MOD1 – DNA ENGINEERING

Engelward, Spring 2008

Day 3

About the experiments in Mod1

-how is recombination used to fix double strand breaks

-how your two-plasmid assay works

Agarose Gels – How do we 'look' at DNA?

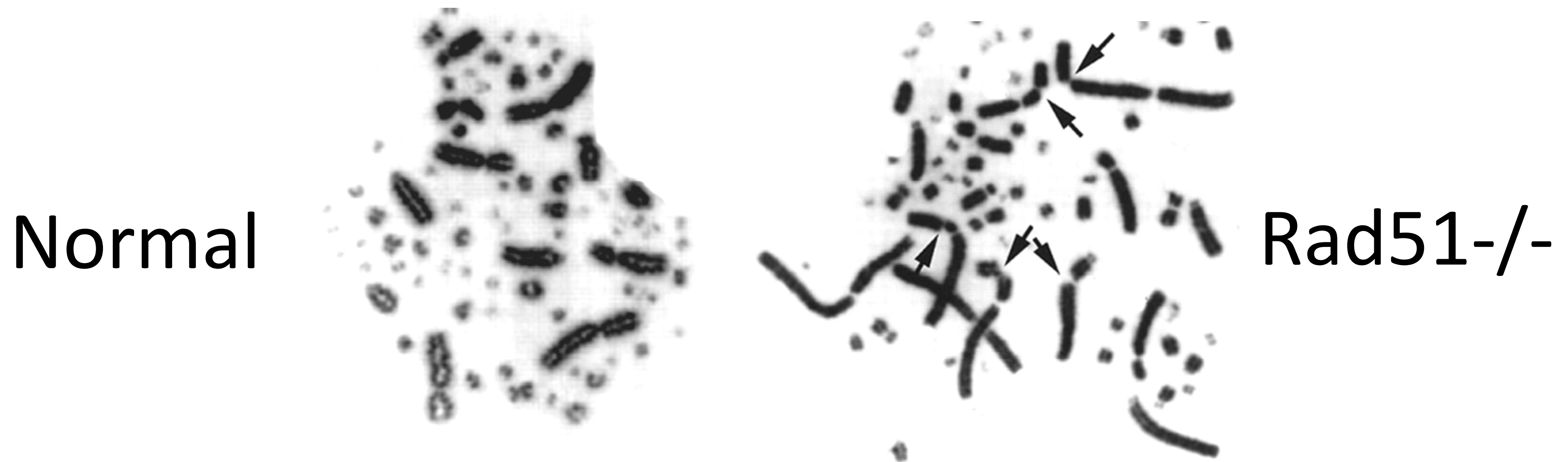
Anticipating Potential Problems & Pitfalls

Background & Significance:

“Homology-Directed Repair” for double strand breaks

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

Why you owe Your Life to Homologous Recombination…

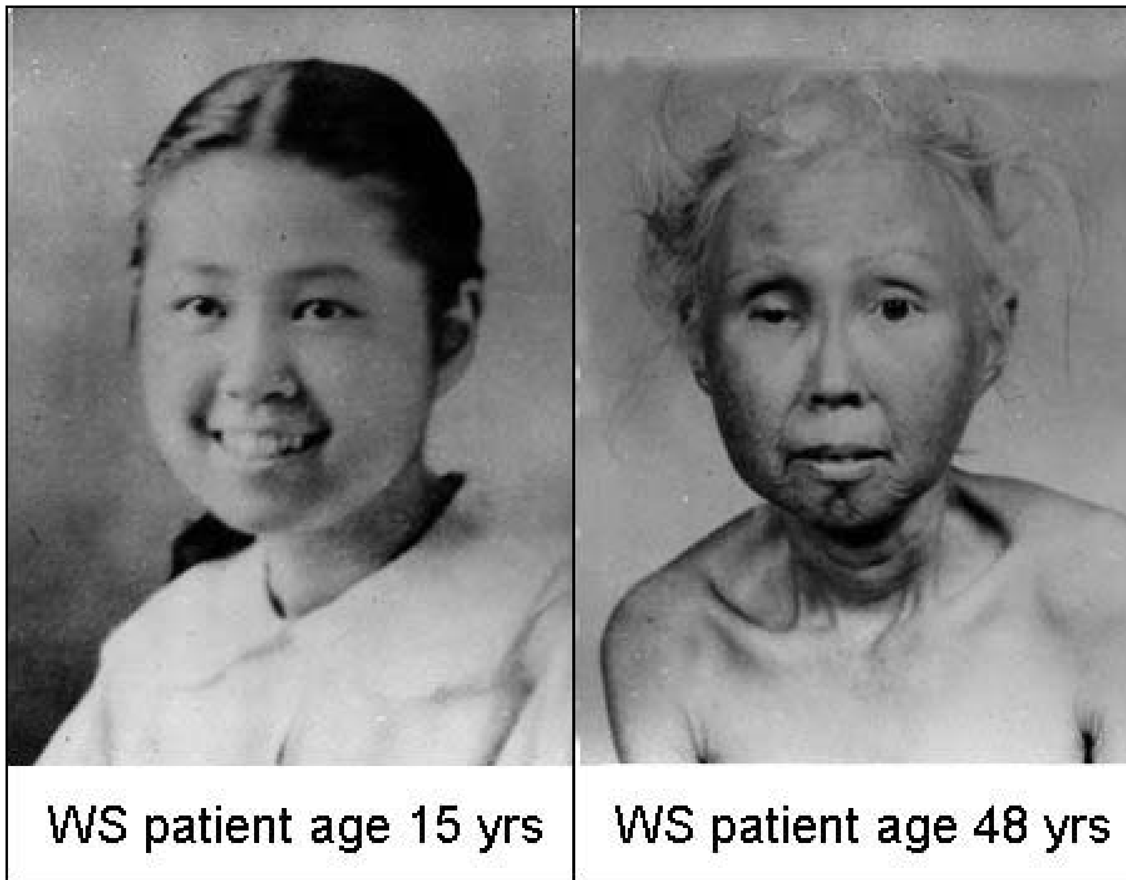


Turn Off Homologous Recombination
→ Chromosomes Fall Apart

Sonada *et al*., *EMBO J.* **17,** 598–608 (1998).

Why you owe Your Youthfulness to Homologous Recombination…

Loss of Helicase → Faulty Recomb.

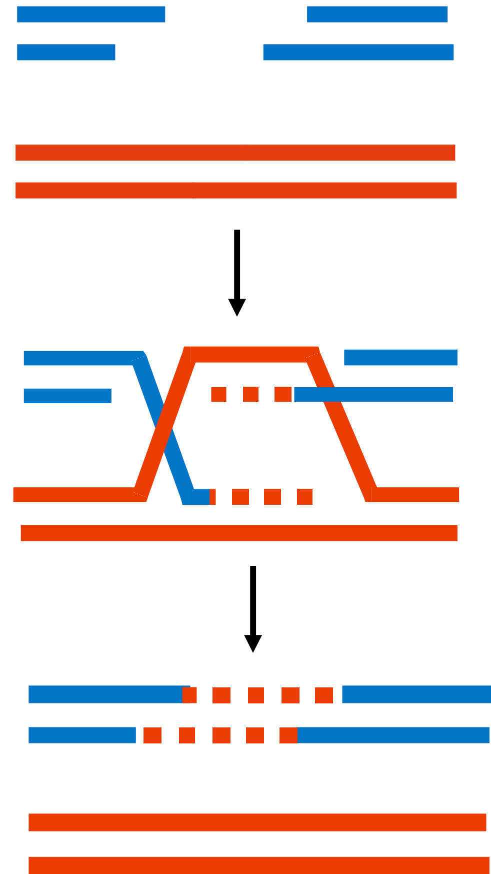


Werner's Syndrome

Double Strand Breaks

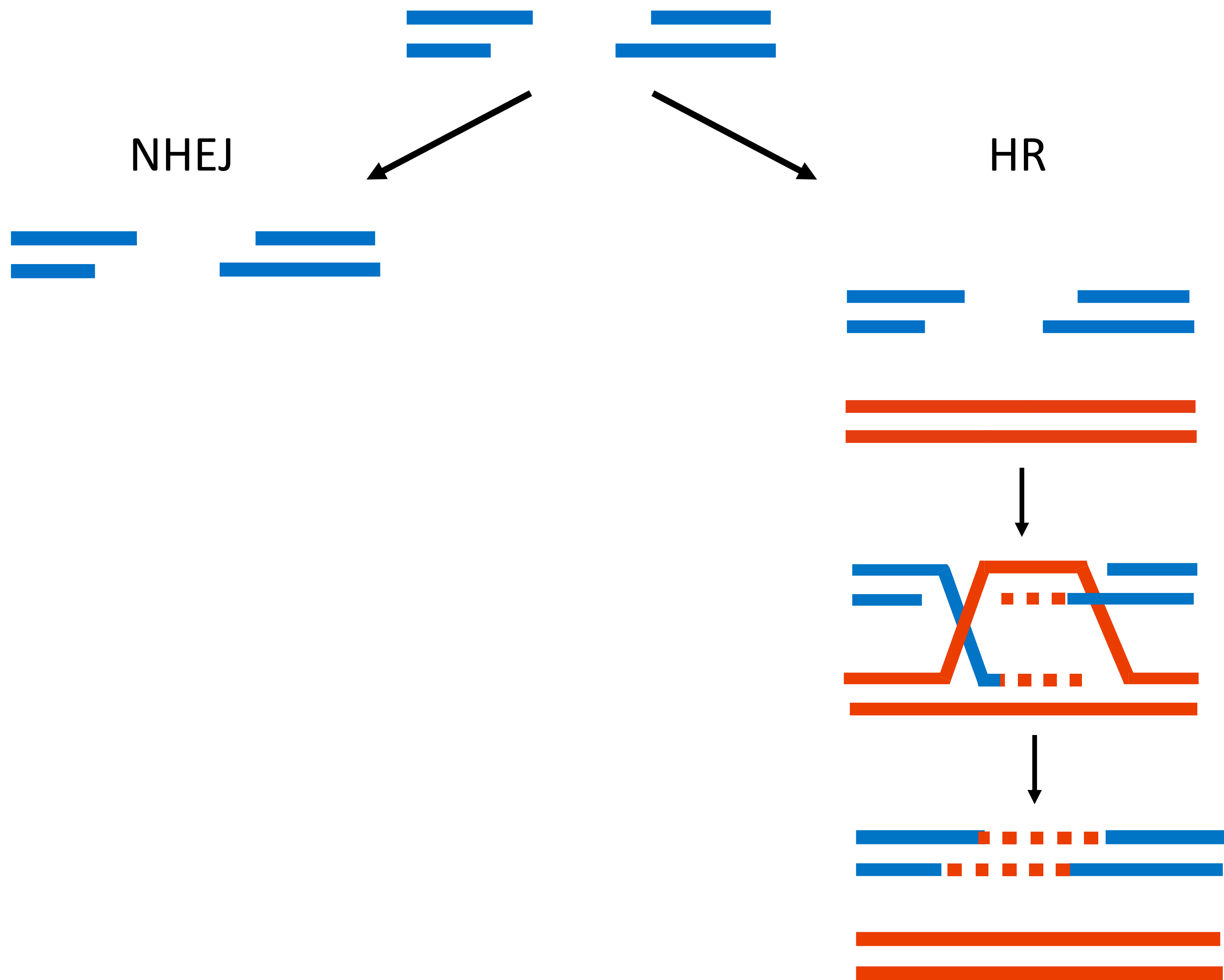
NHEJ

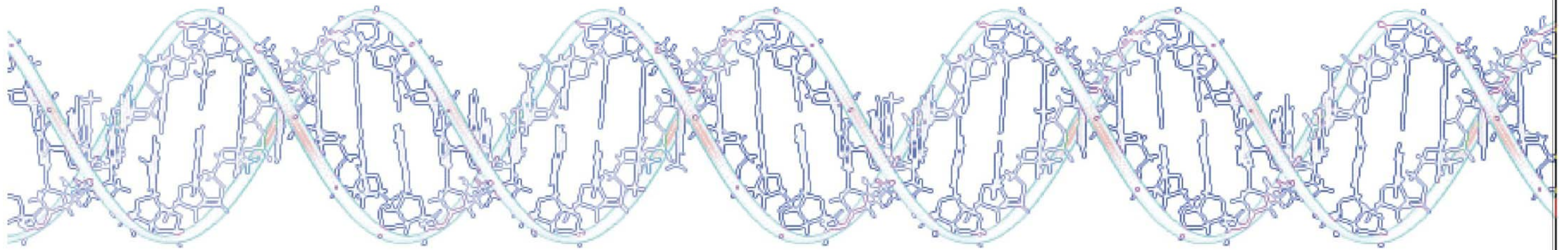
HR



KU heterodimer
(Ku70&80)
DNA-PKcs
DNA ligase IV
XRCC4

See NHEJ Animation by Justin Lo


Double Strand Breaks
NHEJ
HR



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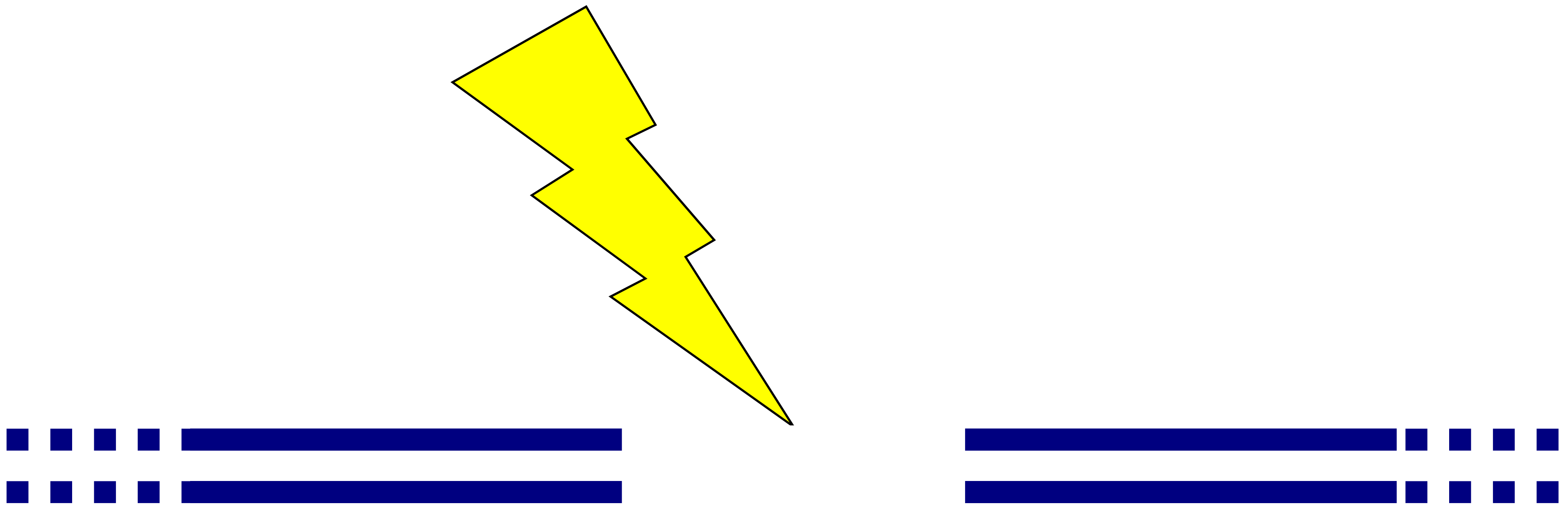
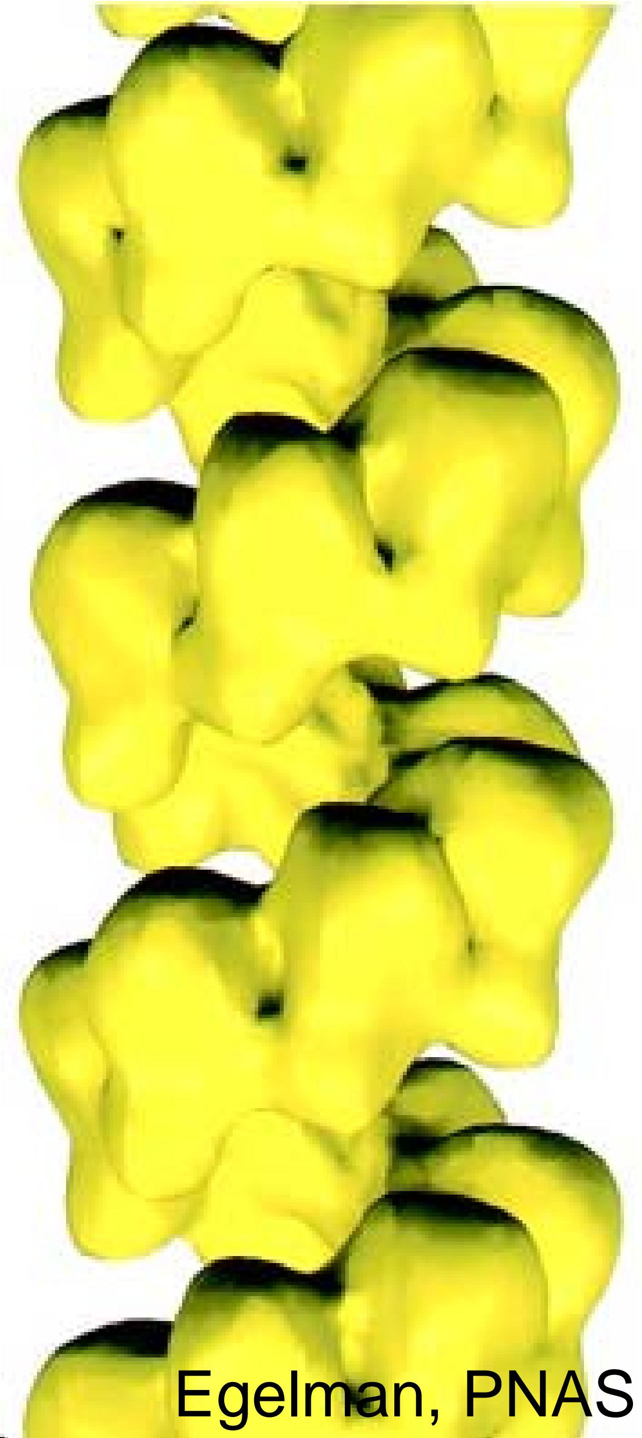


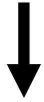
Imagine HR is initiated by the fragment on the left….

Step 1: A double stranded end has been created

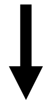
Step 2: Resect the end to create a 3’ overhang

Step 3: Create a nucleoprotein filament capable of homology searching

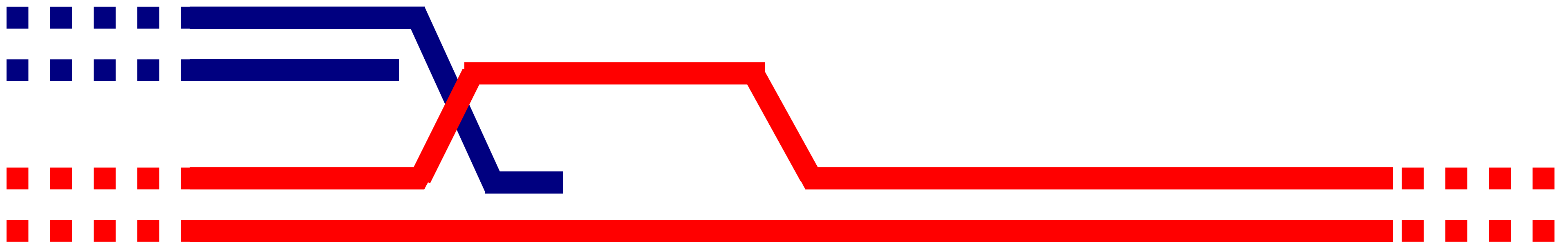




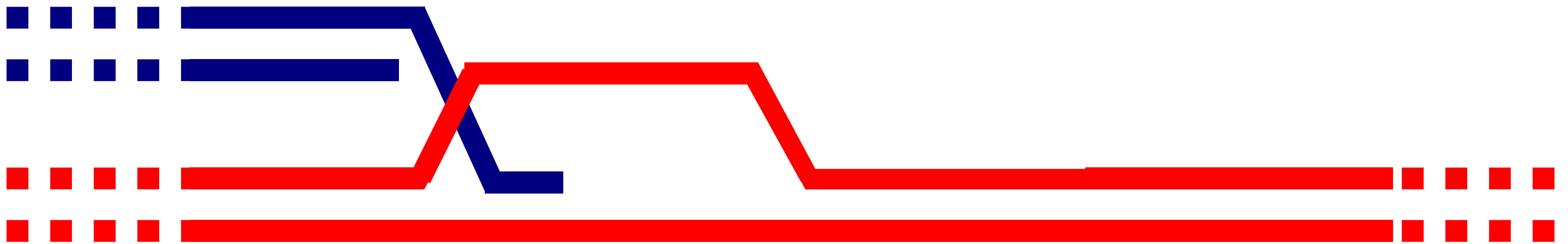
Step 4: Search and Invade



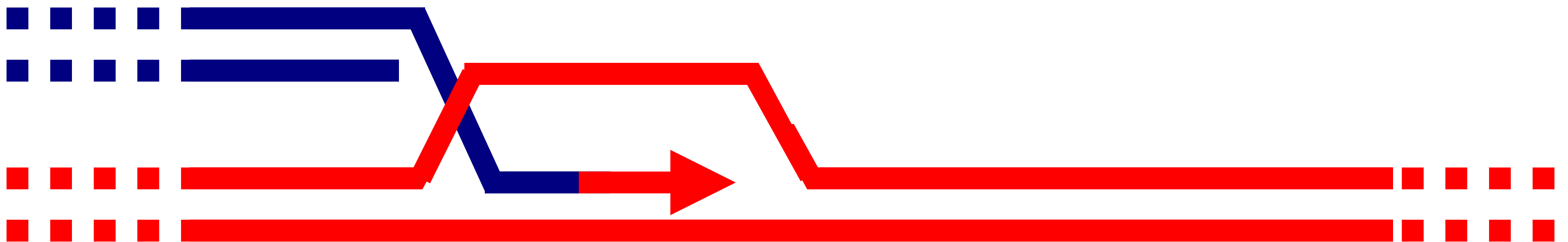
Step 4: Search and Invade

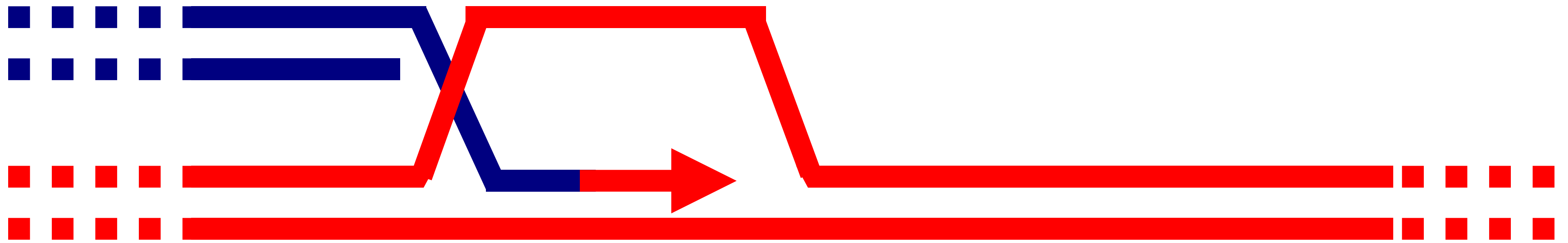


Step 4: Search and Invade

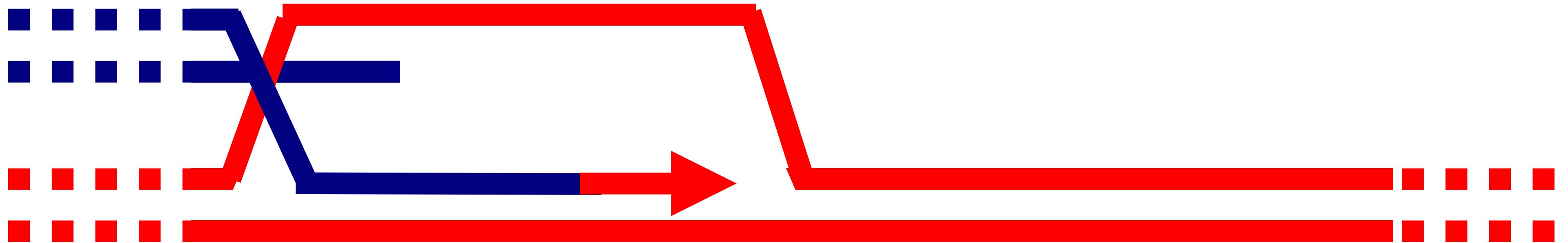


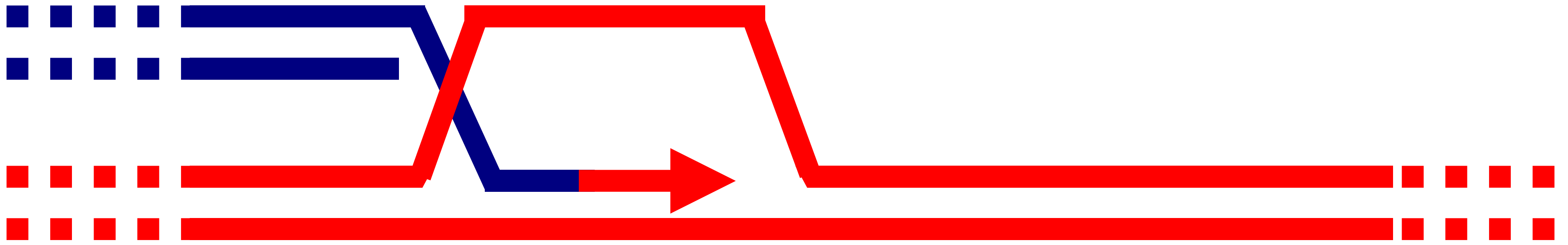
Step 5: Polymerize DNA using invading strand with 3’OH as a primer and the homologous donor DNA as a template



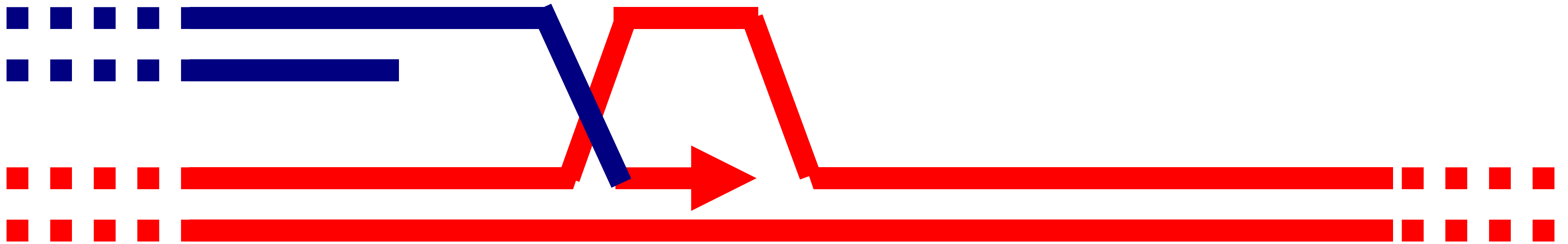


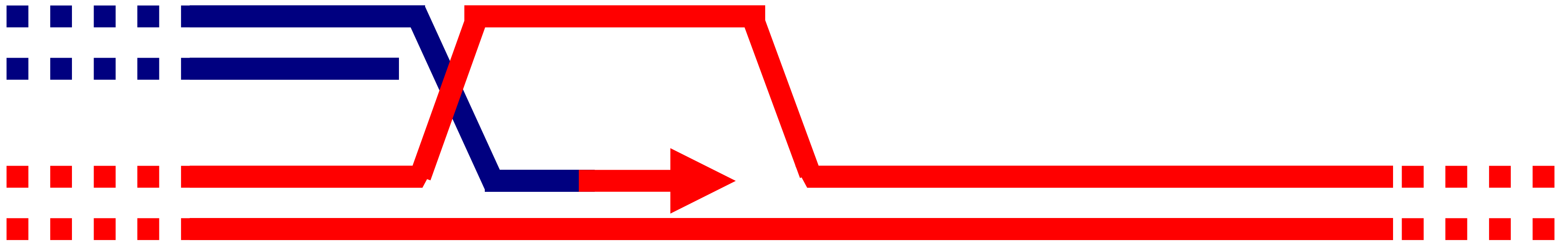
Step 6: Branch Migration (Backwards)



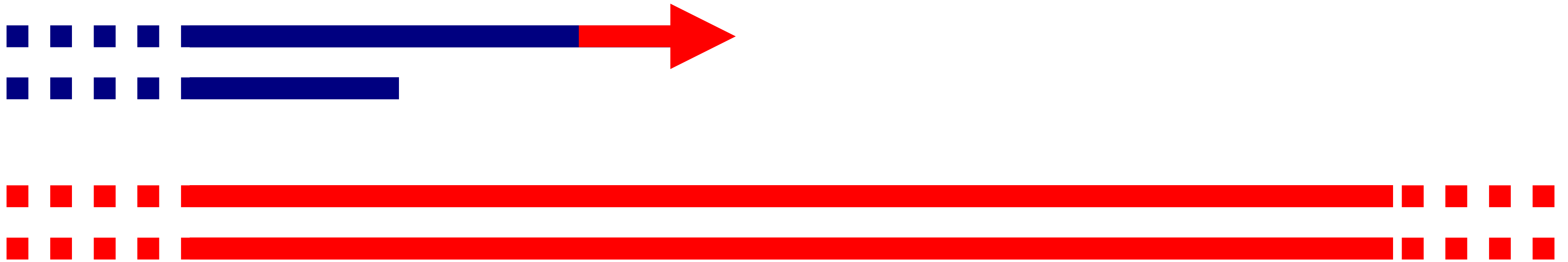


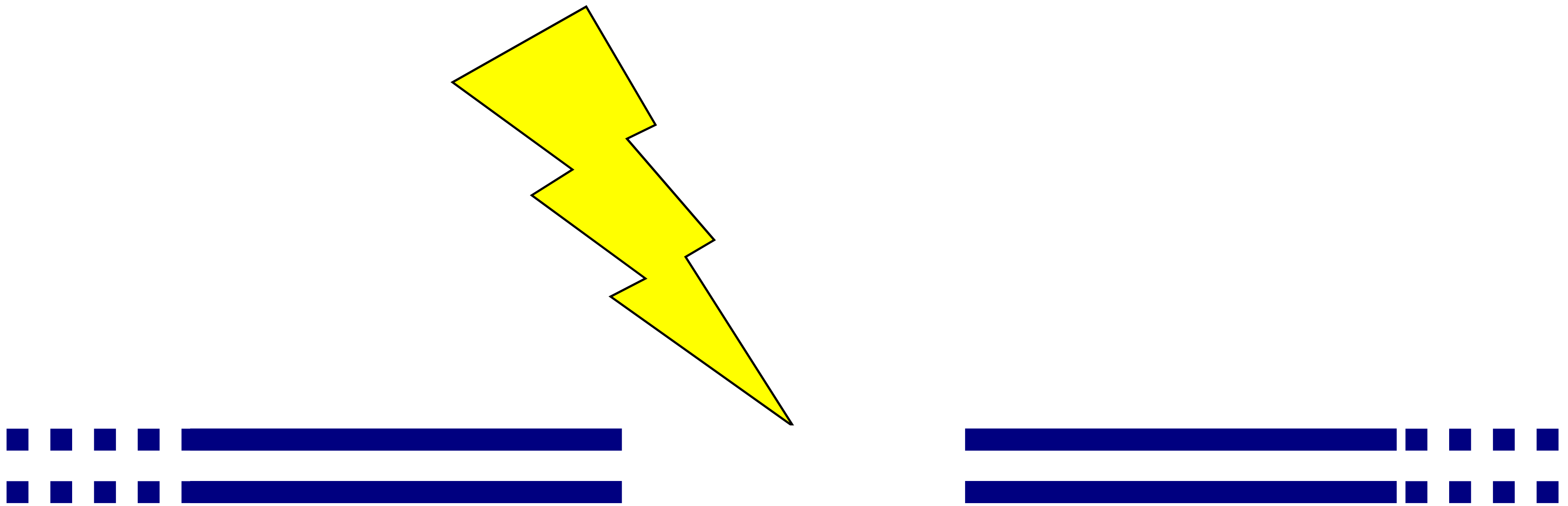
Step 6: Branch Migration (Forwards)





Step 6: Possible Release



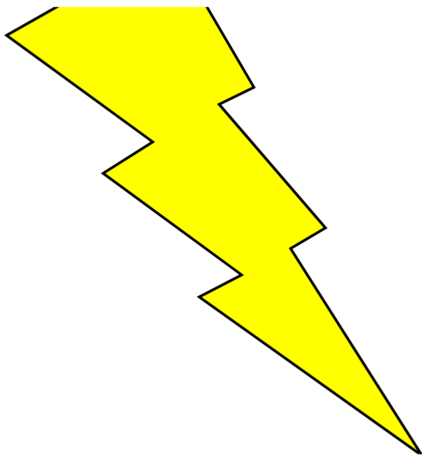


This process started with a two-ended DSB...

Now let's imagine the same thing happened at the other end…

Annealing

Final Steps: Filling, Trimming, Ligating



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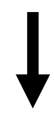
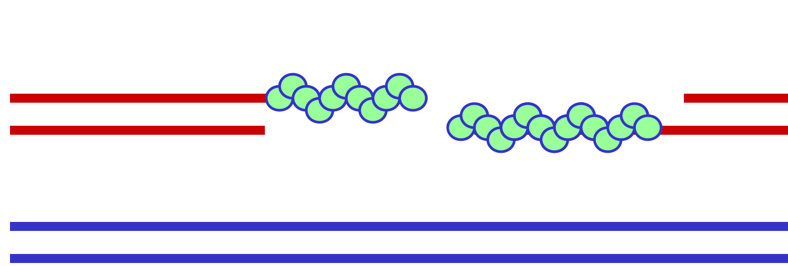


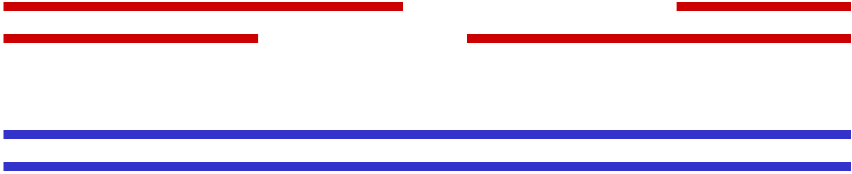
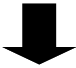
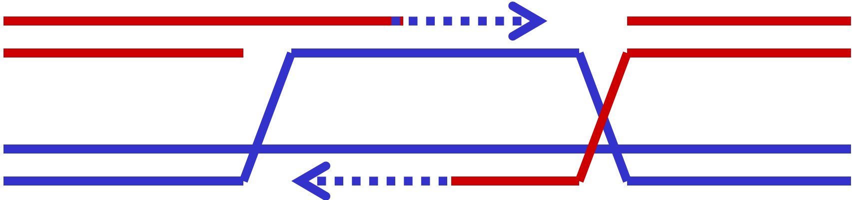
See SDSA Animation
by Justin Lo

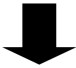
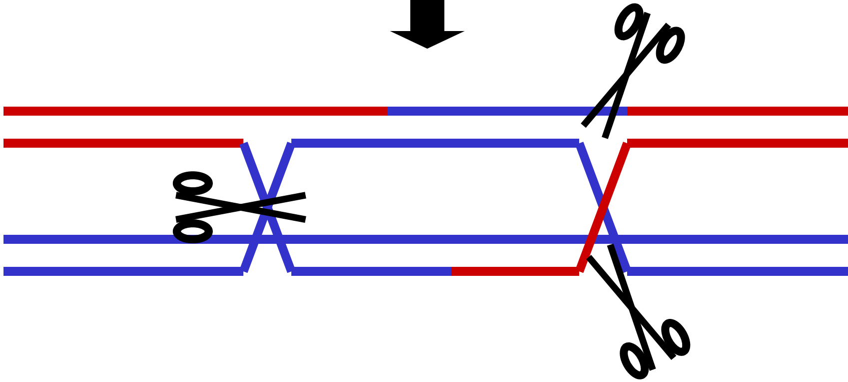


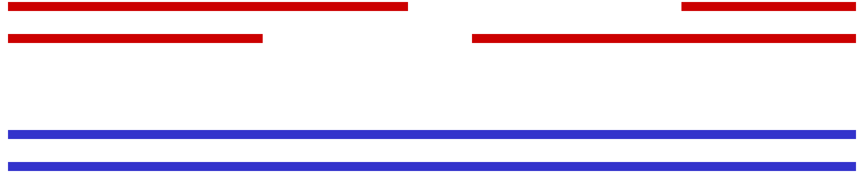
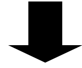
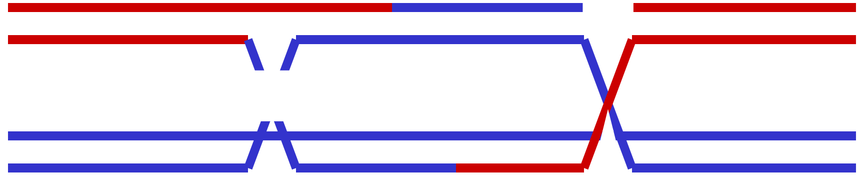
E. coli: RecA*

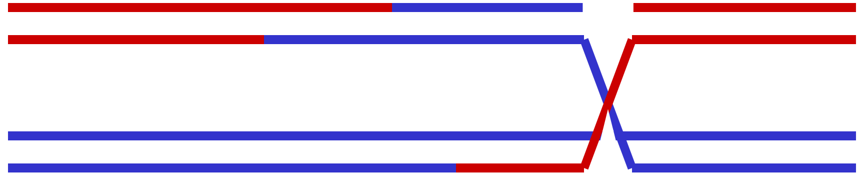
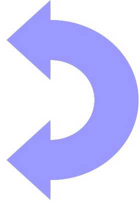
Yeast: Rad51

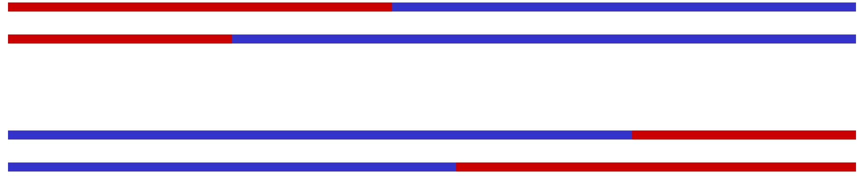
Mammals: RAD51

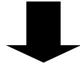




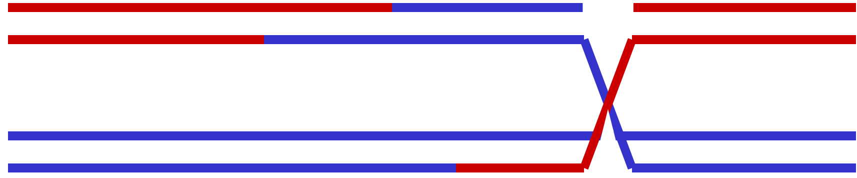



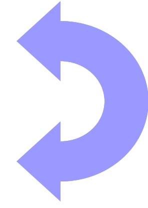




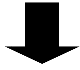



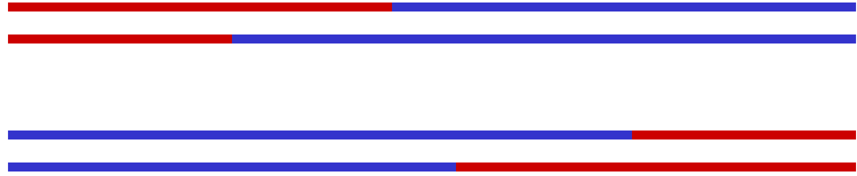






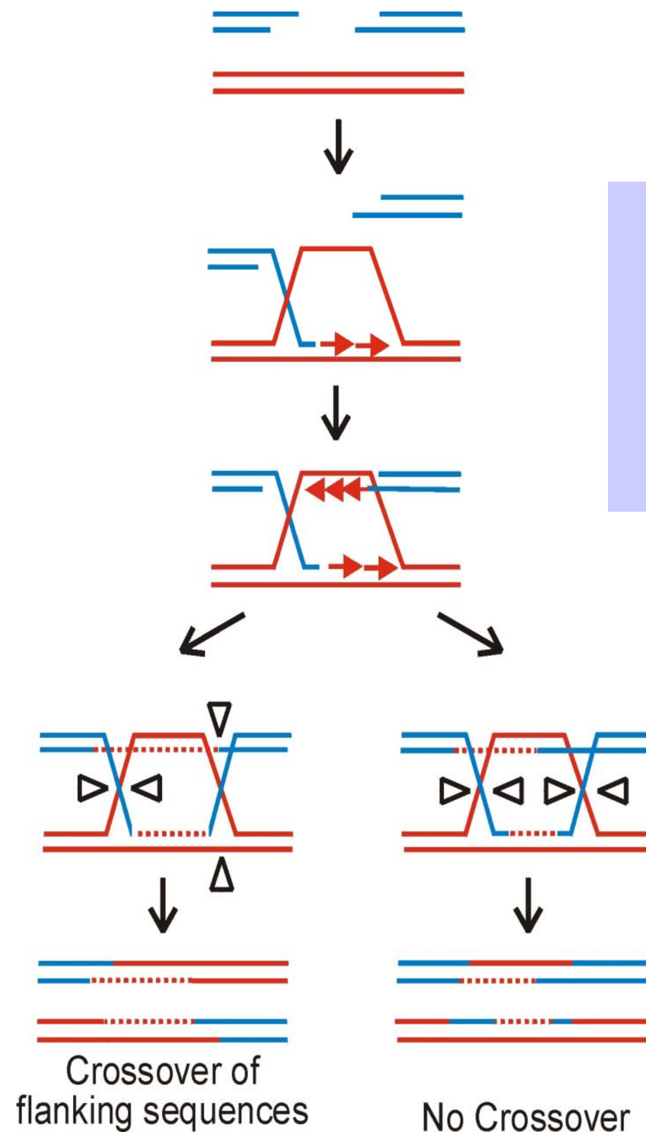






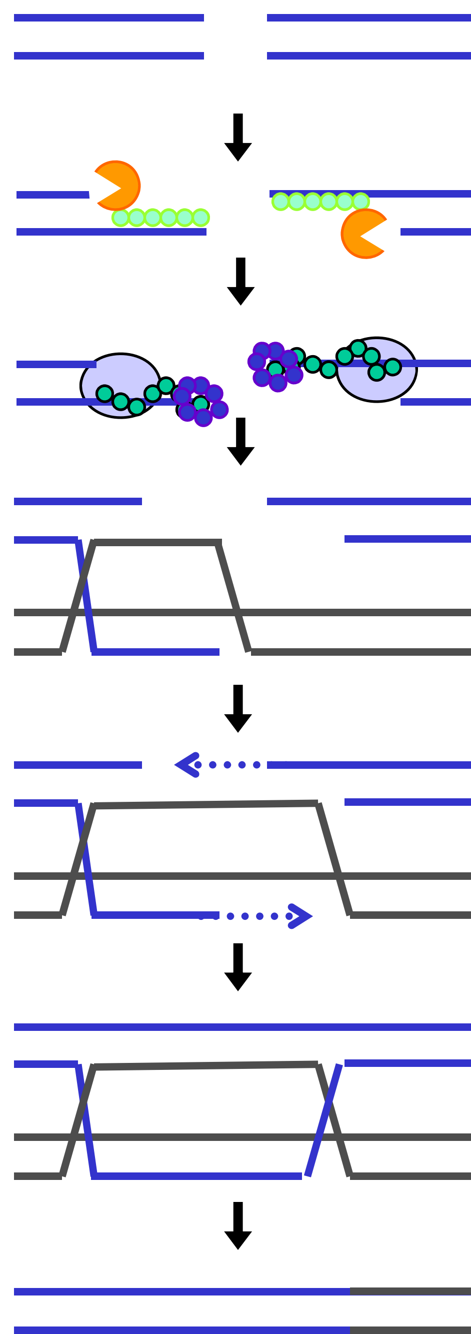
See Prototypic Model Animation
by Justin Lo

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

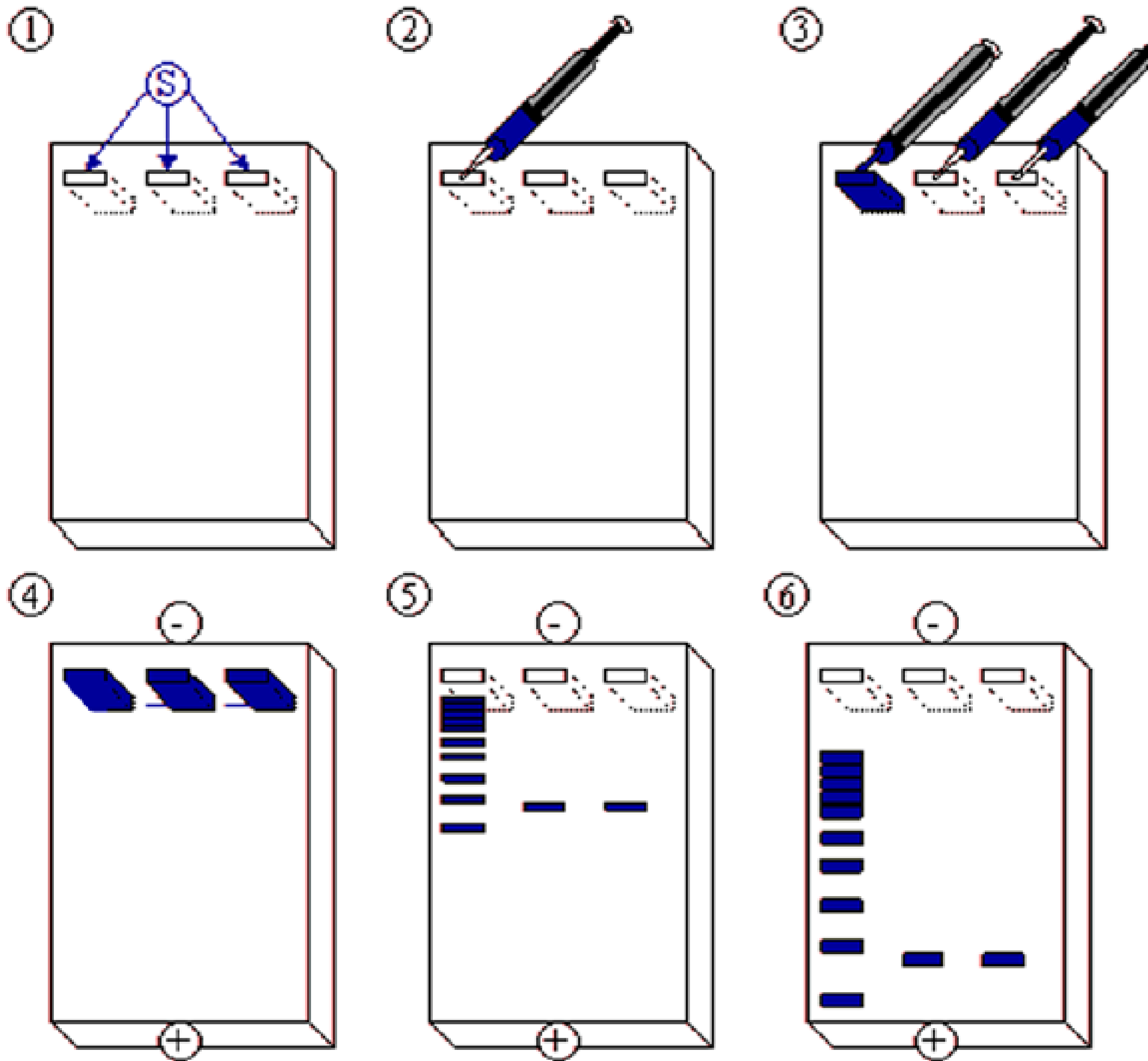
NOTE: BREAKPOINT TURNS FROM BLUE TO RED



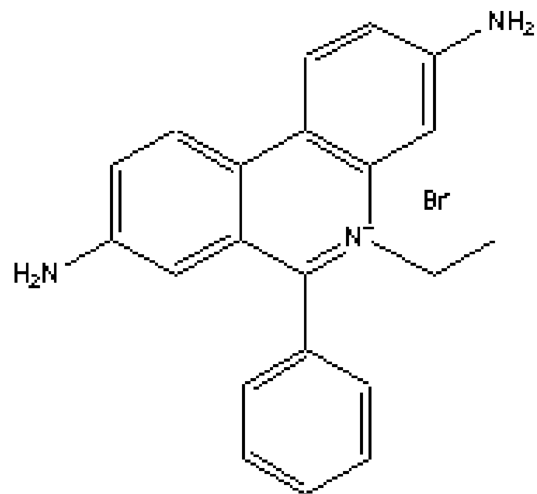
Step	Proteins
Decision to initiate HDR, resection of DNA ends	ATM, ATR, cAbl, Chk1, p53, BRCA1, Fanconi genes, Mre11, Rad50, Nbs1, Exonucleases
Displacement of RPA & Loading Rad51	RPA, Rad52, BRCA2, Rad51, Rad51B, Rad51C, Rad51D, XRCC2, XRCC3
Homology searching, histone remodeling, & invasion	Rad54, Rad54B, Rad52
Holliday junction migration, inhibition by mismatches	MMR, WRN, BLM, Rad54, p53
Repair synthesis, Holliday junction migration, Possible resolution without junction cleavage	Polymerase(s), topoisomerase(s) WRN, BLM, Rad54
Junction resolution Repair of mismatches	Rad51C & possible additional proteins; possible resolution by topoisomerases; MMR

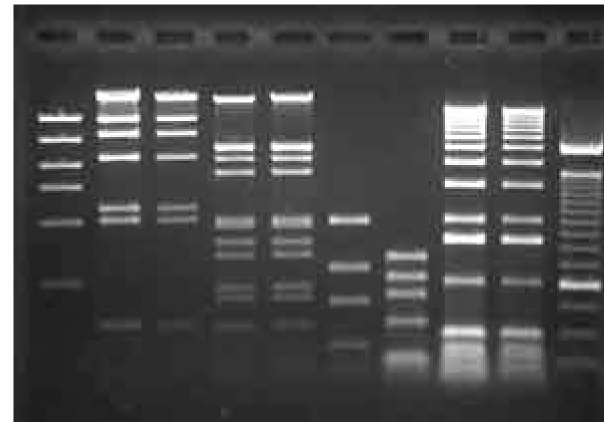
Agarose Gels & Gel Purification

- How do we ‘look’ at DNA?
- How do we get our DNA out of a gel?

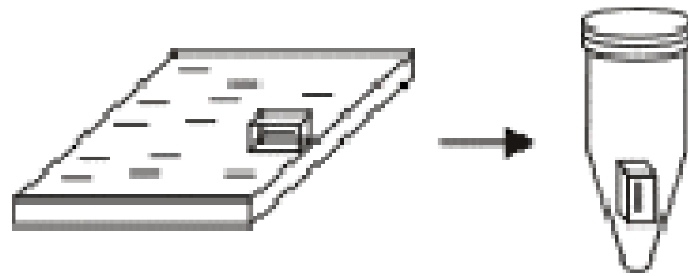


How can you see your DNA?


NH$_2$
Br$^-$
N$^+$
H$_2$N

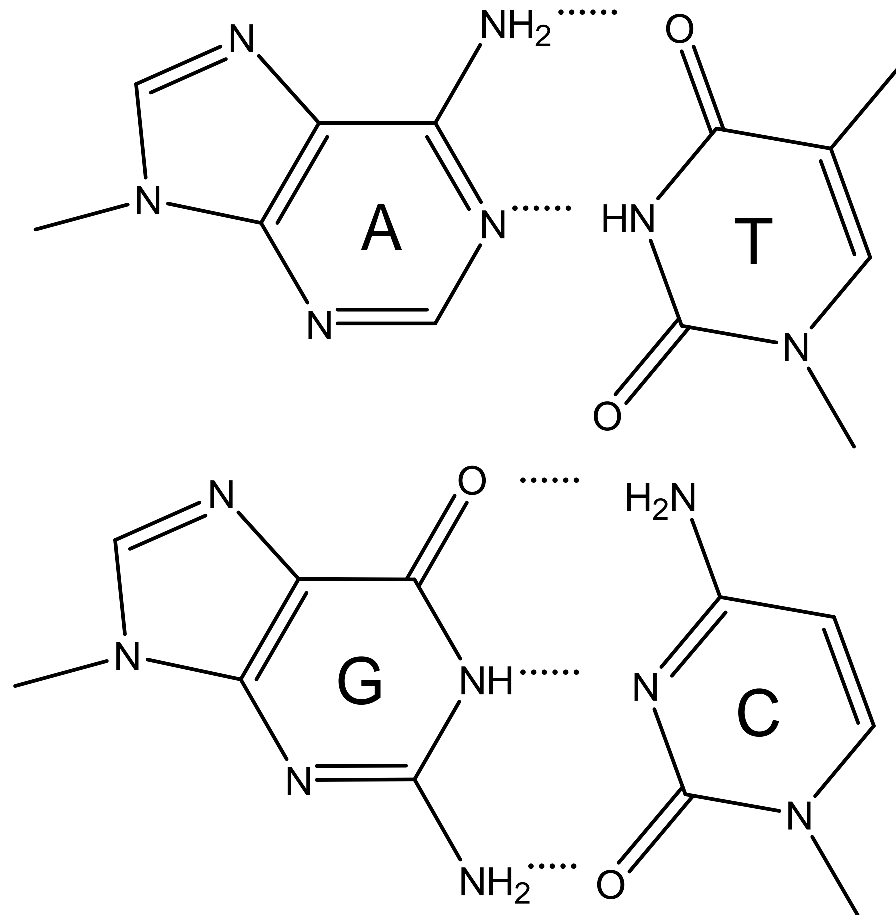


Gel Purification



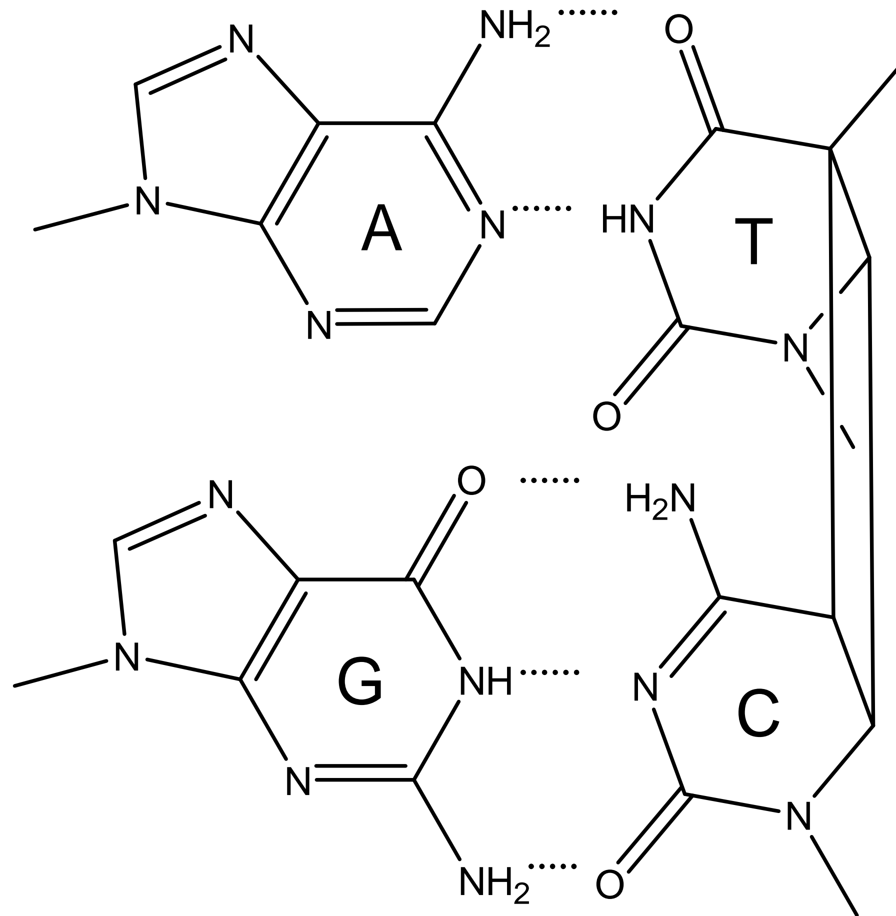
Why do you need to cut out your band fairly quickly?

Sunlight Damages DNA



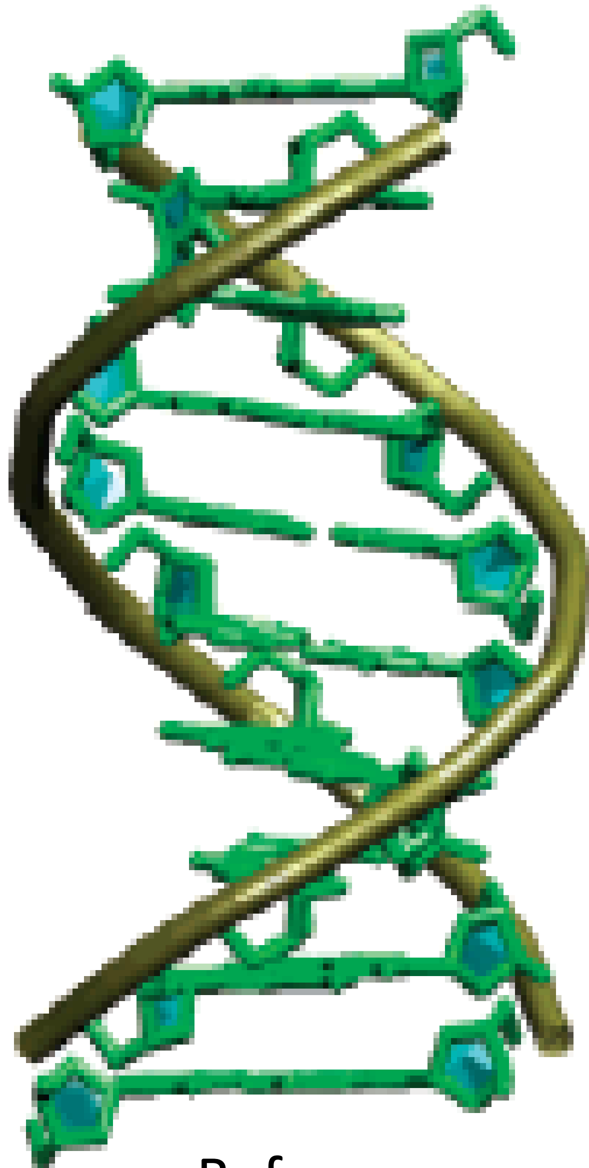
Normal Base Pairs

Sunlight Damages DNA

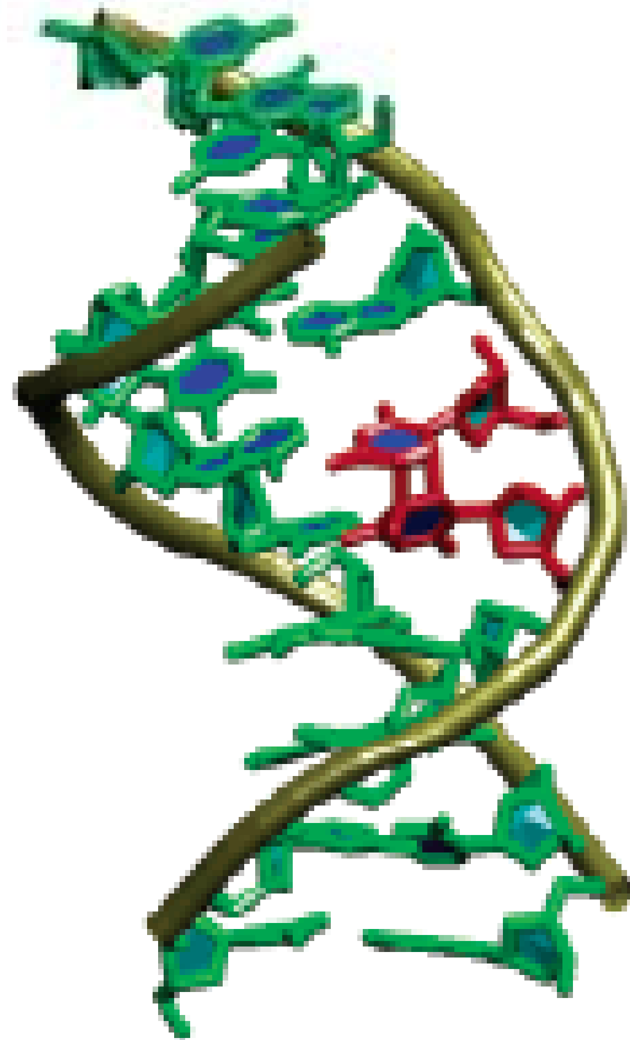


Sunlight-Induces Crosslinks Between Bases

Sunlight Damages DNA

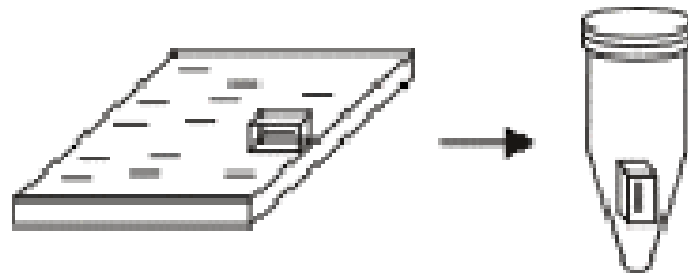


Before



After

Gel Purification

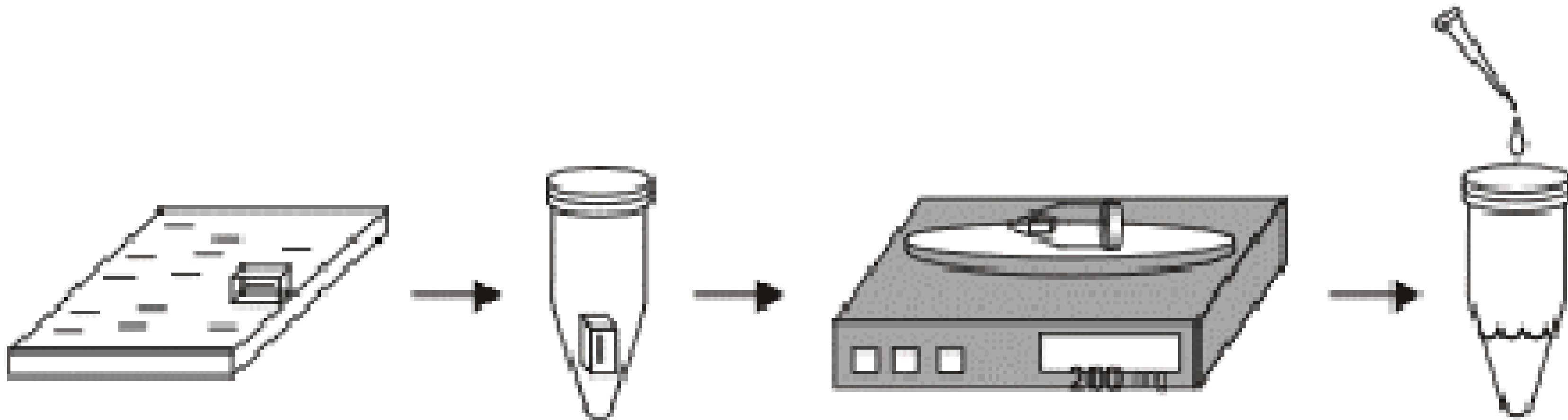


Why do you need to cut out your band fairly quickly?

You will need to dissolve the gel to get the DNA out.. You do this by adding 3 volumes of a gel-dissolving solution.

What does it mean to ‘add 3 volumes’?

How can you estimate the volume of your gel slice?



200 mg

Agarose Gels – How do we 'look' at DNA?

-Loading

-Standards

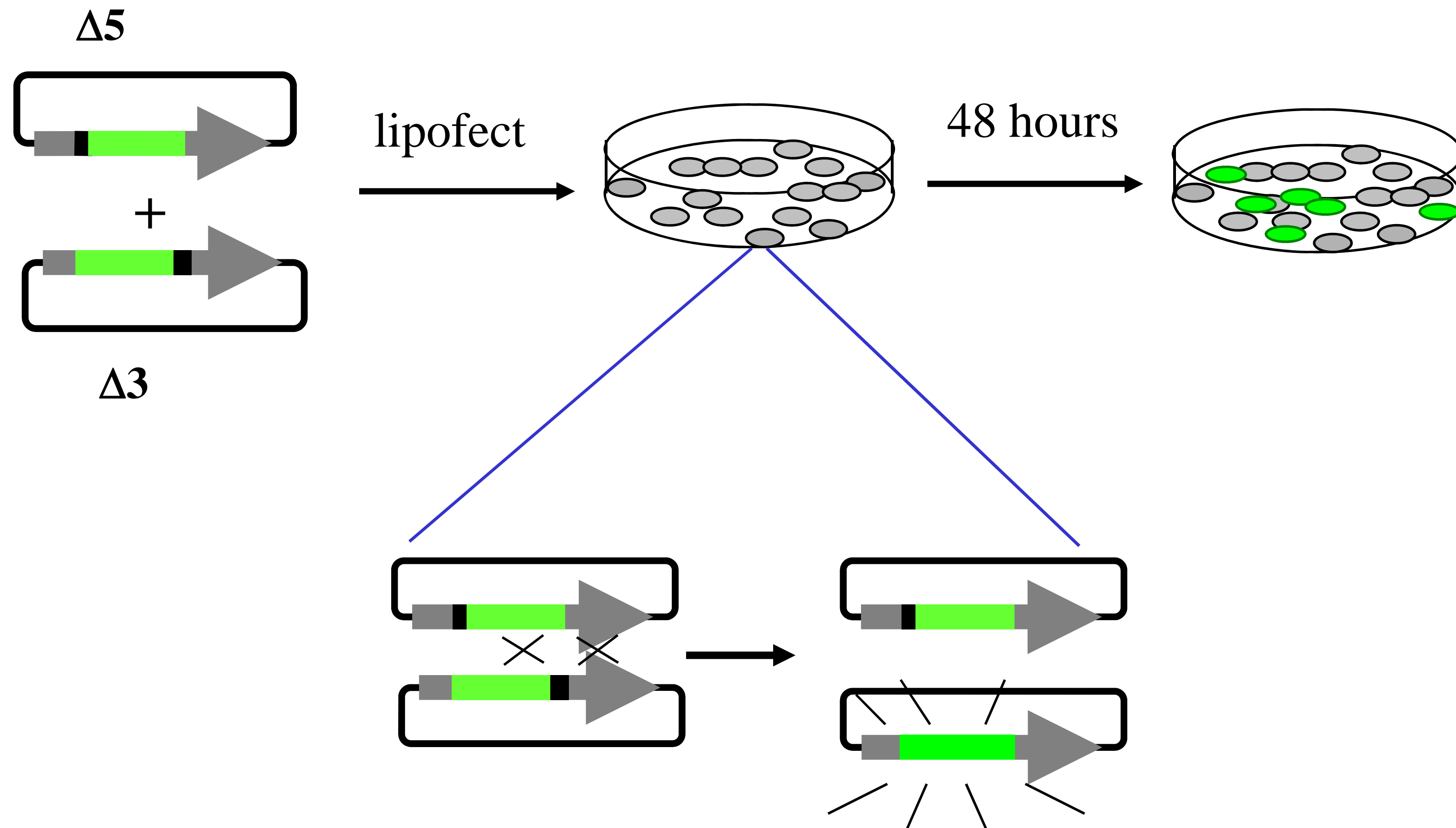
-Parameters that affect migration

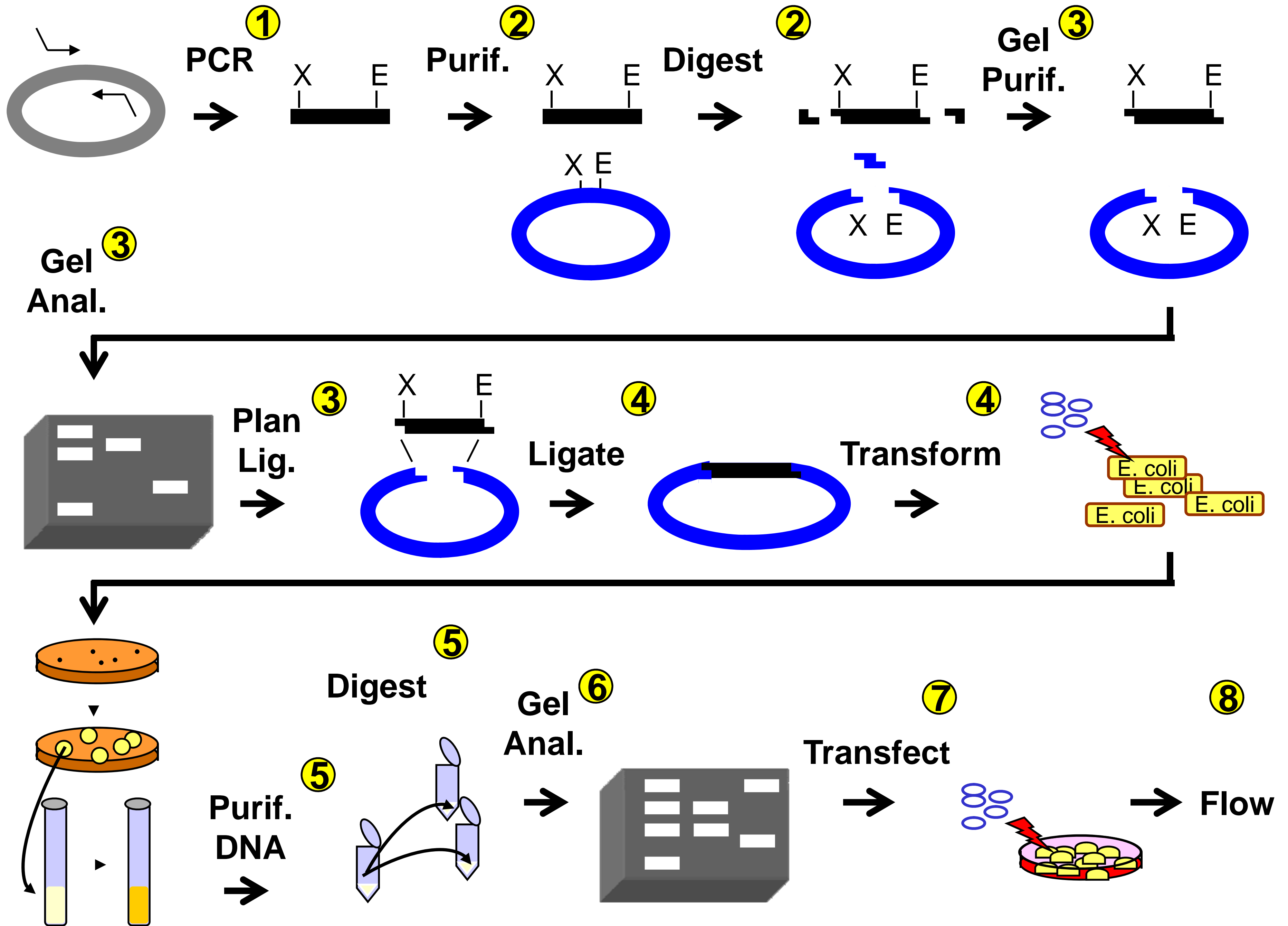
- -gel concentration
- -length of DNA
- -tertiary structure
- -effects of overloading

Overview of the Experiments in Mod1

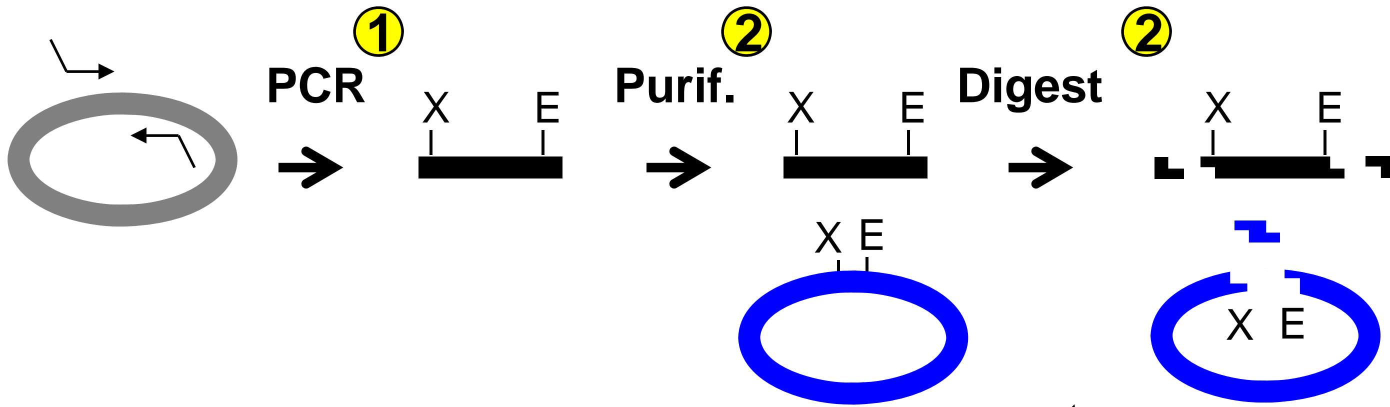
Where you are,
and where you are going

A Plasmid-Based Assay for Homologous Recombination in Mammalian Cells

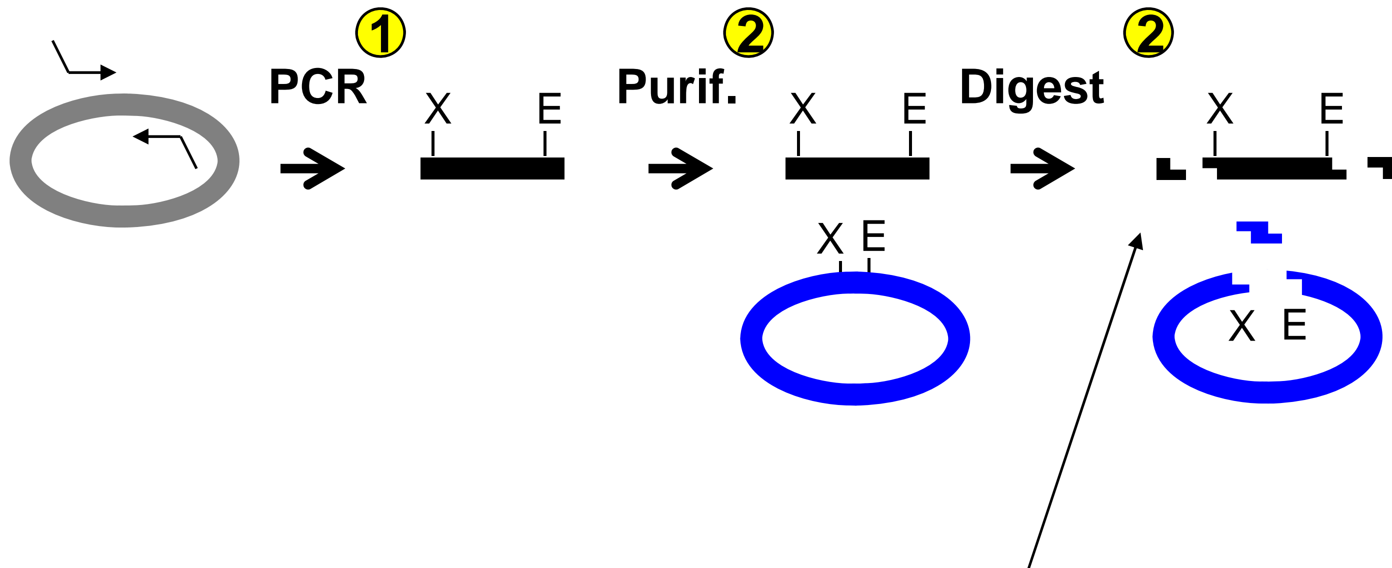




1
PCR
X
E
2
Purif.
X
E
2
Digest
X
E
3
Gel
Purif.
X
E
X E
X E
X E
Gel
Anal.
3
Plan
Lig.
3
X
E
Ligate
4
Transform
4
E. coli
E. coli
E. coli
E. coli
Purif.
DNA
5
Digest
5
Gel
Anal.
6
Transfect
7
Flow
8

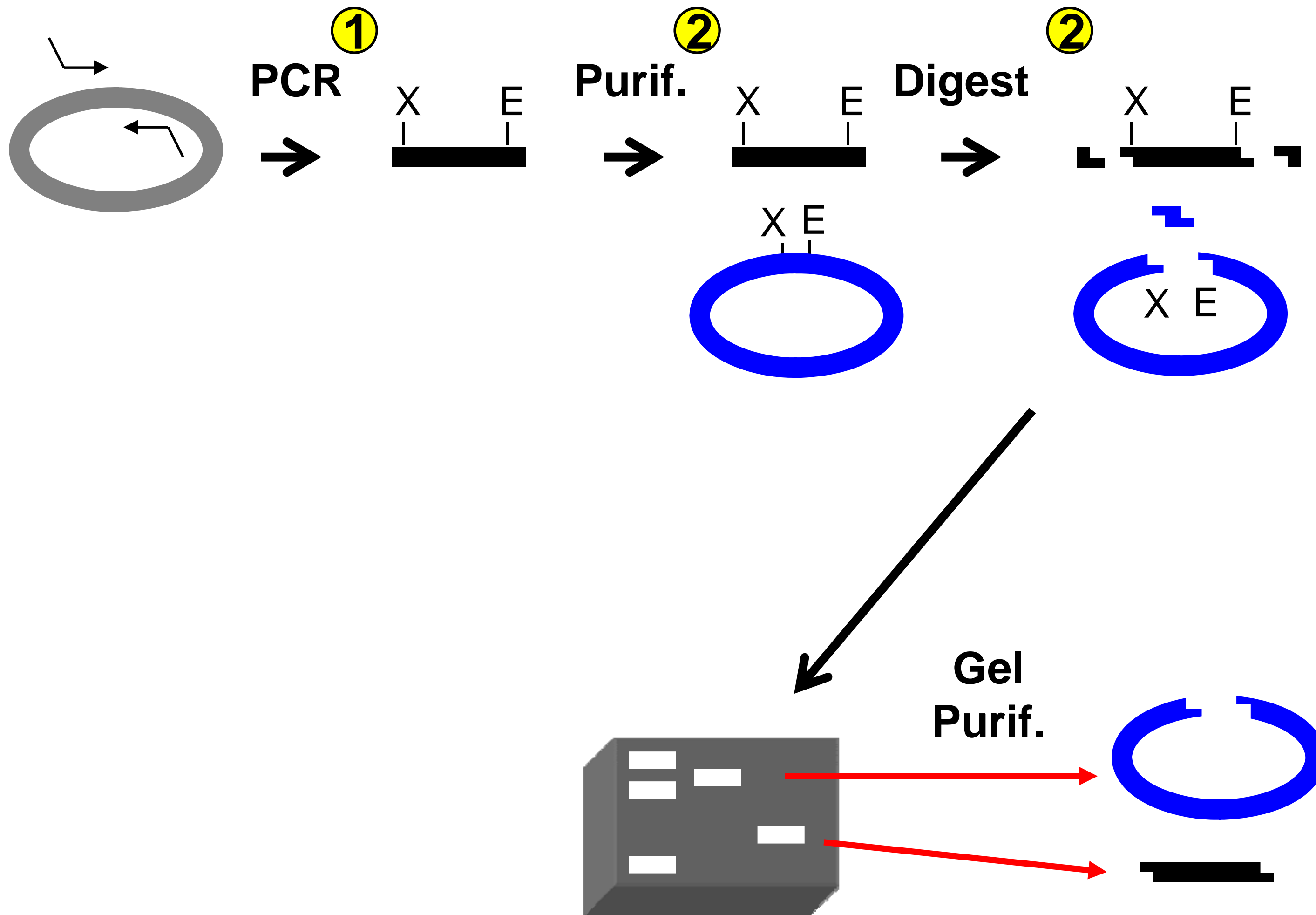


How do you know that your restriction enzymes actually cut the DNA?

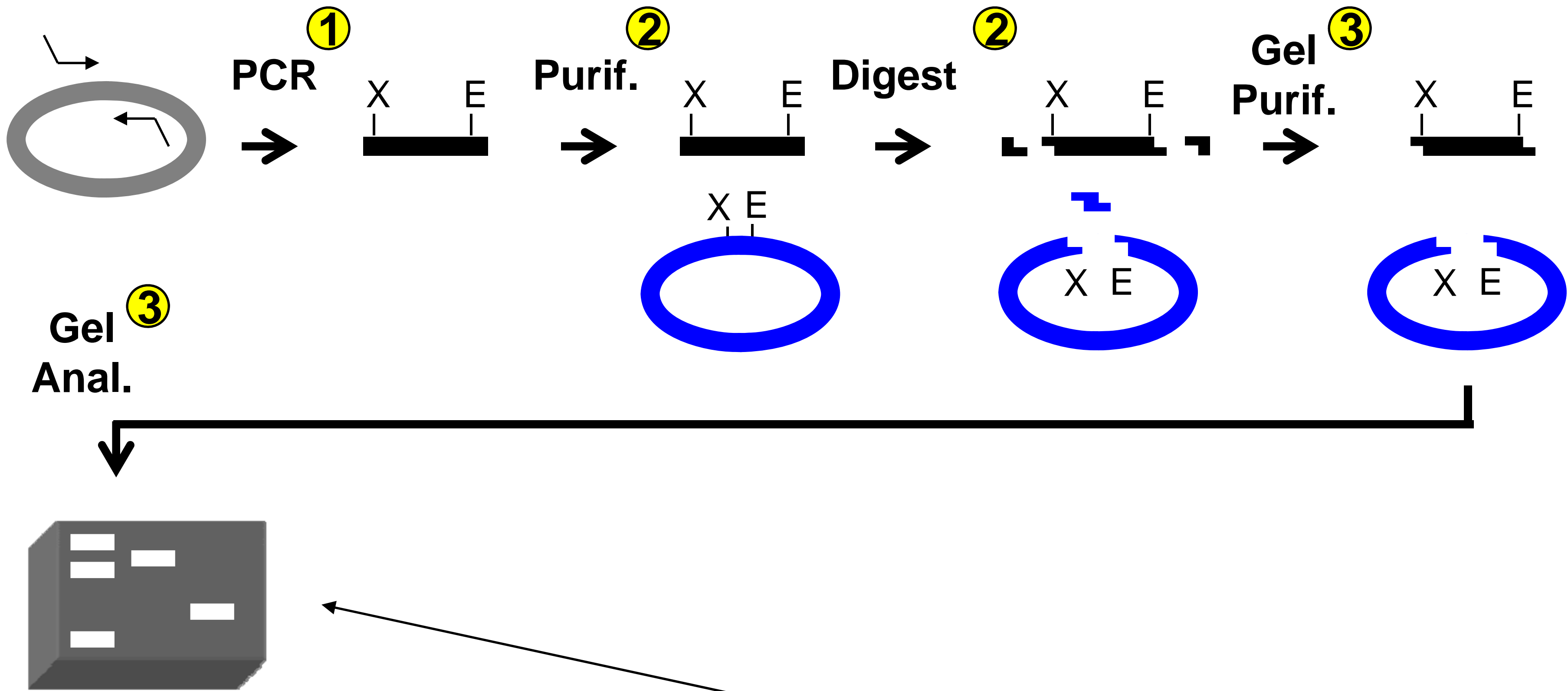


What else is in the reaction with the digested PCR product?

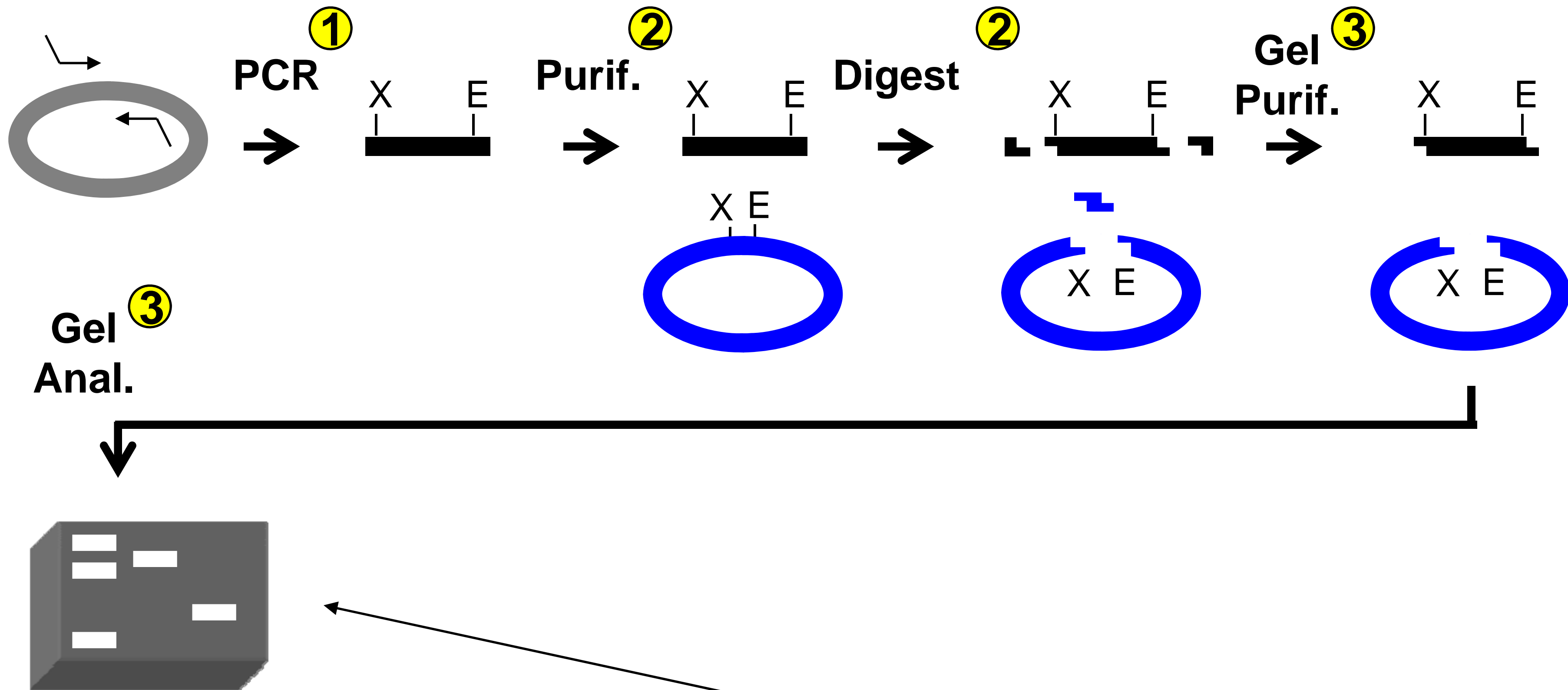
What effect could it have?



Why is it important to excise the DNA from the gel relatively quickly?

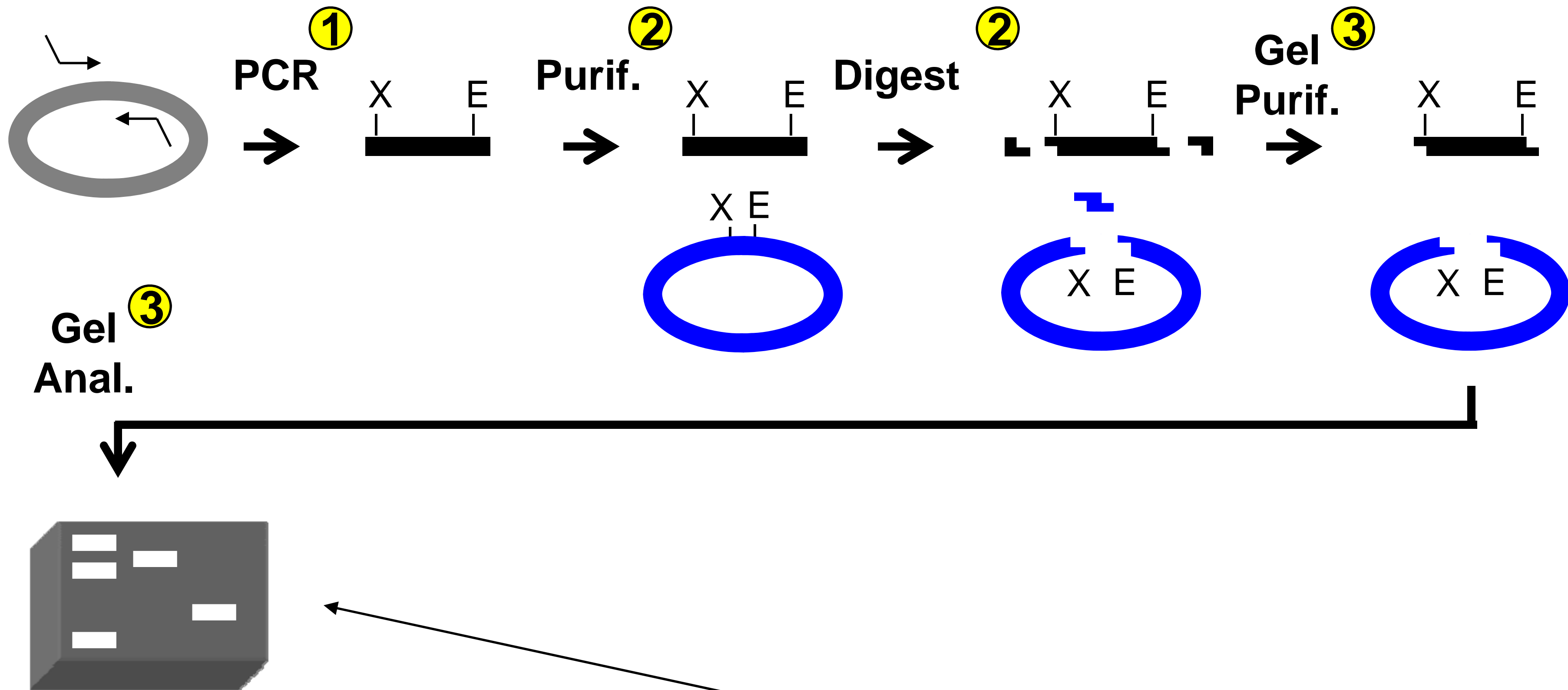


Why run this gel?



Your objective is a 1:4 vector:insert ratio – Why?

What if it was 1:100?
What if it was 100:1?



How do you figure out how to get a 1:4 molar ratio?

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

Agarose Gels – How do we 'look' at DNA?

- -what is a gel and how do you load it?
- -what happens to your DNA when it is exposed to UV?
- -parameters that affect migration

Anticipating Potential Problems & Pitfalls

- -Getting the right DNA ratios