MOD1 – DNA ENGINEERING

Engelward, Spring 2008

Day 2

About the experiments in Mod1

- -how is recombination used to fix double strand breaks
- -how your two-plasmid assay works
- -overview of the experiments you will be doing

Key Concepts for PCR

- -oligonucleotides
- -sequence specific binding of probe & target
- -melting temperature vs annealing temperature
- -non-specific binding

Restriction Enzymes

- -basics restriction enzymes
- -buffer conditions
- -principles of the clean-up kit

Anticipating Potential Problems & Pitfalls

- -what controls are needed and why?

Mod1 - What you will do:

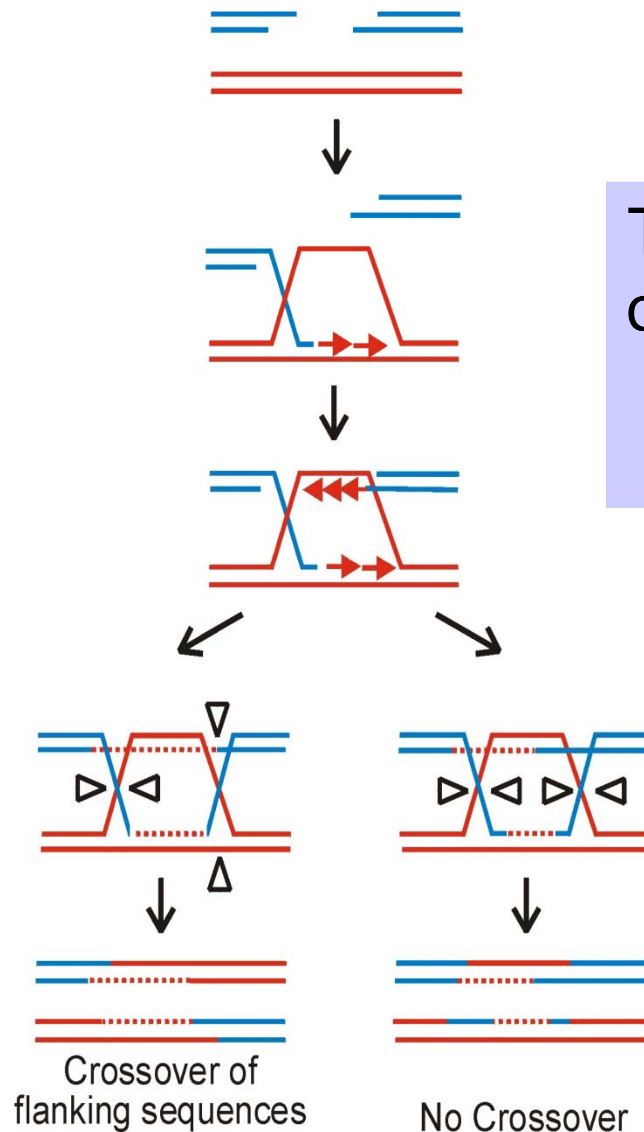
In this module, you will create a plasmid that will be used in an assay to measure homologous recombination activity in mammalian cells.

Background & Significance:

“Homology-Directed Repair” for double strand breaks

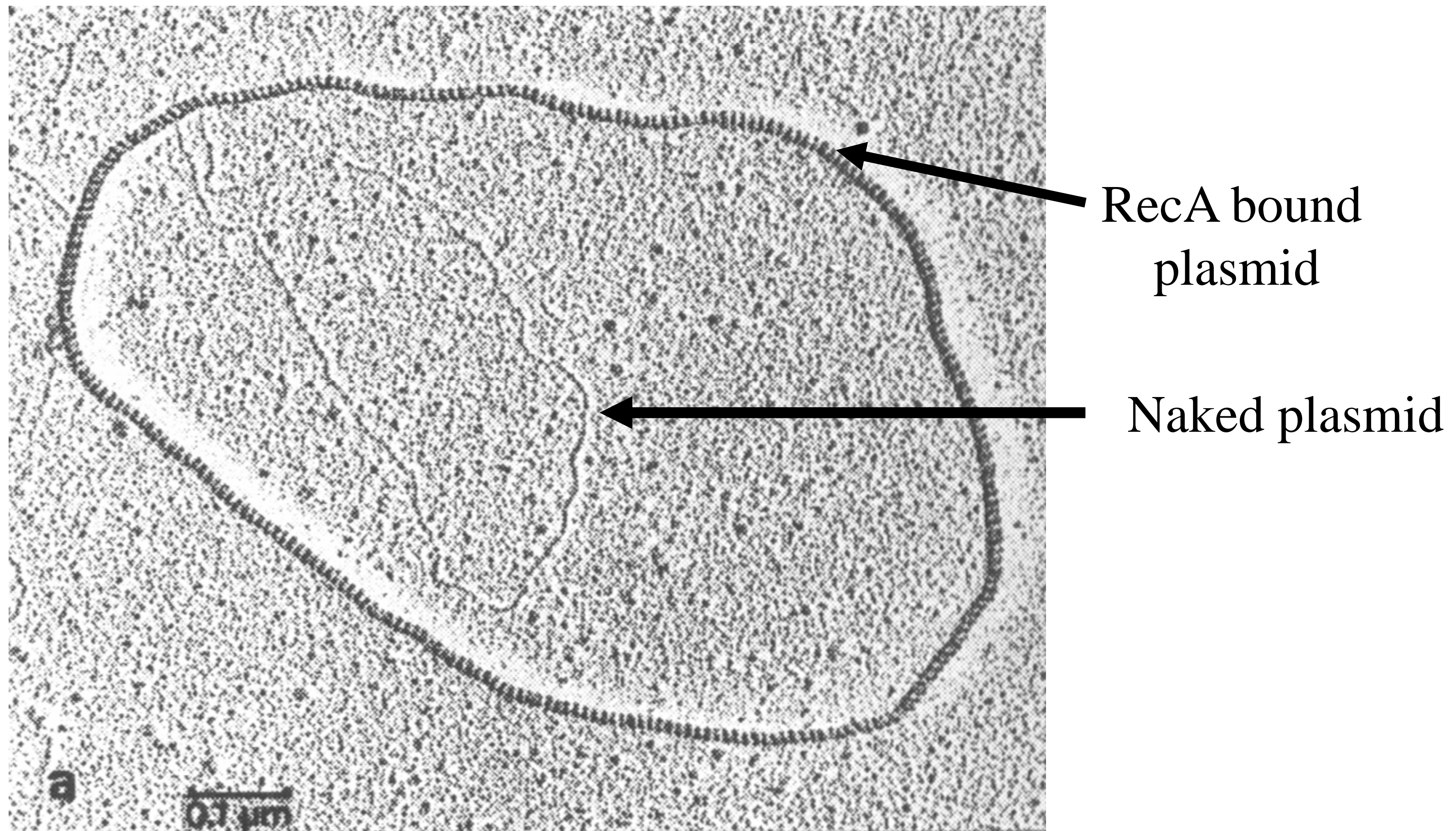
You will need to understand this material in order to write your final report.

DNA Damage can be repaired by **Homology Directed Repair (HDR)**



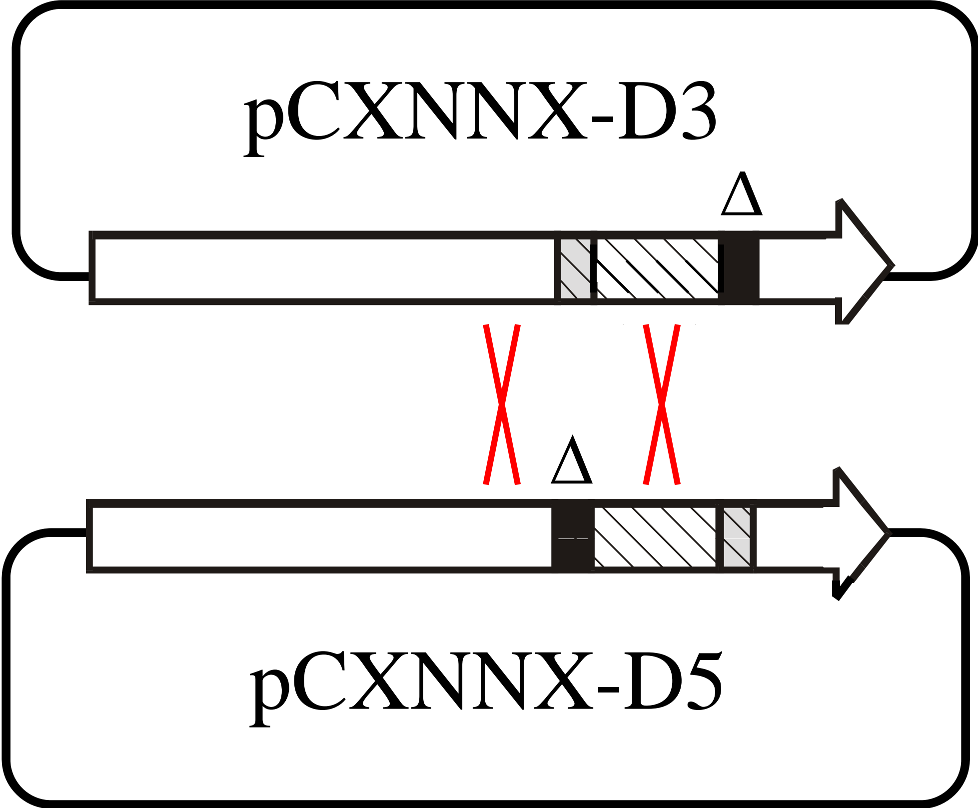
This is the 'prototypic' model of repair of how homologous recombination can repair a double strand break

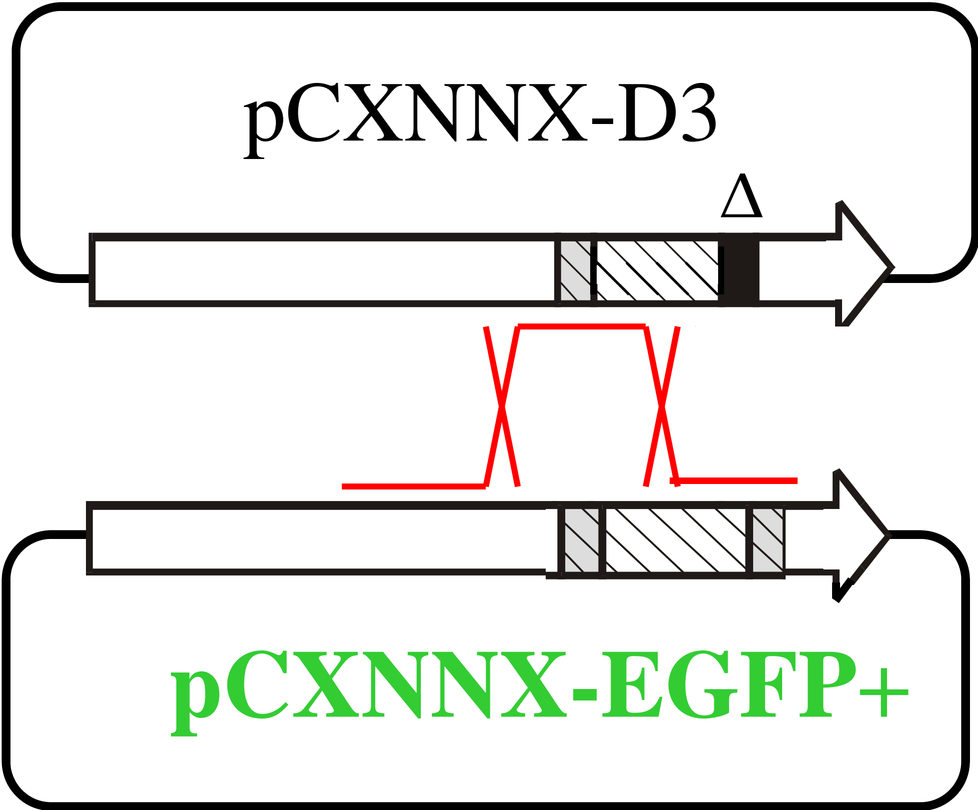
NOTE: BREAKPOINT TURNS FROM BLUE TO RED



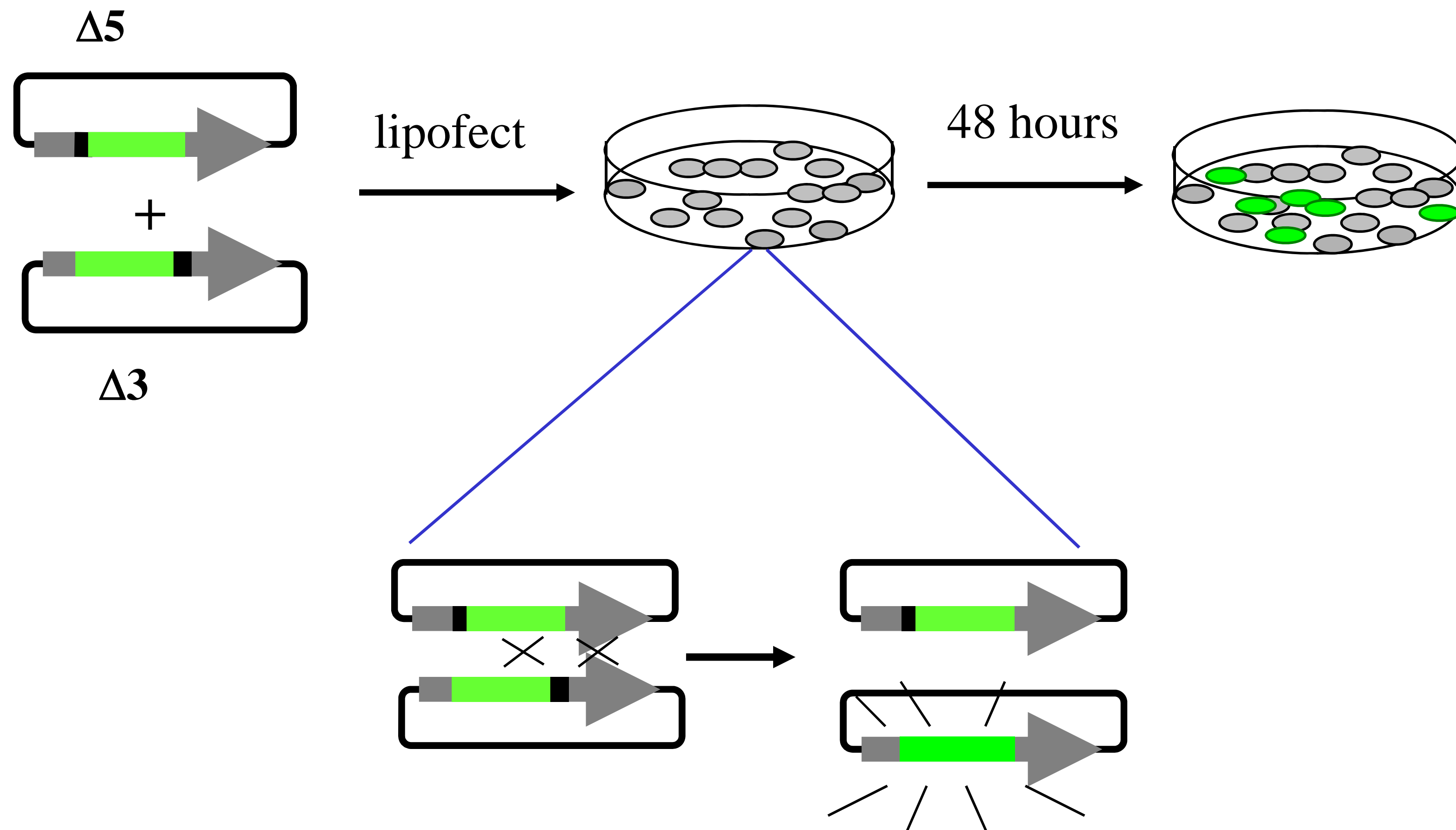
Relaxed circular duplex DNA covered with recA. Naked plasmid of same length lying within.
From Stasiak et al., Nature 299: 185-186 (1982).

Your Assay for
Homologous Recombination


pCXNNX-D3
Δ
Δ
pCXNNX-D5


pCXNNX-D3
Δ
pCXNNX-EGFP+

A Plasmid-Based Assay for Homologous Recombination in Mammalian Cells



Overview of the Experiments in Mod1

Where you are,
and where you are going

Construction of the Δ5 Plasmid

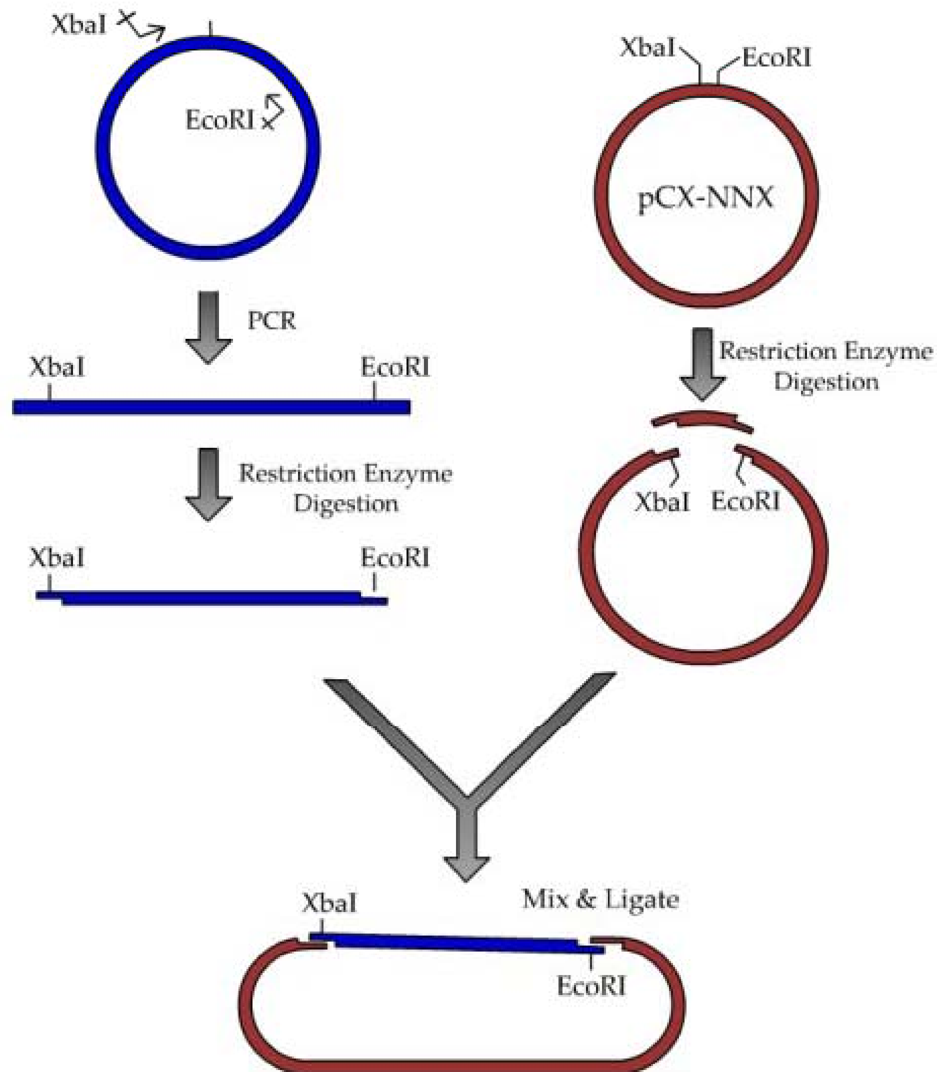
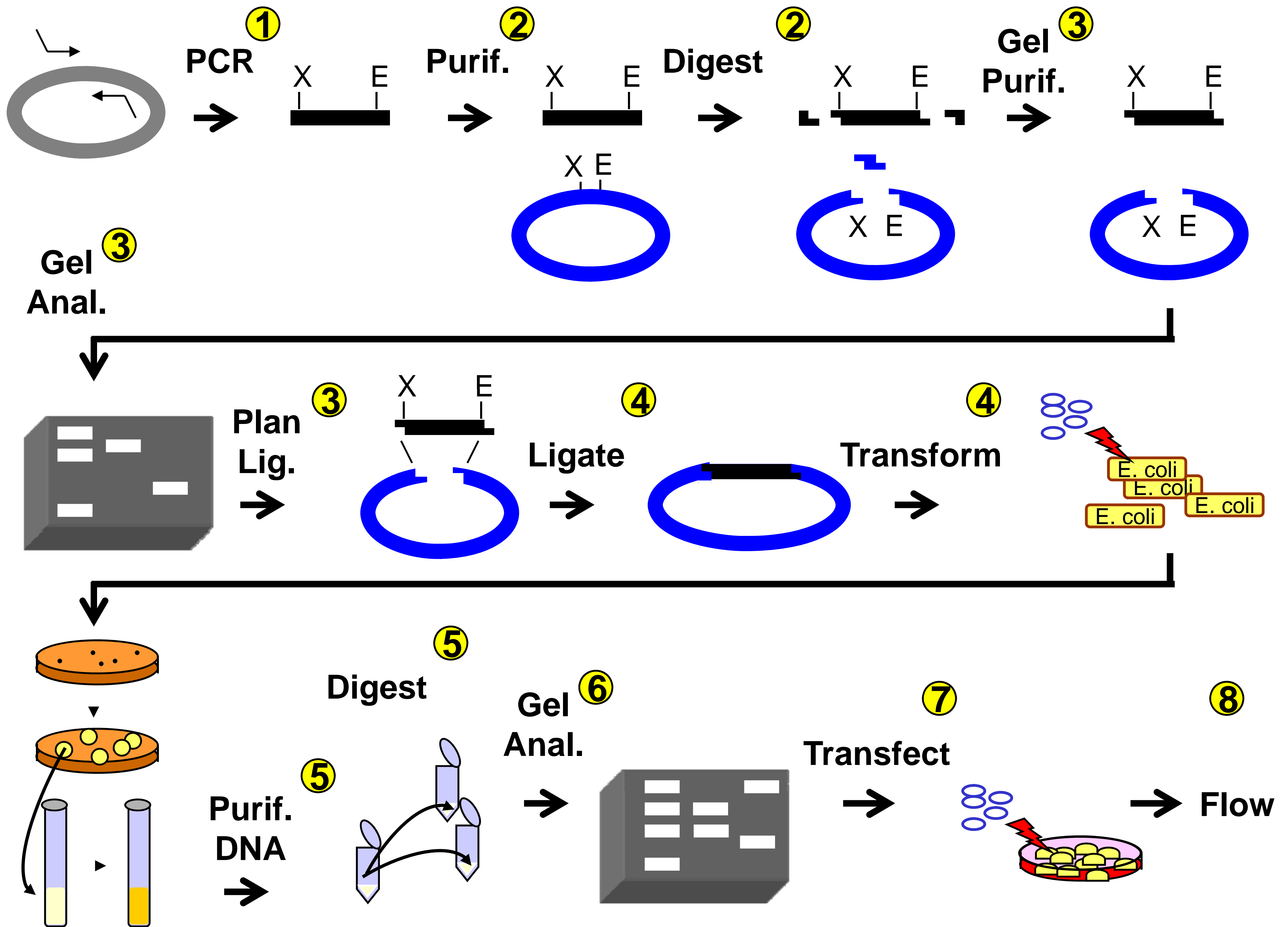
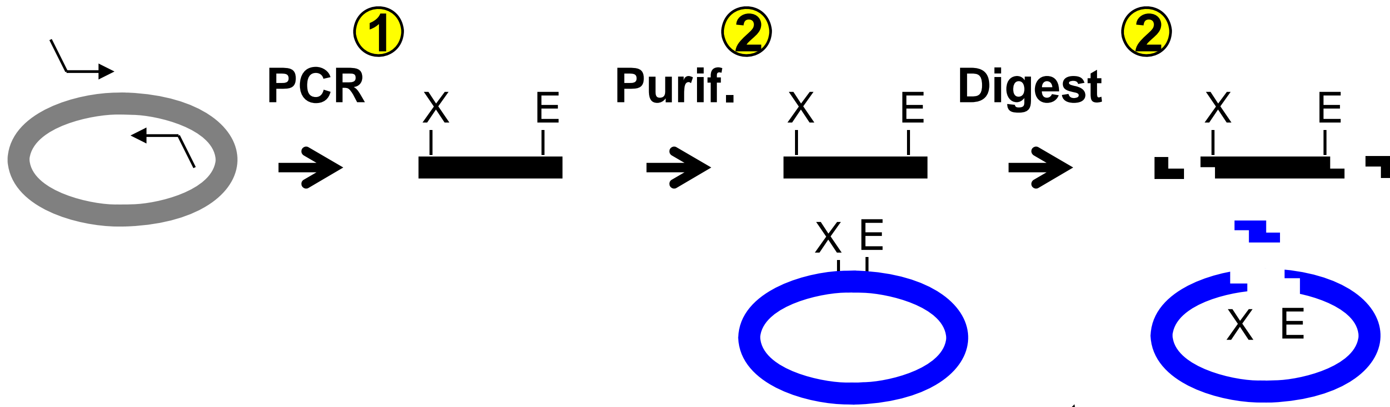


Figure by Justin Lo


1
PCR
X
E
2
Purif.
X
E
X E
2
Digest
X
E
X E
Gel
3
Purif.
X
E
X E
Gel
3
Anal.
Plan
3
Lig.
X
E
Ligate
4
Transform
4
E. coli
E. coli
E. coli
E. coli
5
Digest
Gel
6
Anal.
7
Transfect
8
Flow
Purif.
5
DNA



How do you know that your restriction enzymes actually cut the DNA?

Key Concepts for PCR

-biochemistry
-fidelity
-reaction conditions
-primer design
-stringency

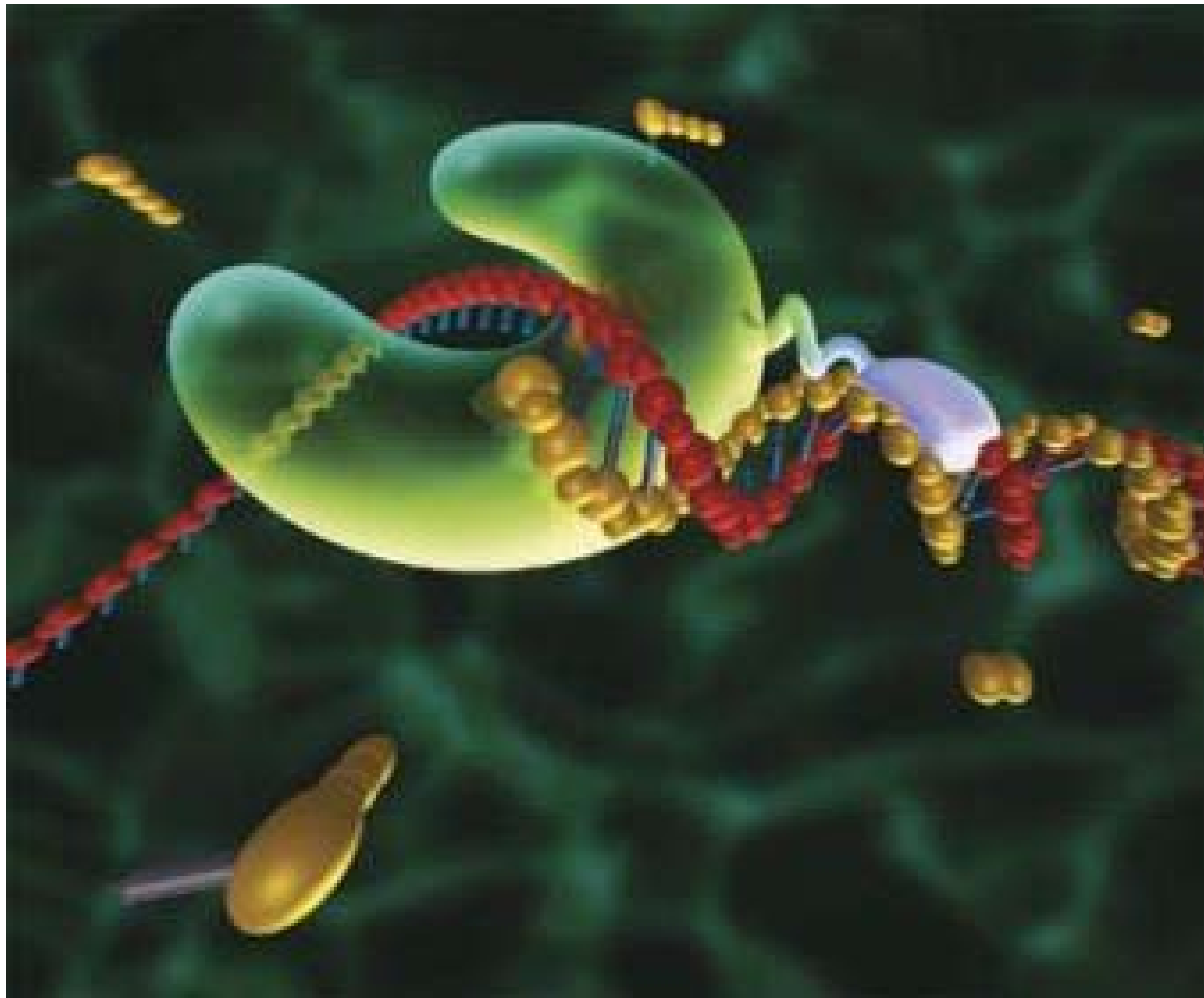
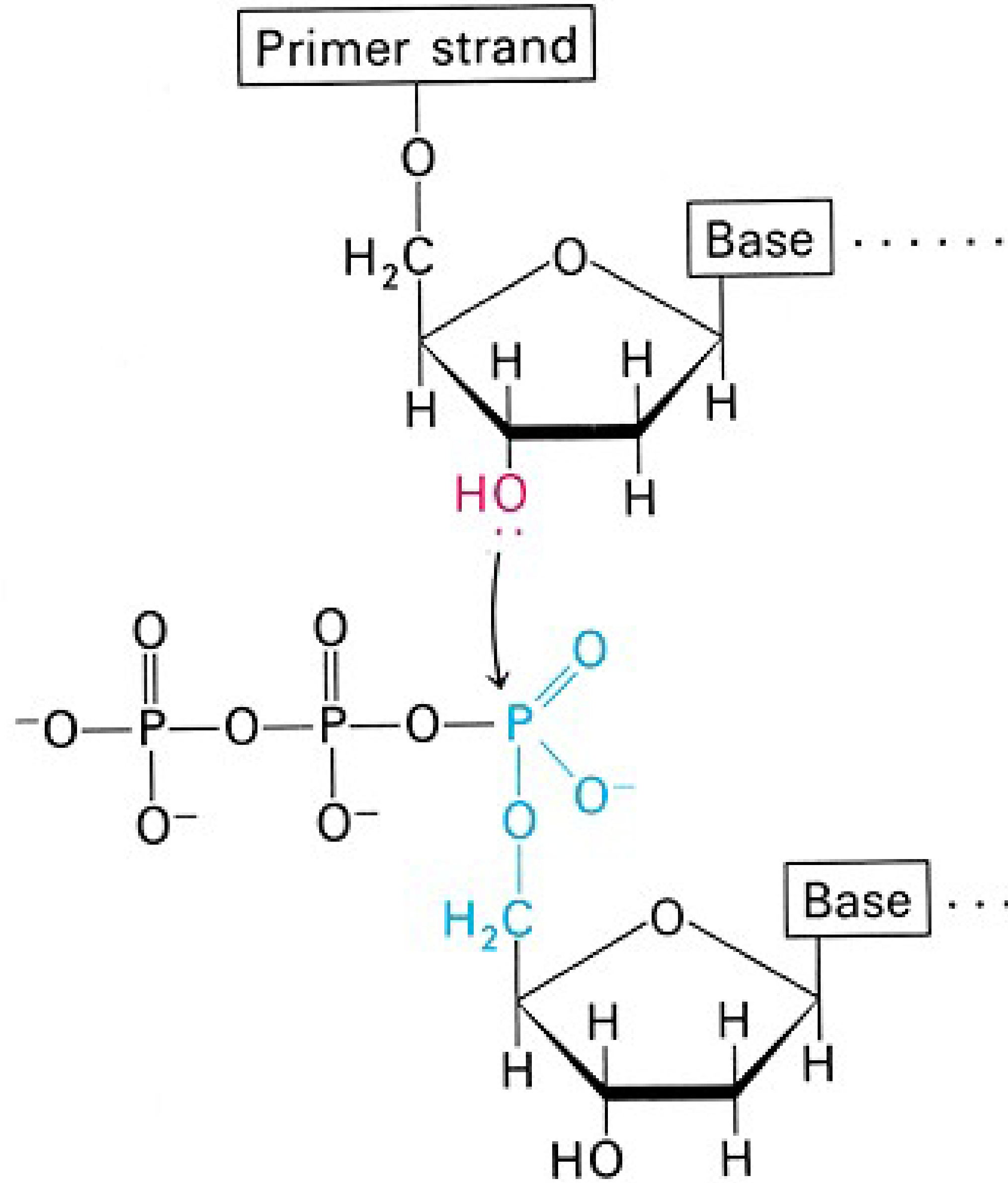
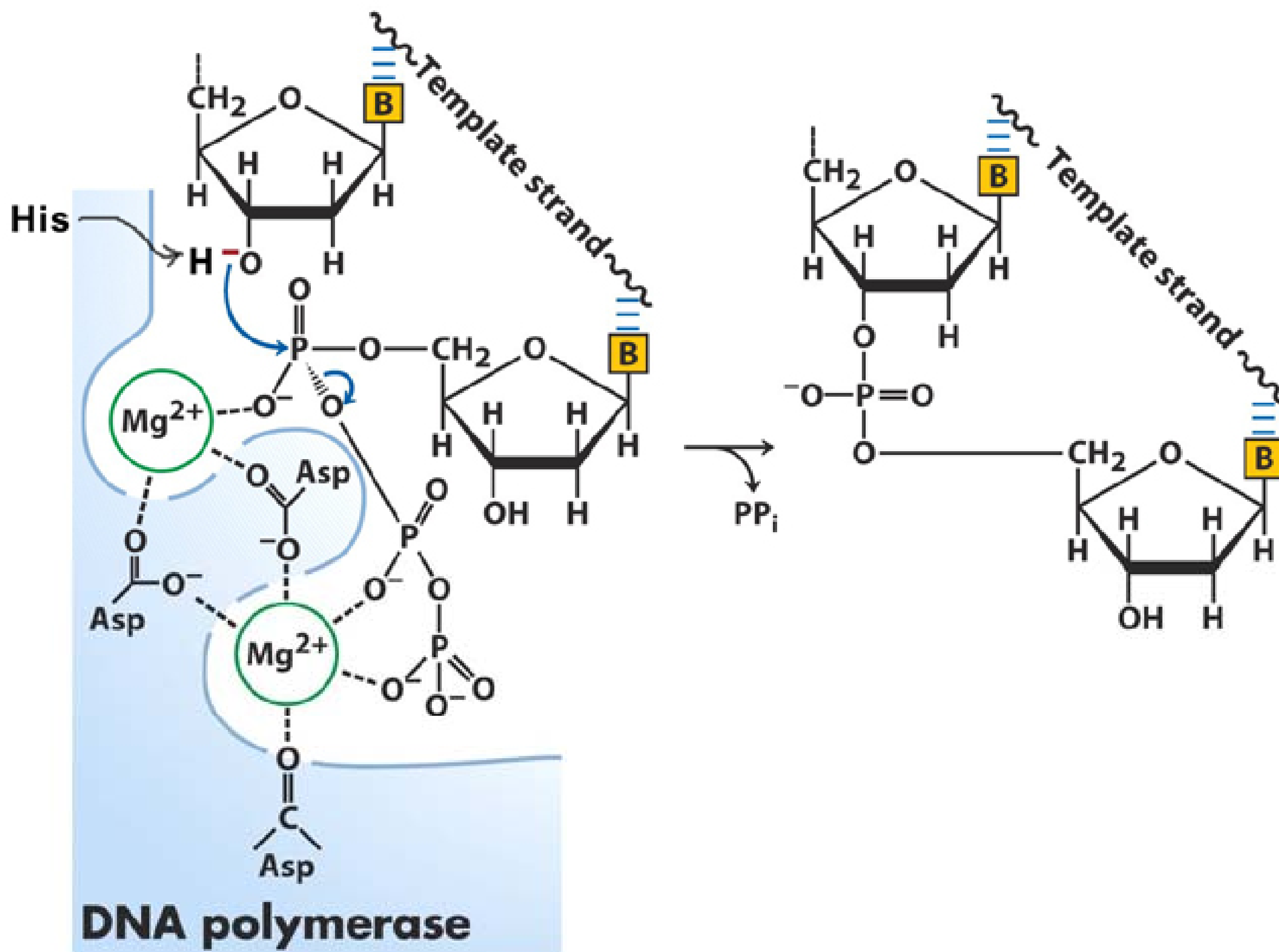


Image from BioPro


Primer strand
Base
Base
Template strand



Template strand
His
Mg2+
Asp
PPi
DNA polymerase

Error Rates

Taq

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

Pfu

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

What do you need in your test tube to perform PCR?

1) Polymerase
2) Template
3) Primer (3'OH)
4) dNTPs
5) Mg++ ($MgCl_2$)
6) Correct pH (Tris buffer)
7) Correct temperature
8) Correct salt concentration (KCl)
9) Sometimes people add DMSO and BME

General Advice on Primer Design

1. 17-28 bases
2. 50-60% (GC)
3. Melting Temps should be ~65-80°C
4. 3'-ends of primers should not be complementary to each other (why?)
5. Hairpins should be avoided (why?)
6. Check for ‘accidental’ annealing elsewhere in your target.

Which variables are most important for getting PCR to work?

You can avoid these common problems...

- Quality of template DNA
- Correct annealing temperature
- Sufficiently long elongation step
- Appropriate Mg++ concentration
- Correct primers (!)

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

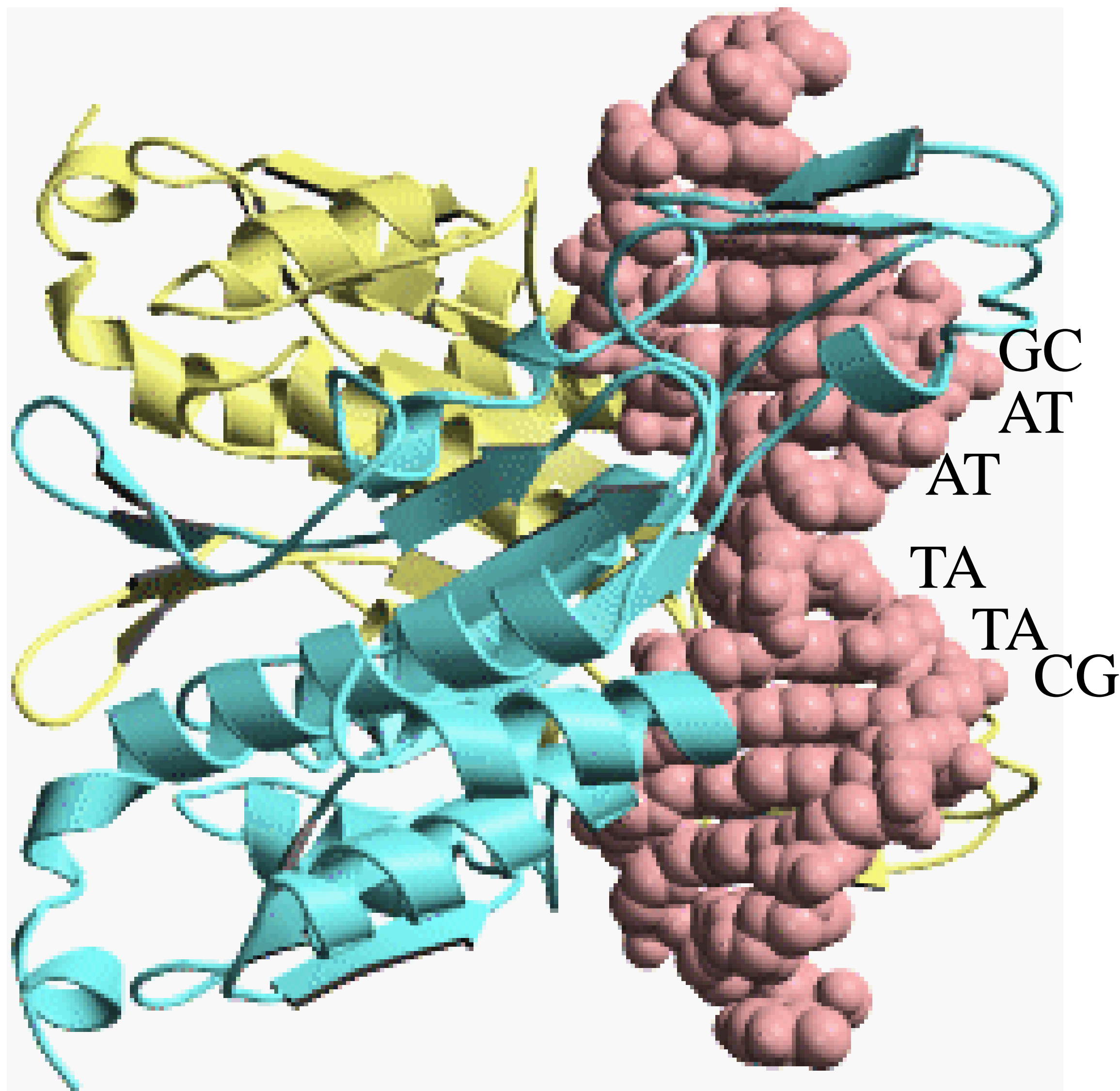
What would happen if there was a mismatch at the 5’ end of the primer? ...the 3’ end of the primer?

What would happen if the annealing temperature was too low?

Additional Concepts:
Gradient PCR, Touch Down, Hot start

Restriction Enzymes

-where they come from
-what they do
-how cells protect themselves
-how to use them



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

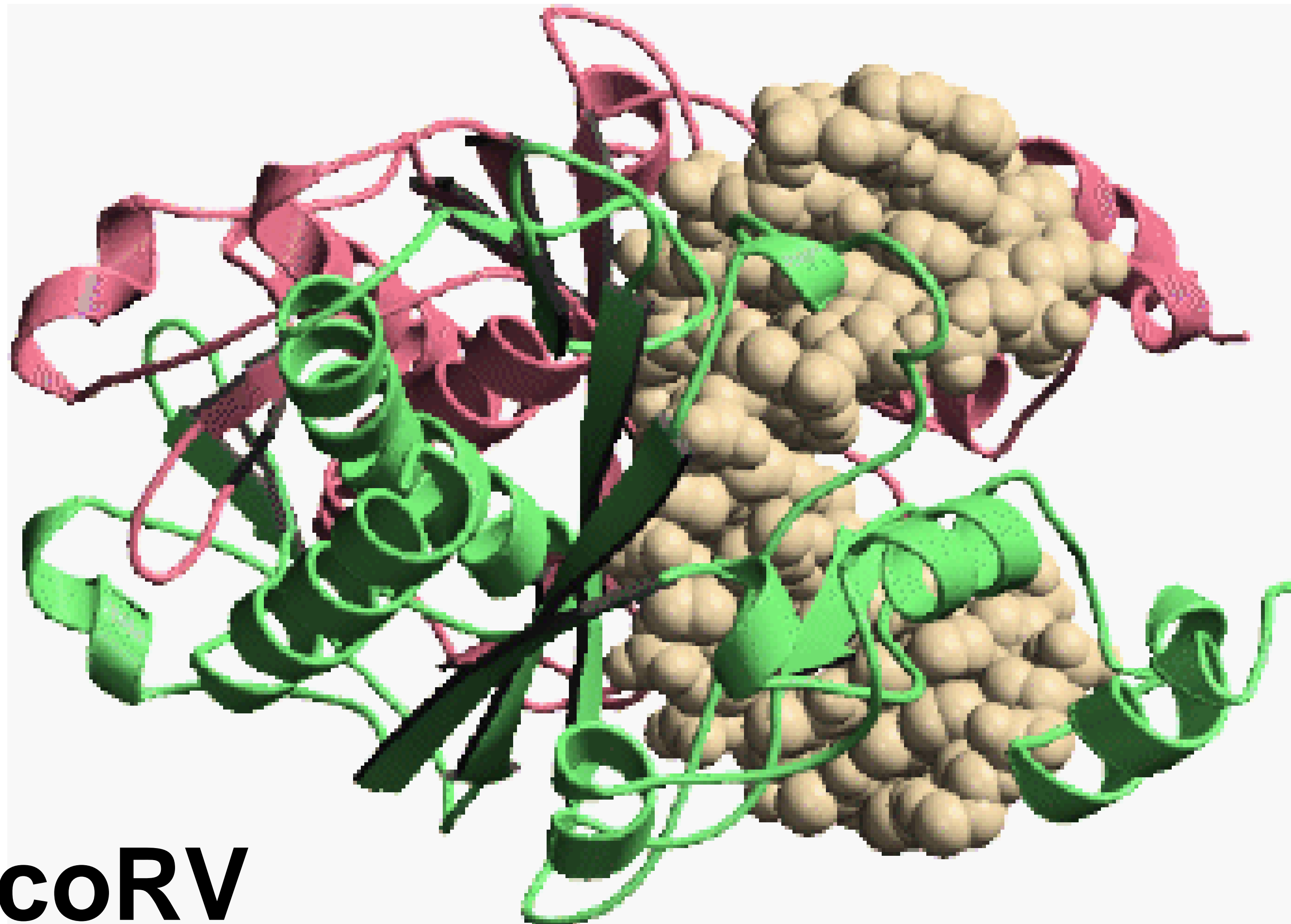
EcoRI

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

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

EcoRI

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



EcoRV

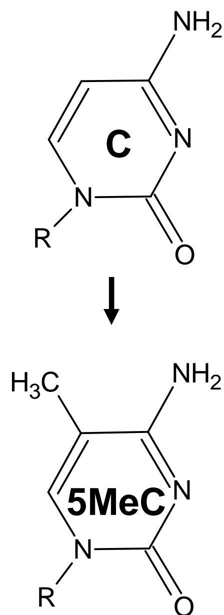
Structure from: Winkler *et al.*, EMBO J., 12, 1781-1795 (1993)

How do bugs keep from chopping themselves up?

“Cognate Methyltransferases”

M.HaeIII

5′-GG**C**C-3′
3′-C**C**GG-5′



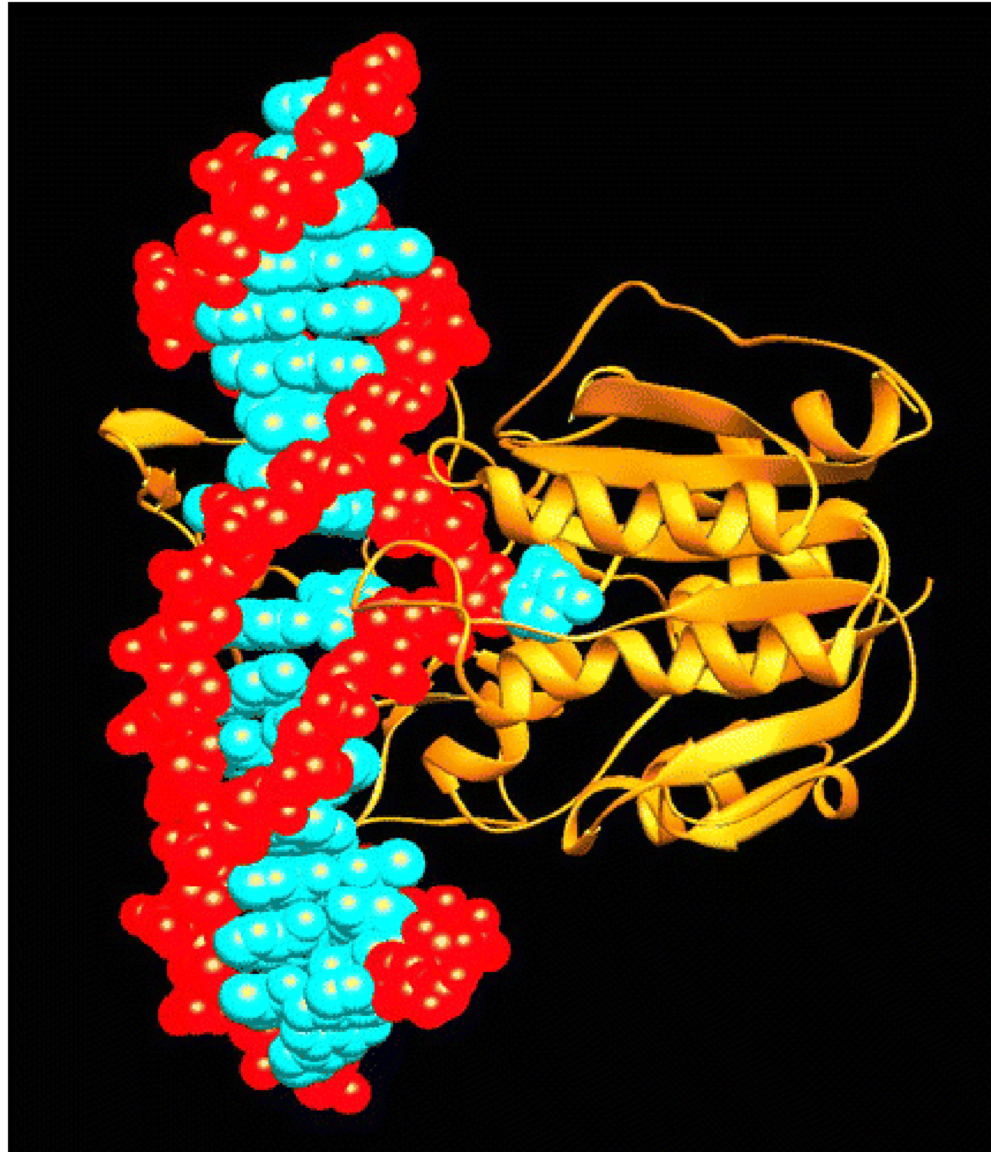
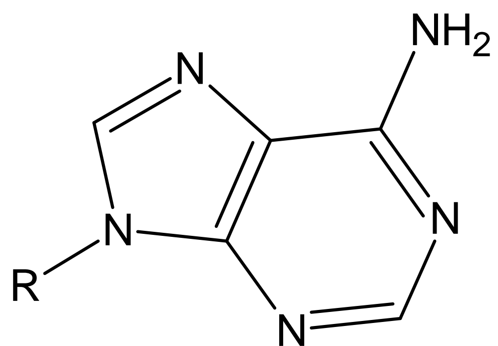
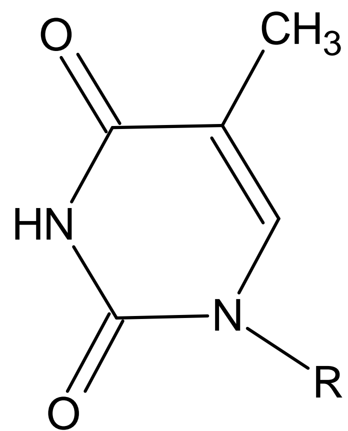
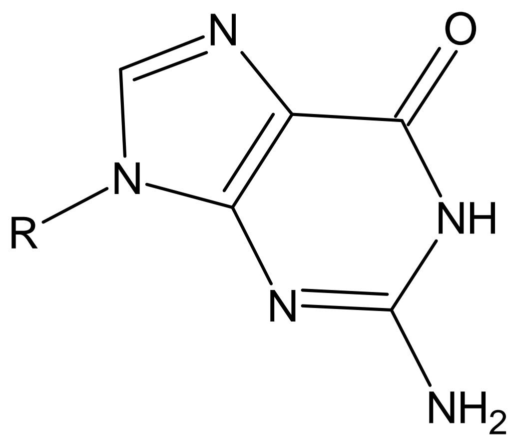
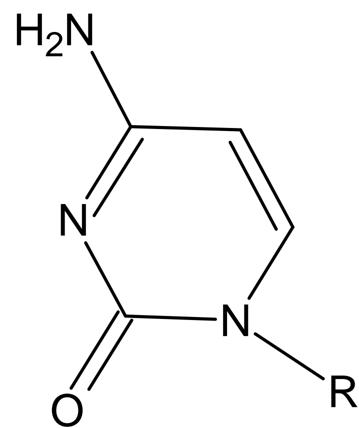
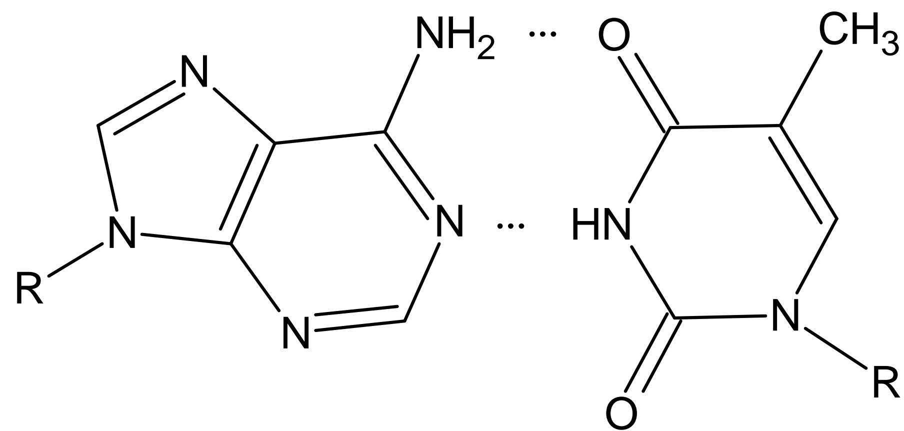
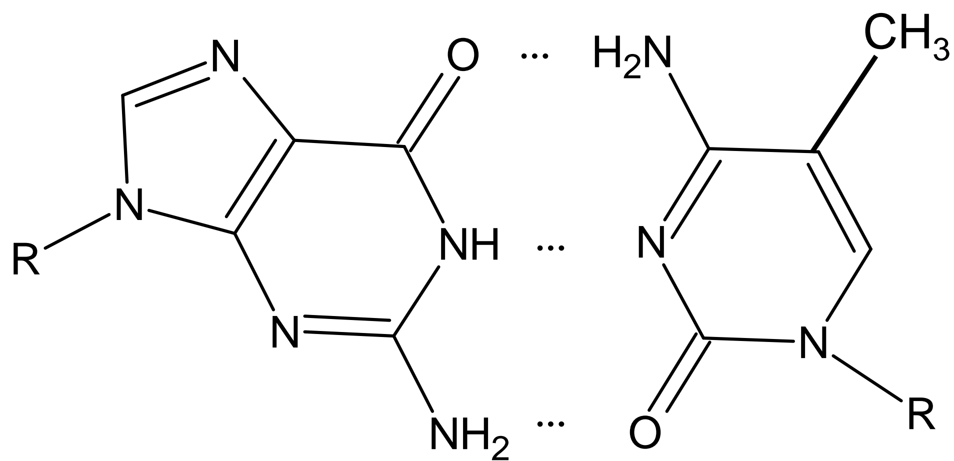


Figure from R. Roberts, Annual Review of Biochemistry (1998) 67: 181-198.

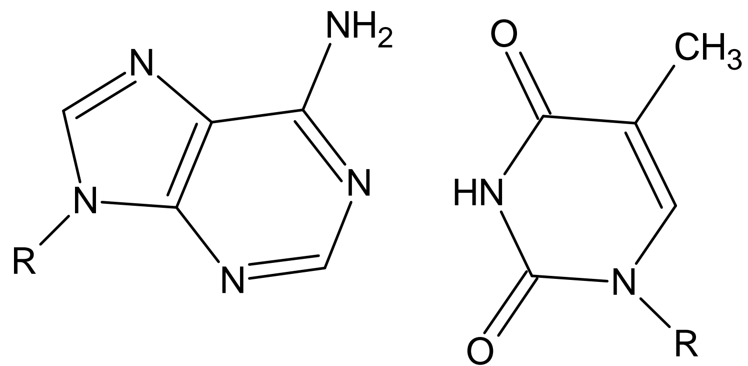

NH2
N
N
N
N
R

O
CH3
HN
N
R
O

N
O
N
NH
R
N
NH2

H2N
N
N
R
O

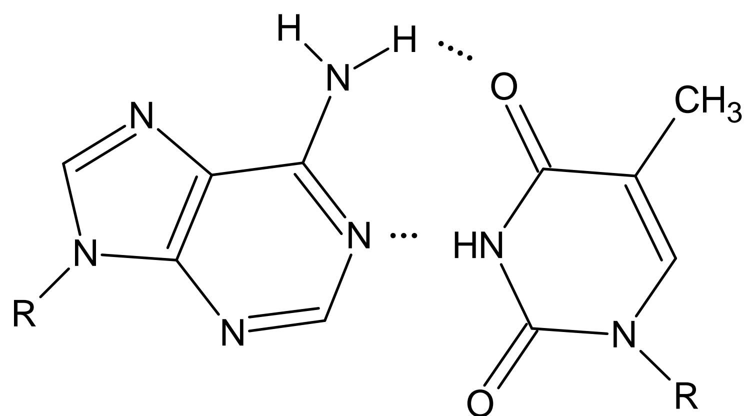




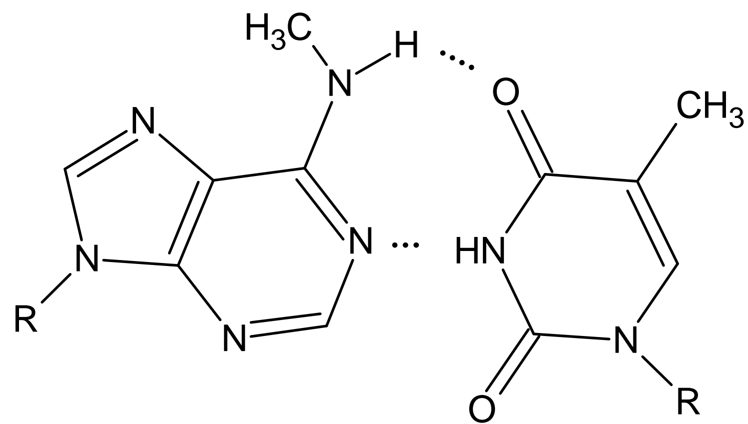
5MeC

Normal A:T Base Pairs





6MeA



On a practical level…
Using Restriction Enzymes

-Different lengths of recognition sequences
-Different kinds of restriction enzymes (blunt/OH/distal)
-Shared recognition sequences
-Shared overhangs

-Buffer conditions
-Enzyme compatibility
-Storing and diluting your restriction enzymes
-Reaction conditions (time & temp)
-Specificity (potential pitfalls!)
-Lack of activity (host cells & potential pitfalls)

Get to know your tool box!

Anticipating Problems & Pitfalls:

What might go wrong in your experiment?

Incomplete Reactions

Controls: How can you tell if your DNA has actually been cut?

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