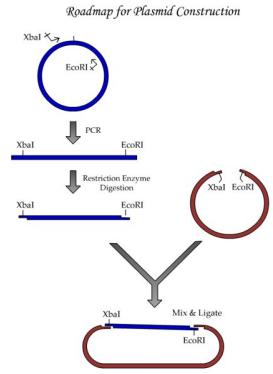
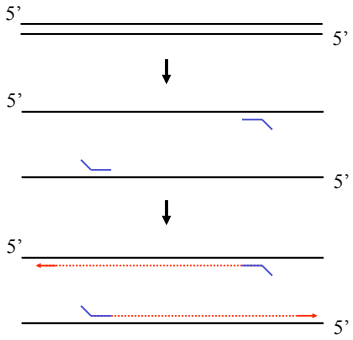


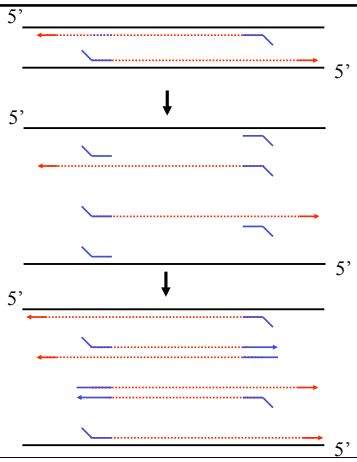
Figure by Justin Lo

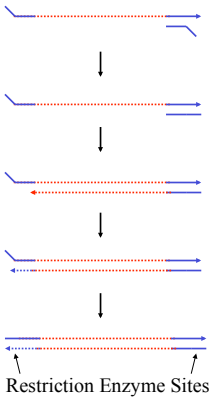
How do you add sticky ends to your PCR product?

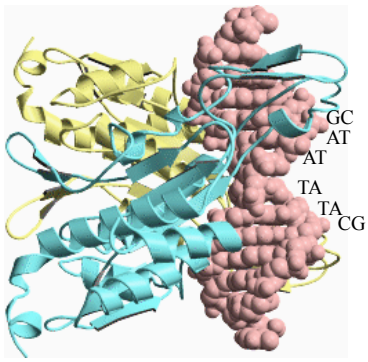
PCR

Basic Principles:









5' - G A A T T C - 3'
3' - C T T A A G - 5'

EcoRI

Why do you need to add extra sequence to the 5' end of your primer?

Image from: Rosenberg, J. M. Curr. Opin. Struct. Biol. 1: 104-110 (1991)

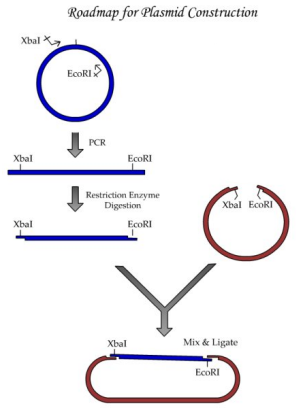


Figure by Justin Lo

Why do you need a "no template control"?

When should you prepare this control?

About this Module

Goals for this Module

Brief background: Homologous recombination is not just for meiosis!

Overview of the Experiment

Today's Experiment: **Design Primers and Perform PCR**

Review of DNA Replication

Chemistry of nucleotide addition (5' vs 3' end)

PCR - Cycling

How you can use PCR to add a restriction site to your PCR product