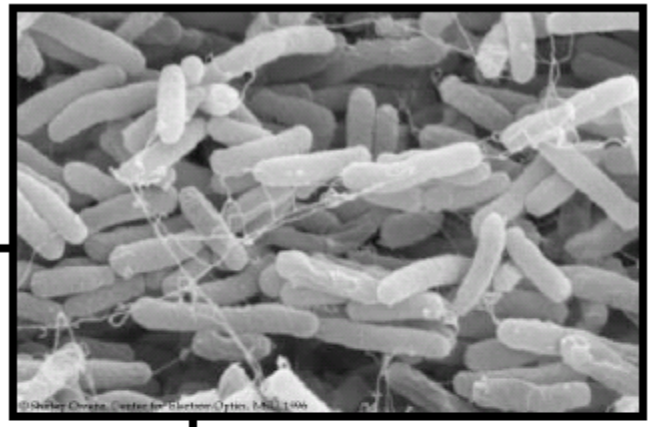
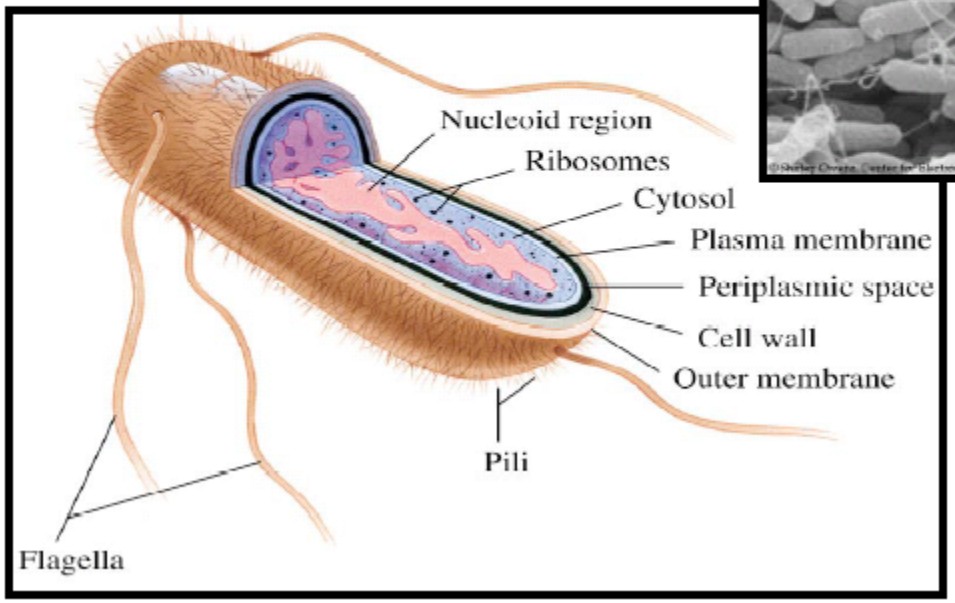
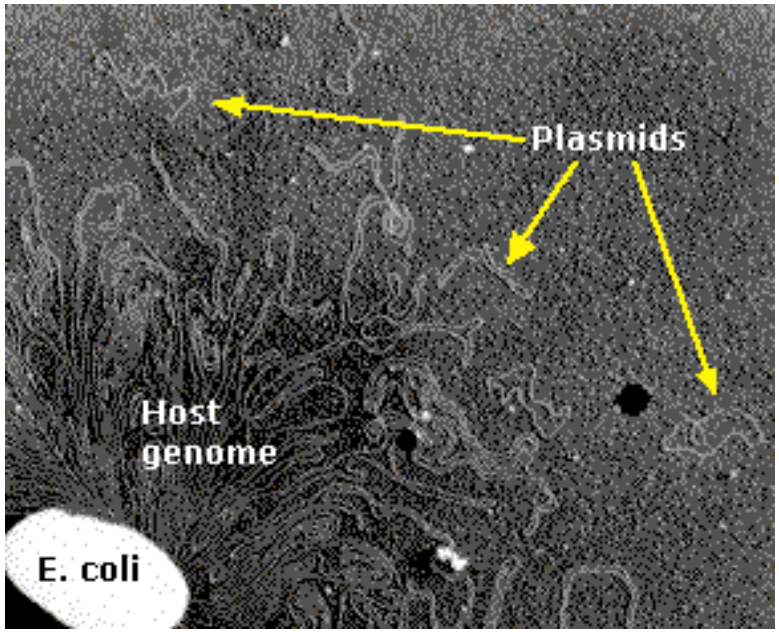
E. Coli as a host organism in molecular biology



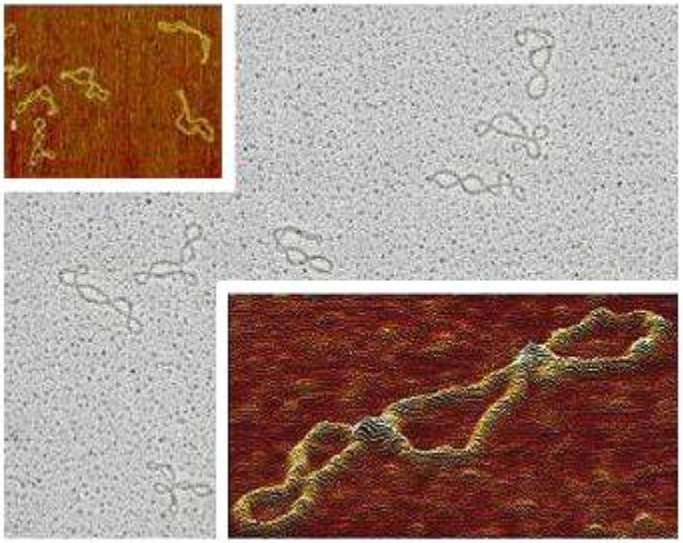


Electron micrograph of an E. coli cell ruptured to release its DNA. The tangle is a portion of a single DNA molecule containing over 4.6 million base pairs encoding approximately 4,300 genes. The small circlets are plasmids.

(Courtesy of Huntington Potter and David Dressler, Harvard Medical School, accessed at http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/R/RecombinantDNA.html)



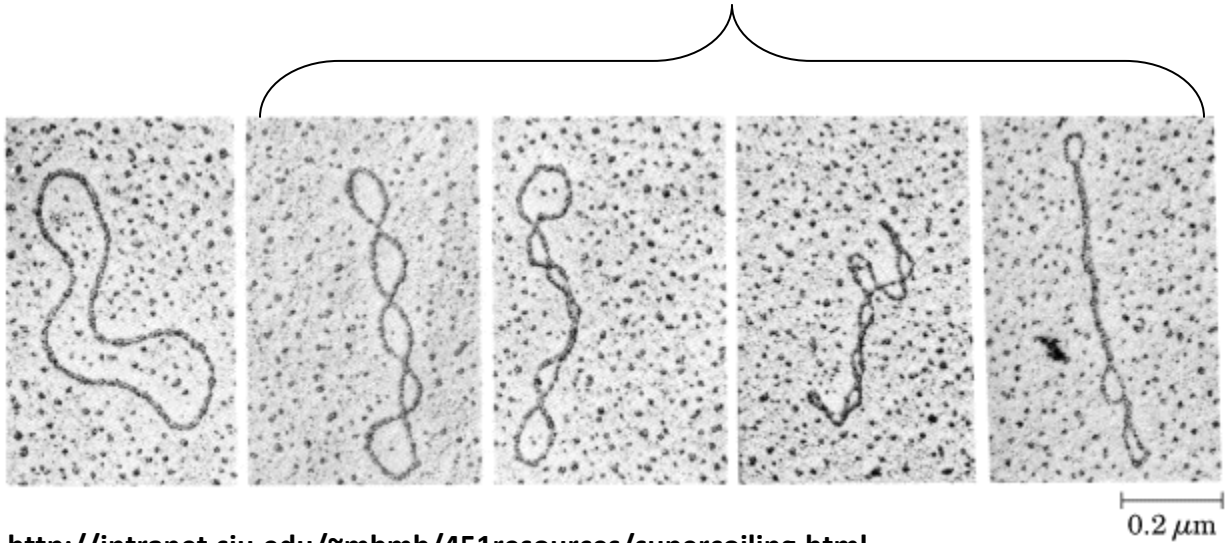
Plasmid DNA: extrachromosomal circular molecules in the bacterial cell



http://w3.uniroma1.it/centricnr-can/edocs/gme3.htm

relaxed

supercoiled



http://intranet.siu.edu/~mbmb/451resources/supercoiling.html

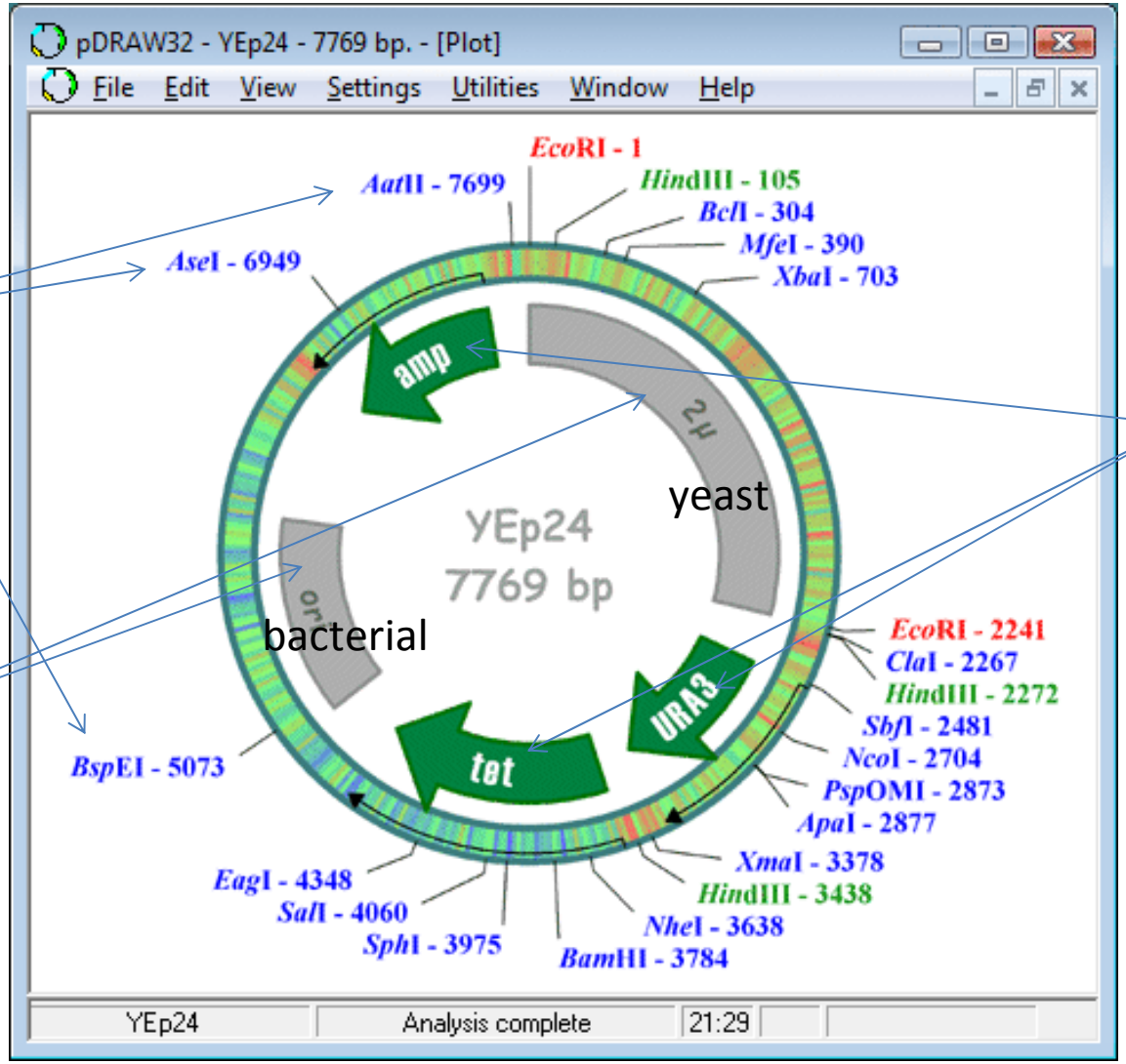
Shigella Outbreaks in Japan, 1953-1960: occurrence of antibiotic-resistant strains

Year	# Strains tested	Sm	Tc	Cm	Sm + Cm	Sm + Tc	Cm + Tc	Sm, Cm & Tc
1953	4900	5	2	0	0	0	0	0
1954	4876	11	0	0	0	0	0	0
1955	5327	4	0	0	0	0	0	1
1956	4399	8	4	0	0	0	1	0
1957	4873	13	46	0	2	2	0	37
1958	6563	18	20	0	7	2	0	193
1959	4071	16	32	0	71	0	0	74
1960	3396	29	36	0	61	9	7	308

(From http://www.mun.ca/biochem/courses/4103/topics/plasmids.html)

- **Plasmids confer antibiotic resistance to strains harboring them!**
- **Plasmids can be "shared" between strains by lateral transfer!**

Another View: Plasmid Maps



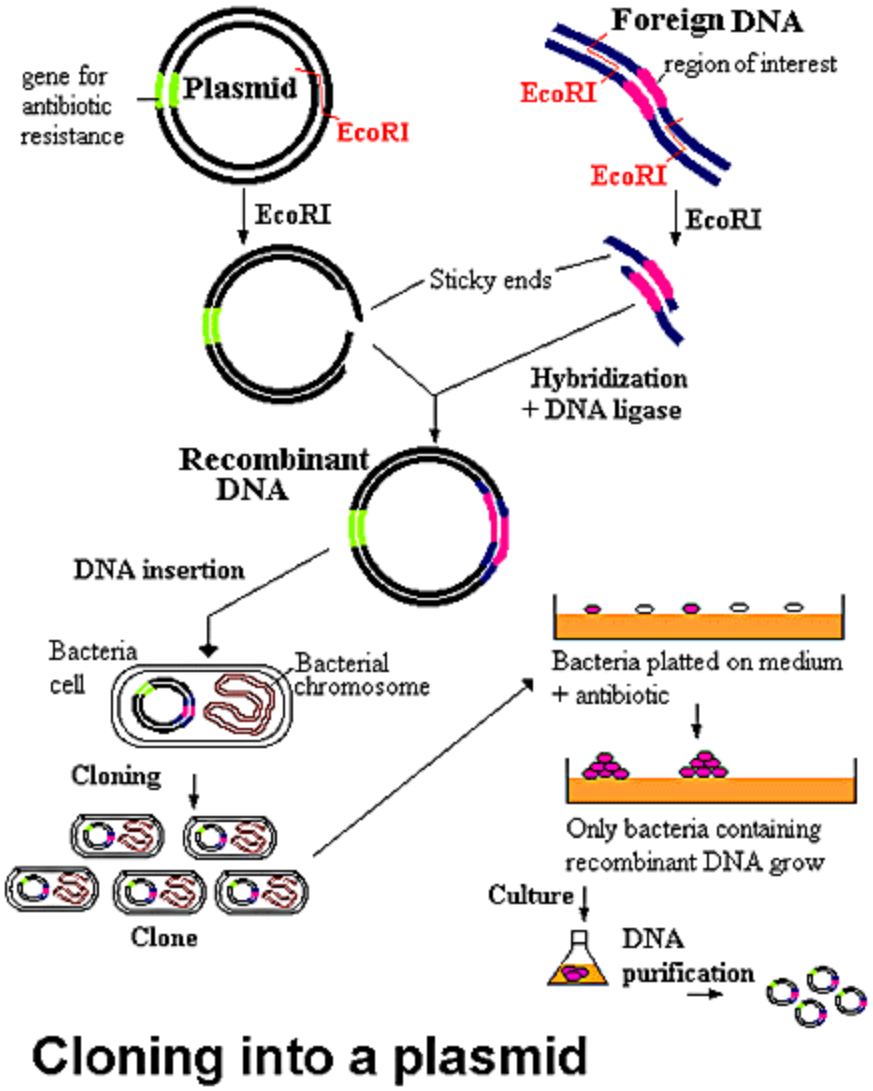
Restriction Sites

Genes

Replication Origin

This plasmid is a bacterial:yeast shuttle vector!

Plasmids can be used as vectors to carry a gene of interest

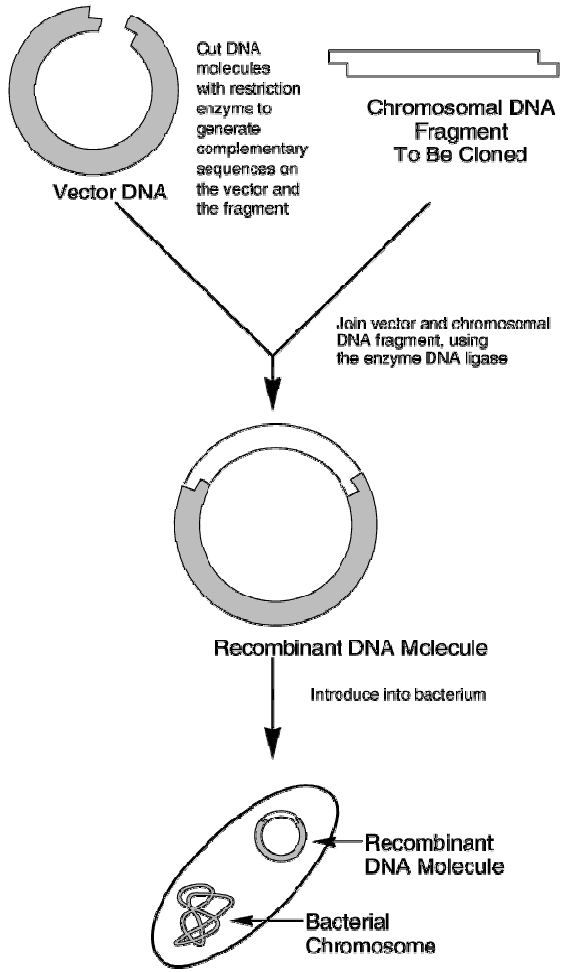


Cloning into a plasmid

(from http://www.accessexcellence.org/RC/VL/GG/plasmid.html)

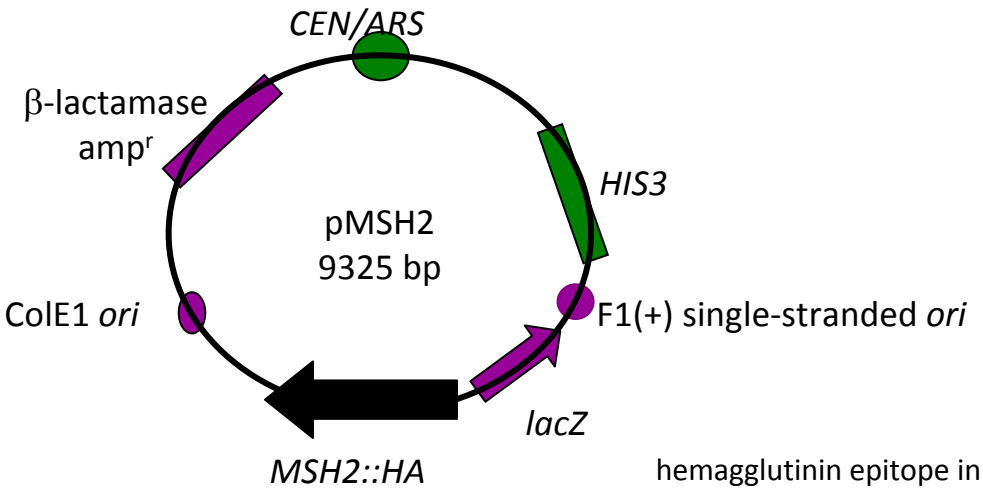
Gene Cloning I

- Provides an easily replicated source of a gene.
- Enables detailed analysis of gene structure.
- Permits production of large quantities of the gene product for functional and structural analysis.



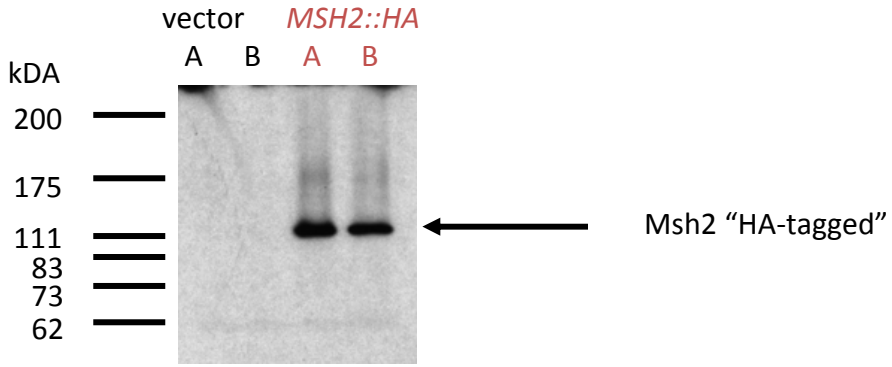
Taking a closer look at our plasmid, pMSH2

MSH2 construct for mutagenesis and functional assays



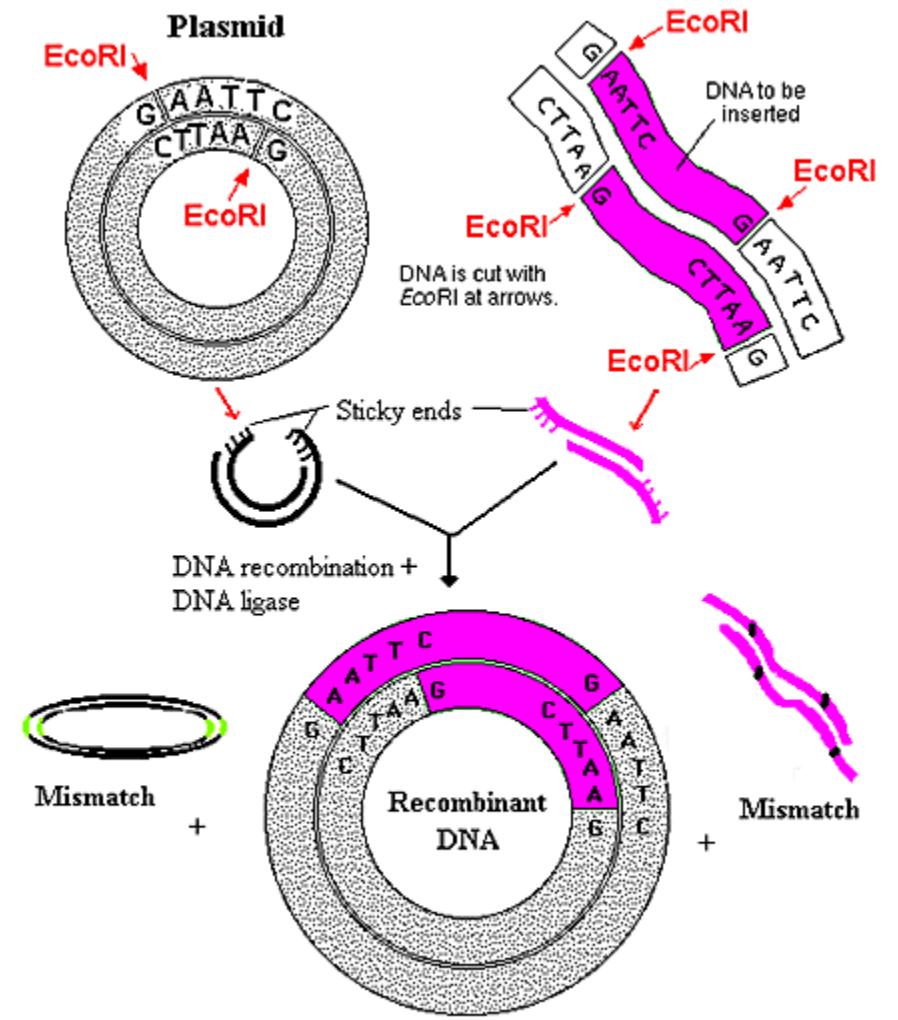
hemagglutinin epitope in the *MSH2* coding region

-produces a protein product of the expected molecular weight



-complements *msh2*Δ defects, therefore the construct is functional

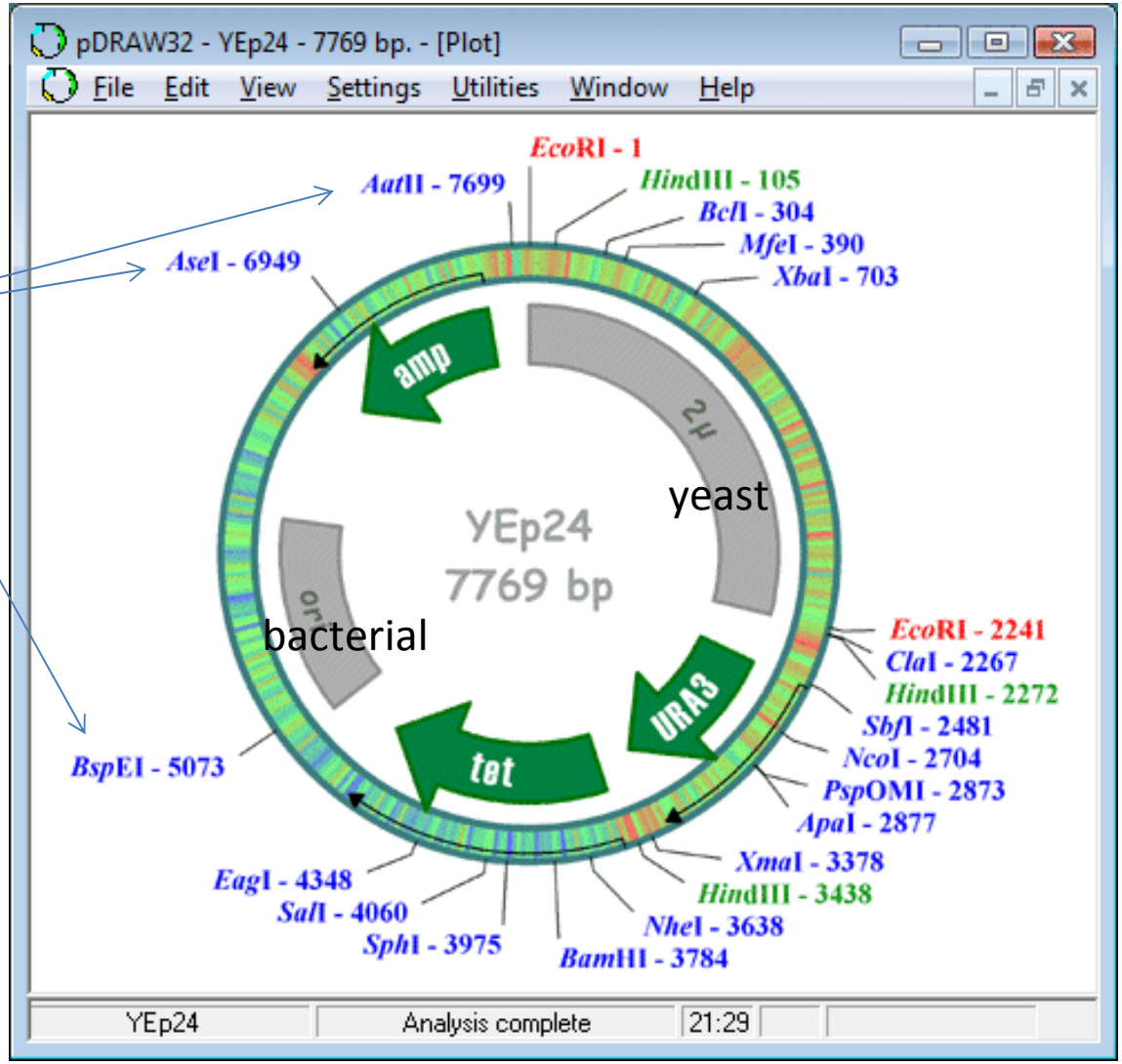
Strategy for subcloning DNA fragments into a plasmid vector



Inserting a DNA Sample into a Plasmid

Another View: Plasmid Maps

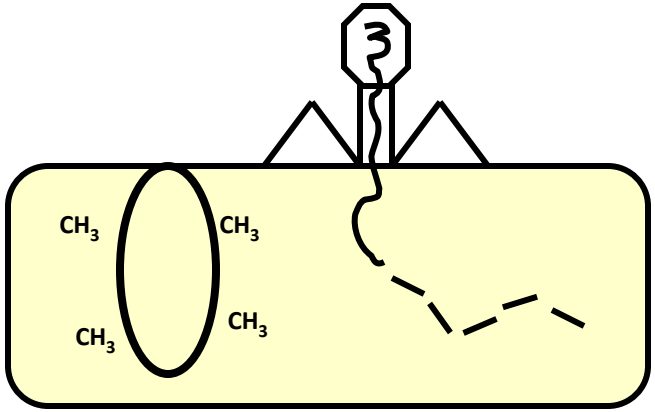
Restriction Sites



What are restriction sites for??

Biological function of restriction endonucleases is to protect cells from foreign DNA

Infecting DNA is cleaved (restricted) by the restriction enzymes, preventing it from successfully replicating and parasitizing the cell



most lab strains are completely "domesticated" (R-M systems have been inactivated)

some examples in the genotypes:

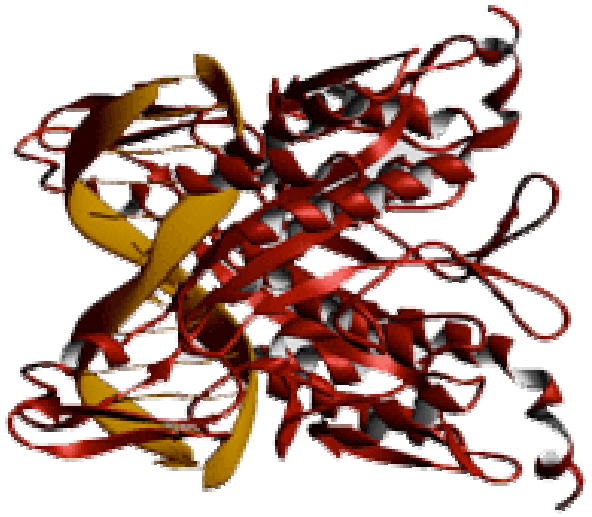
- *hsd*
- *mcr*A
- *mcr*BC

Restriction Endonucleases

- Over 10,000 bacteria and archaea have been screened for restriction enzymes

- Restriction enzymes are not confined exclusively to bacteria

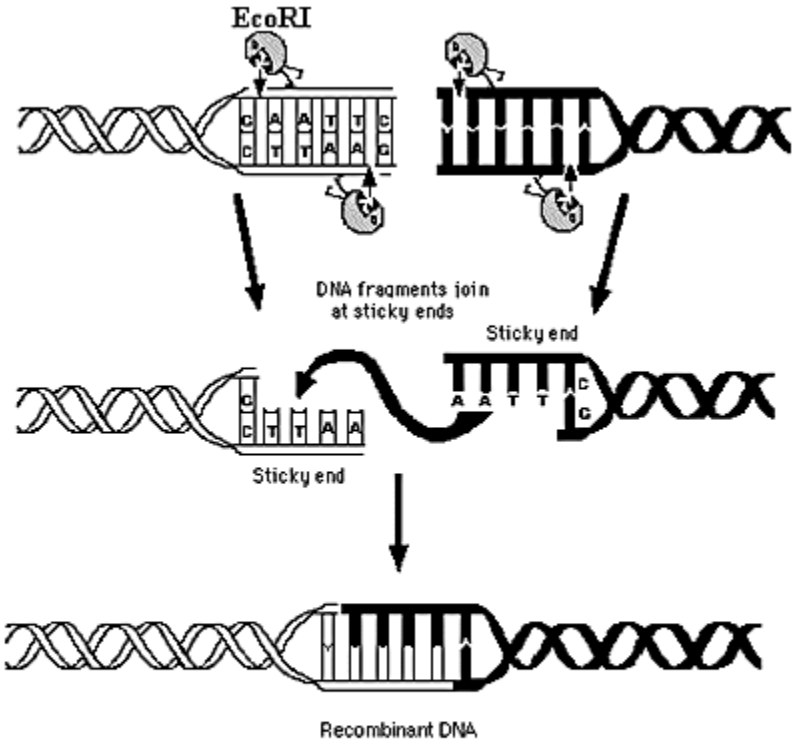
- Nearly 3,000 enzymes have been found, exhibiting over 200 different specificities (many of the 3000 are isoschizomers; different enzyme, same recognition site)



***Eco*RI binding DNA**

http://www.biophysics.pitt.edu/john_m_rosenberg.htm

Activity of a typical Restriction Enzyme



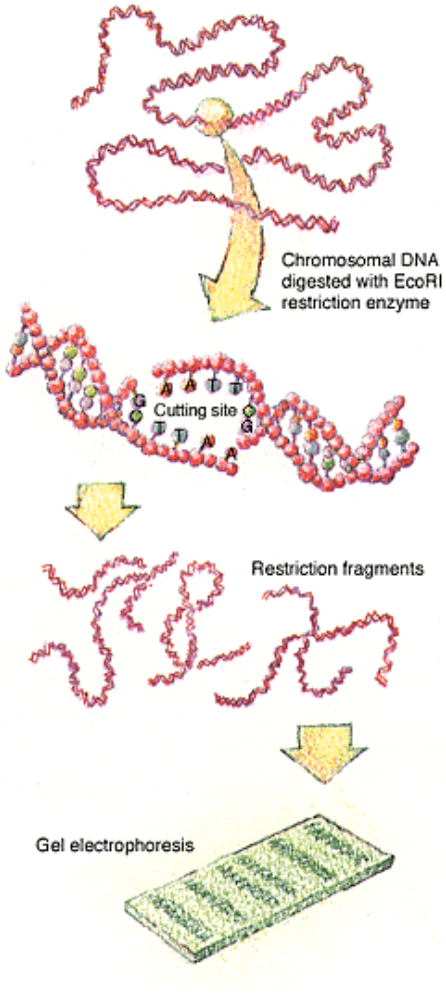
Restriction Enzyme Action of EcoRI

ends generated from a Type II restriction endonucleases

	enzyme	recognition site	end generated		
defined ends	*Pvu*II	5′CAGCTG 3′GTCGAC	5′CAG 3′GTC	CTG GAC	blunt
	*Bam*HI	5′GGATCC 3′CCTAGG	5′G 3′CCTAG	GATCC G	5′ overhang
	*Pst*I	5′CTGCAG 3′GACGTC	5′CTGCA 3′G	G ACGTC	3′ overhang
variable ends	*Xmn*I	5′GAANNNNTTC 3′CTTNNNNAAG	5′GAANN 3′CTTNN	NNTTC NNAAG	blunt
	*Ban*I	5′GGPyPuCC 3′CCPuPyGG	5′G 3′CCPuPyG	GPyPuCC G	5′ overhang
	*Bst*XI	5′CCANNNNNNTGG 3′GGTNNNNNNACC	5′CCANNNNN 3′GGTN	NTGG NNNNNACC	3′ overhang

Restriction Enzymes I

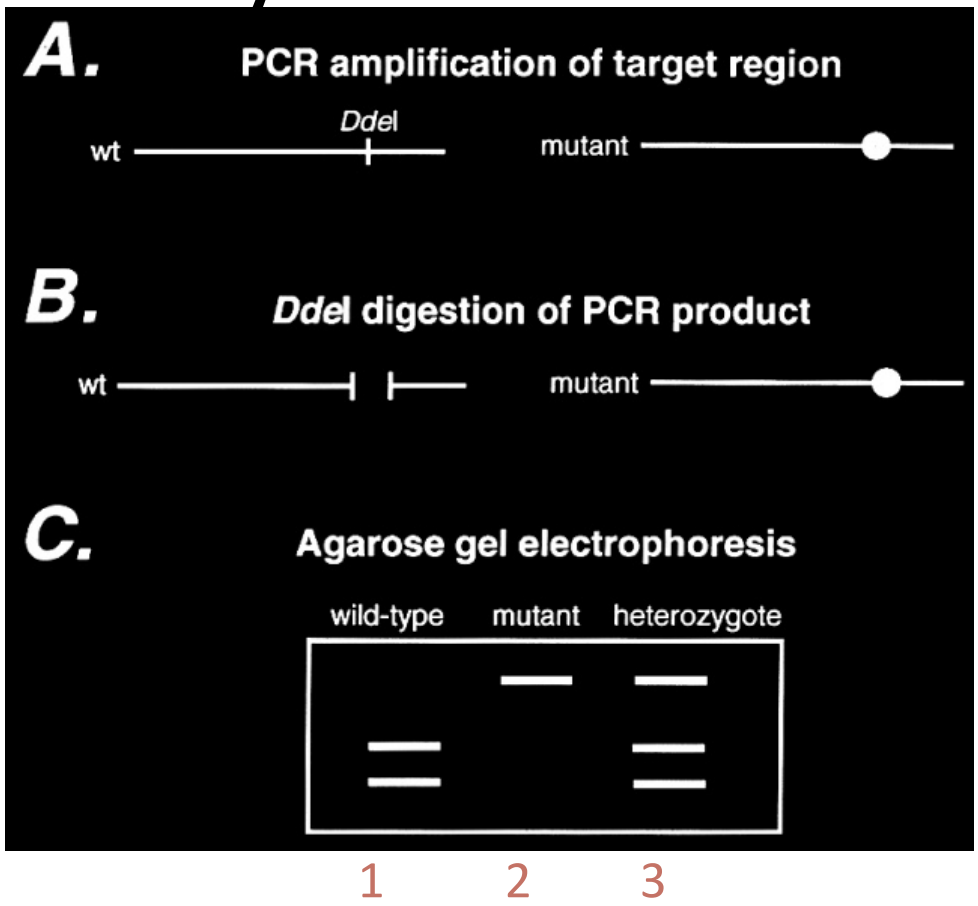
- Restriction enzymes can be used:
  - to cut DNA at specific nucleotide sequences.
    - Example – to cut chromosomes into smaller pieces for analysis by gel electrophoresis.
  - for cloning or the generation of genetic libraries.



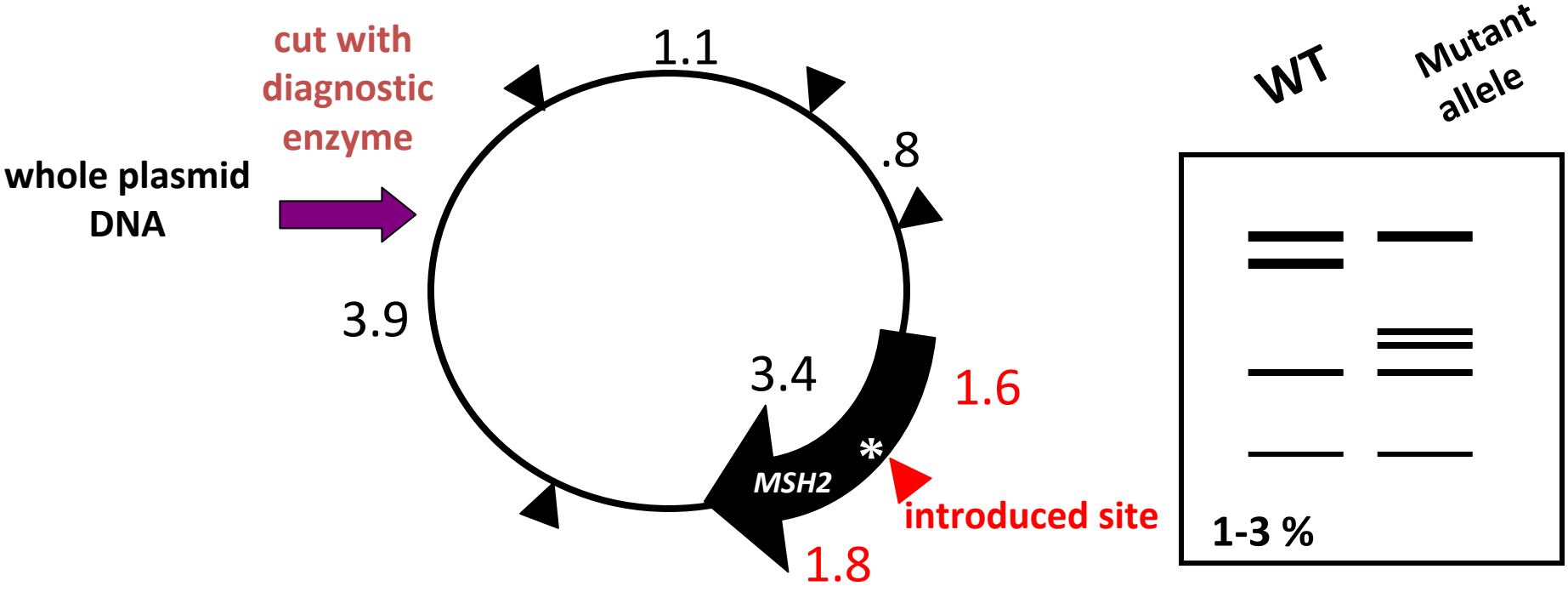
Courtesy of U. S. Department of Energy

Restriction Enzymes II

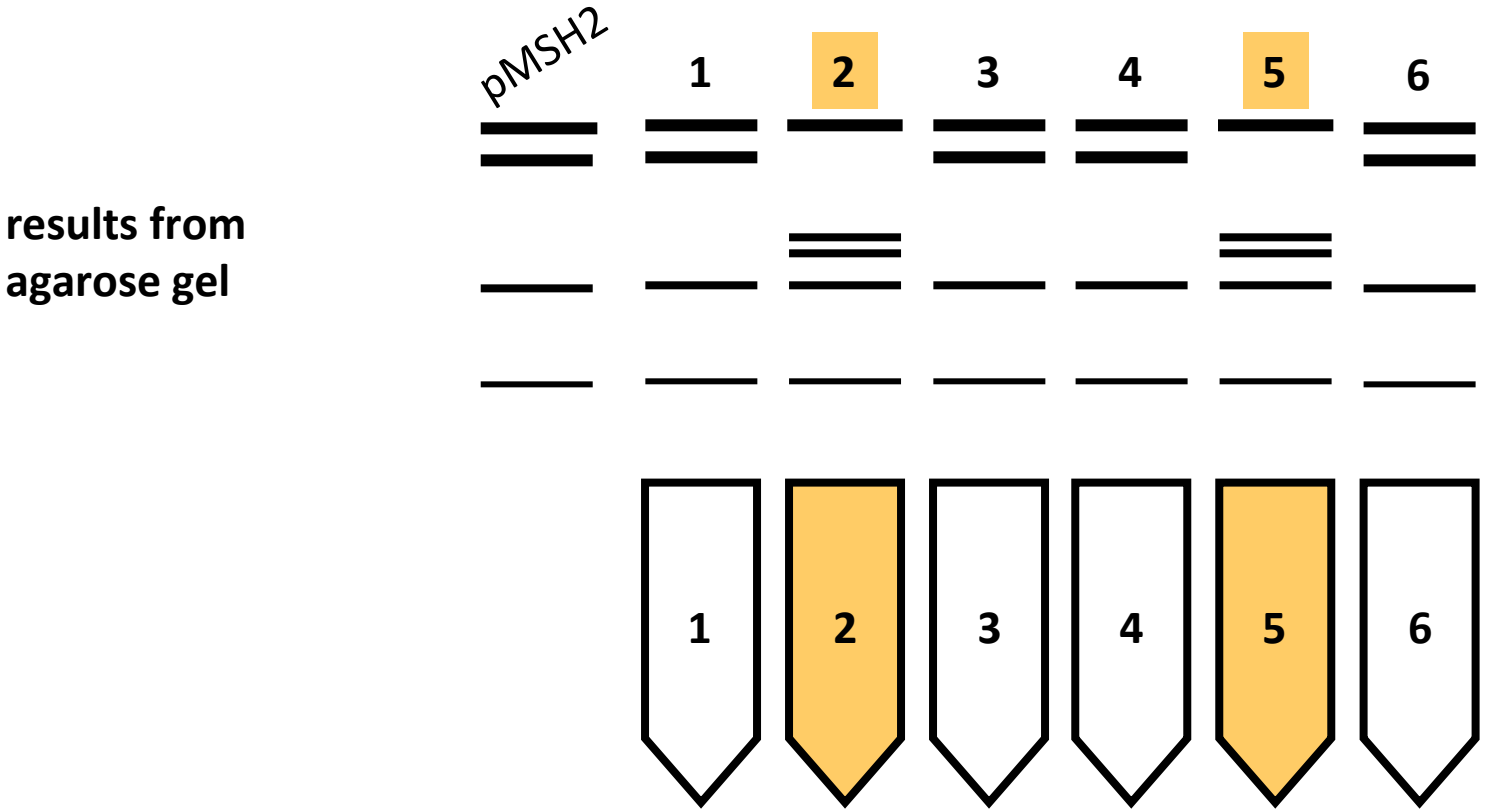
- Can be used to detect mutations in DNA.
  - Example – The enzyme called DdeI can identify the mutation that causes sickle cell anemia.
  - The mutation changes the DNA sequence so that DdeI cannot cut the DNA if the mutation is present.



We will use restriction digestion to distinguish between WT and mutagenized alleles of our pMSH2 vector



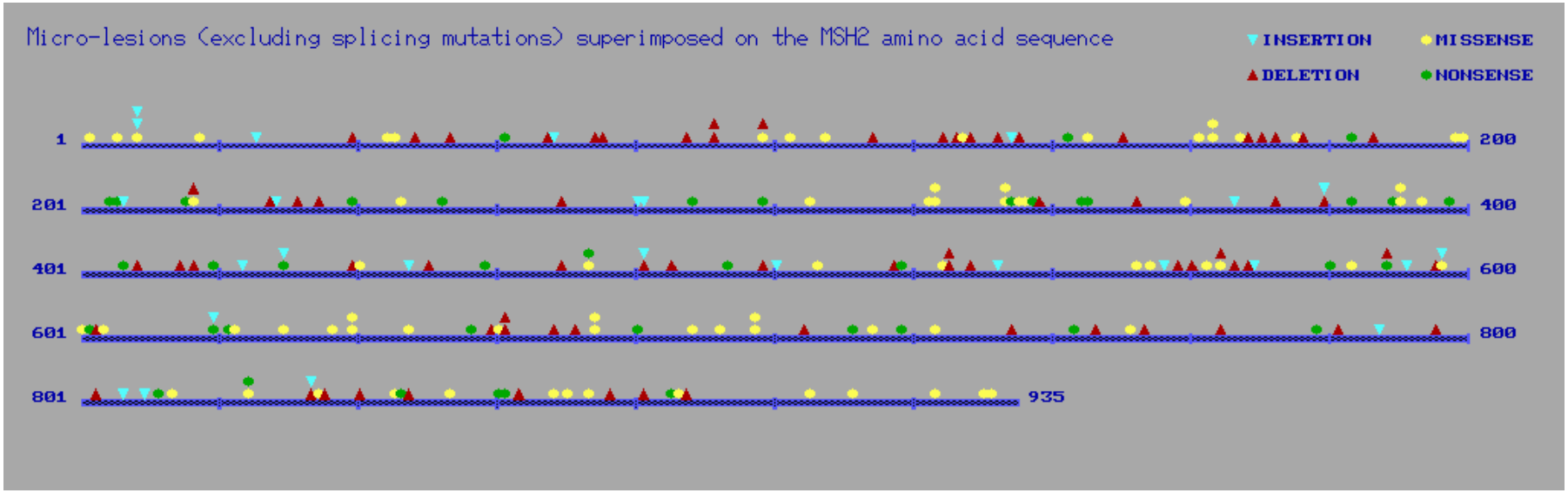
After the diagnostic gel, only proceed with a plasmid showing the altered restriction endonuclease digestion pattern (e.g. 2 or 5)



✓**Inoculate 25 ml of media with the correct bacterial colony for a midi-scale plasmid preparation (anion exchange chromatography)**

✓**Use miniprep DNA to transform yeast to begin the functional analyses**

Project Overview



Manipulate the yeast *MSH2* gene to determine which human missense mutations are likely to be benign or pathogenic in nature.

Examine the defect at a molecular level to determine why the Msh2 variants are dysfunctional.

Locating the codon to be mutagenized

Note that the two sequences don't have corresponding codon numbers

for example Ala 719 in yeast is Ala 700 in humans

```
Yeast 600  INITLTYTPVFEKLSLVLAHLDVIASFAHTSSYAPIPYIRPKLHPMDSERRTHLISSRHP 659
           +NI+  Y    + L+ VLA LD + SFAH S+ AP+PY+RP +     + R  L +SRH
Human 582  VNISSGYVEPMQTLNDVLAQLDAVVSFAHVSNGAPVPYVRPAILE-KGQGRIILKASRHA 640

Yeast 660  VLEMQDDISFISNDVTLESGKGDFLIITGPNMGGKSTYIRQVGVISLMAQIGCFVPCEEA 719
            +E+QD+I+FI NDV  E  K  F IITGPNMGGKSTYIRQ GVI LMAQIGCFVPCE A
Human 641  CVEVQDEIAFIPNDVYFEKDKQMFHIITGPNMGGKSTYIRQTGVIVLMAQIGCFVPCES A 700

Yeast 720  EIAIVDAILCRVGAGDSQLKGVSTFMVEILETASILKNASKNSLIIVDELGRGTSTYDGF 779
           E++IVD IL RVGAGDSQLKGVSTFM E+LETASIL++A+K+SLII+DELGRGTSTYDGF
Human 701  EVSIVDCILARVGAGDSQLKGVSTFMAEMLETASILRSATKDSLIIIDELGRGTSTYDGF 760

Yeast 780  GLAWAIAEHIASKIGCFALFATHFHELTELSEKLPNVKNMHVVAHIEKNLKEQKHDDEDI 839
           GLAWAI+E+IA+KIG F +FATHFHELT L+ ++P V N+HV A           +E +
Human 761  GLAWAISEYIATKIGAFCMFATHFHELTALANQIPTVNNLHVTA---------LTTEETL 811

Yeast 840  TLLYKVEPGISDQSFGIHVAEVVQFPEKIVKMAKRKANELDDLKTNNE----DLKK--AK 893
           T+LY+V+ G+ DQSFGIHVAE+  FP+ +++ AK+KA EL++ +   E    D+ +  AK
Human 812  TMLYQVKKGVCDQSFGIHVAELANFPKHVIECAKQKALELEEFQYIGESQGYDIMEPAAK 871
```

Designing a mutagenic oligonucleotide -
choosing the codon guided by a yeast codon usage table

yeast codon 122 changed from glycine (G) to serine (S)
termed G122S mutation

wild-type *MSH2* coding sequence

```
    A  S  P  G  N  I  E
5′CGCATCTCCAGGGAACATTGAGC
```

missense mutation in *MSH2* coding sequence

```
    A  S  P  S  N  I  E
5′CGCATCTCCATCTAACATTGAGC

    A  S  P  S  N  I  E
5′CGCATCTCCATCAAACATTGAGC

    A  S  P  S  N  I  E
5′CGCATCTCCATCCAACATTGAGC

    A  S  P  S  N  I  E
5′CGCATCTCCAAGTAACATTGAGC
```

top four candidates

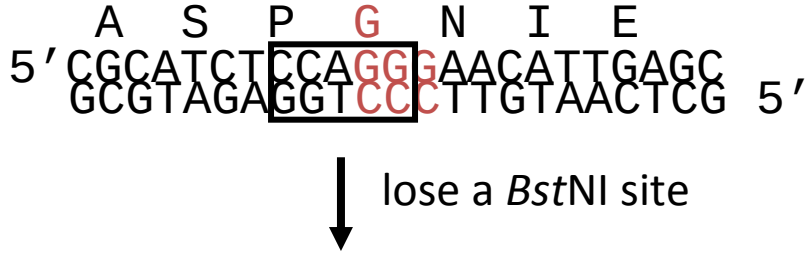
Codon usage in yeast (Appendix of manual)

AmAcid	Codon	Number	/1000	Fraction
Ser	AGT	2411.00	11.14	0.14
Ser	AGC	1599.00	7.39	0.09
Ser	TCG	1411.00	6.52	0.08
Ser	TCA	3316.00	15.33	0.19
Ser	TCT	5495.00	25.40	0.32
Ser	TCC	3198.00	14.78	0.18

Designing a mutagenic oligonucleotide:

choosing the codon to create or destroy a restriction endonuclease site

wild-type *MSH2* coding sequence



missense mutation in *MSH2* coding sequence

✓best codon choice

✓lose a *Bst*NI site

Designing a mutagenic oligonucleotide -
choosing the codon guided by a yeast codon usage table

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missense mutation in *MSH2* coding sequence

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    A  S  P  S  N  I  E
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    A  S  P  S  N  I  E
5′CGCATCTCCATCAAACATTGAGC

    A  S  P  S  N  I  E
5′CGCATCTCCATCCAACATTGAGC

    A  S  P  S  N  I  E
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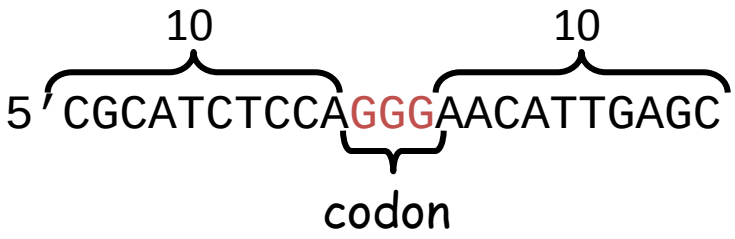
Ser	TCC	3198.00	14.78	0.18

Laboratory Session 3 (Mon/Tue)

designing the mutagenic oligonucleotide

important considerations:

- 23 nucleotide long (for efficient annealing to the template)



- codon preference for expression in yeast
- creating/destroying a restriction site for screening
- strand must be complementary to template