



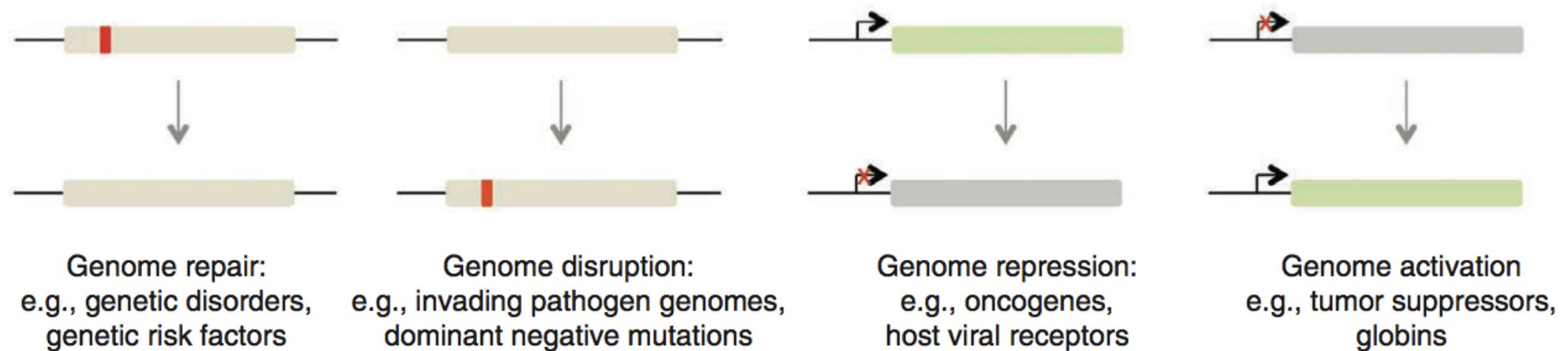
Genetic Manipulation

Samuel Wagner



Principle applications

- Cloning of genes for protein production
- Mutagenesis of organisms
 - Deletions
 - Point mutants
 - Insertions
 - Chimeras
- Reprogramming of gene expression
- Gene-therapy





Part I: Molecular basics

Avery–MacLeod–McCarty experiment

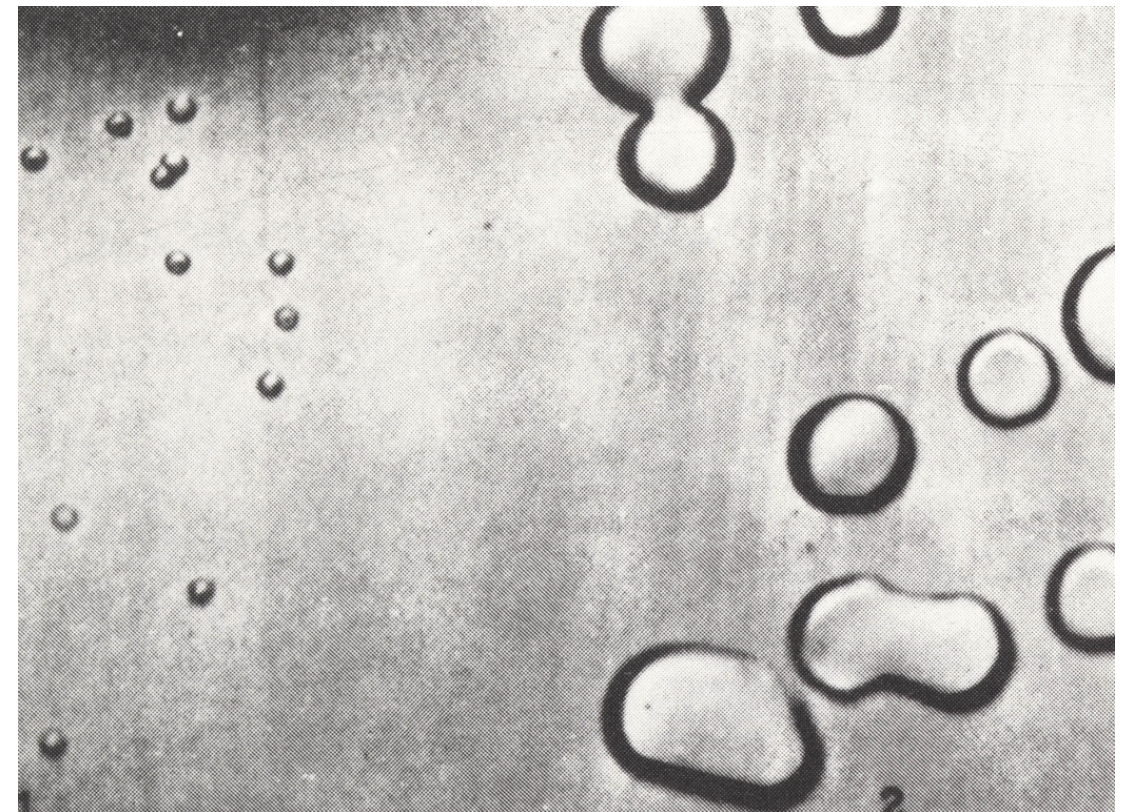
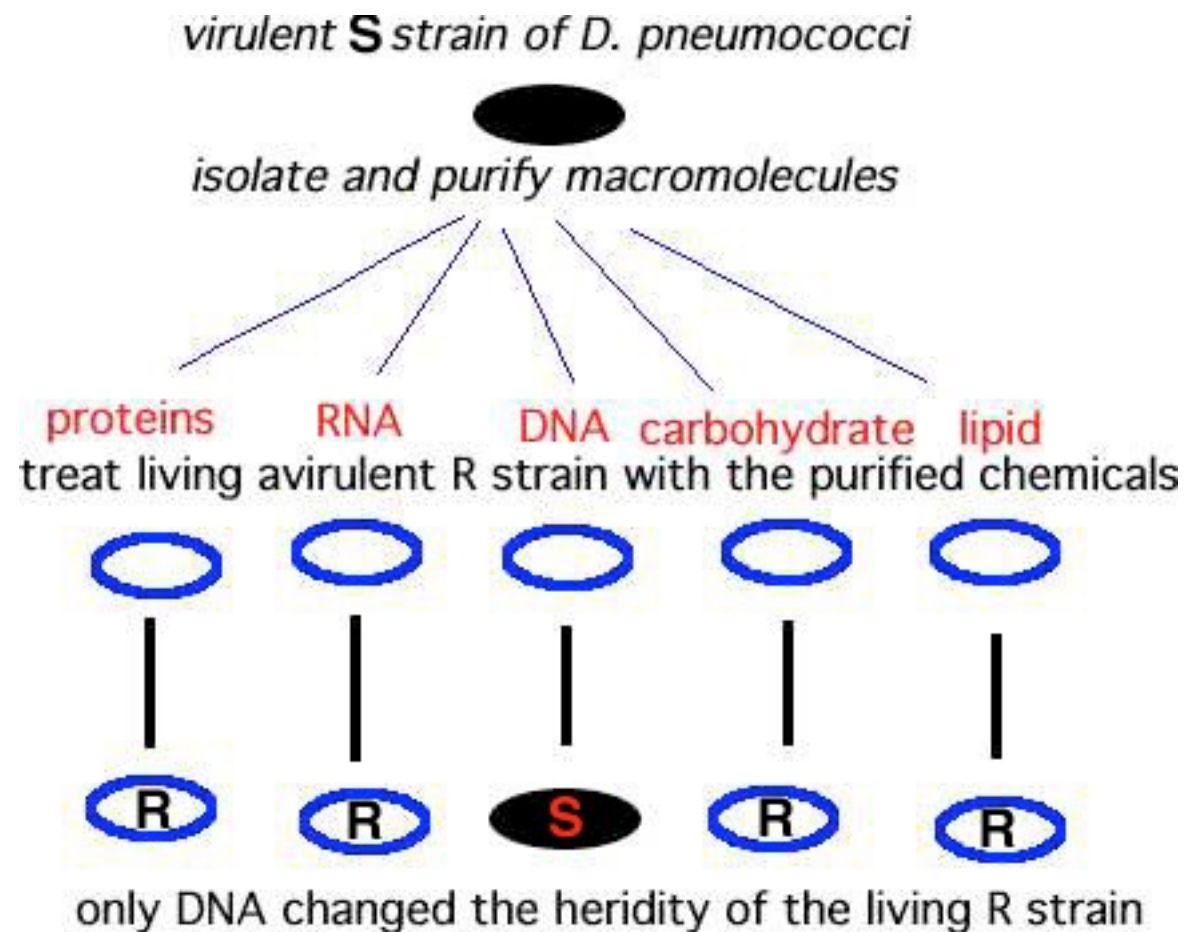


figure: J Exp Med (1944) 79:158

This is naturally occurring genetic “auto-manipulation”

Genetic elements

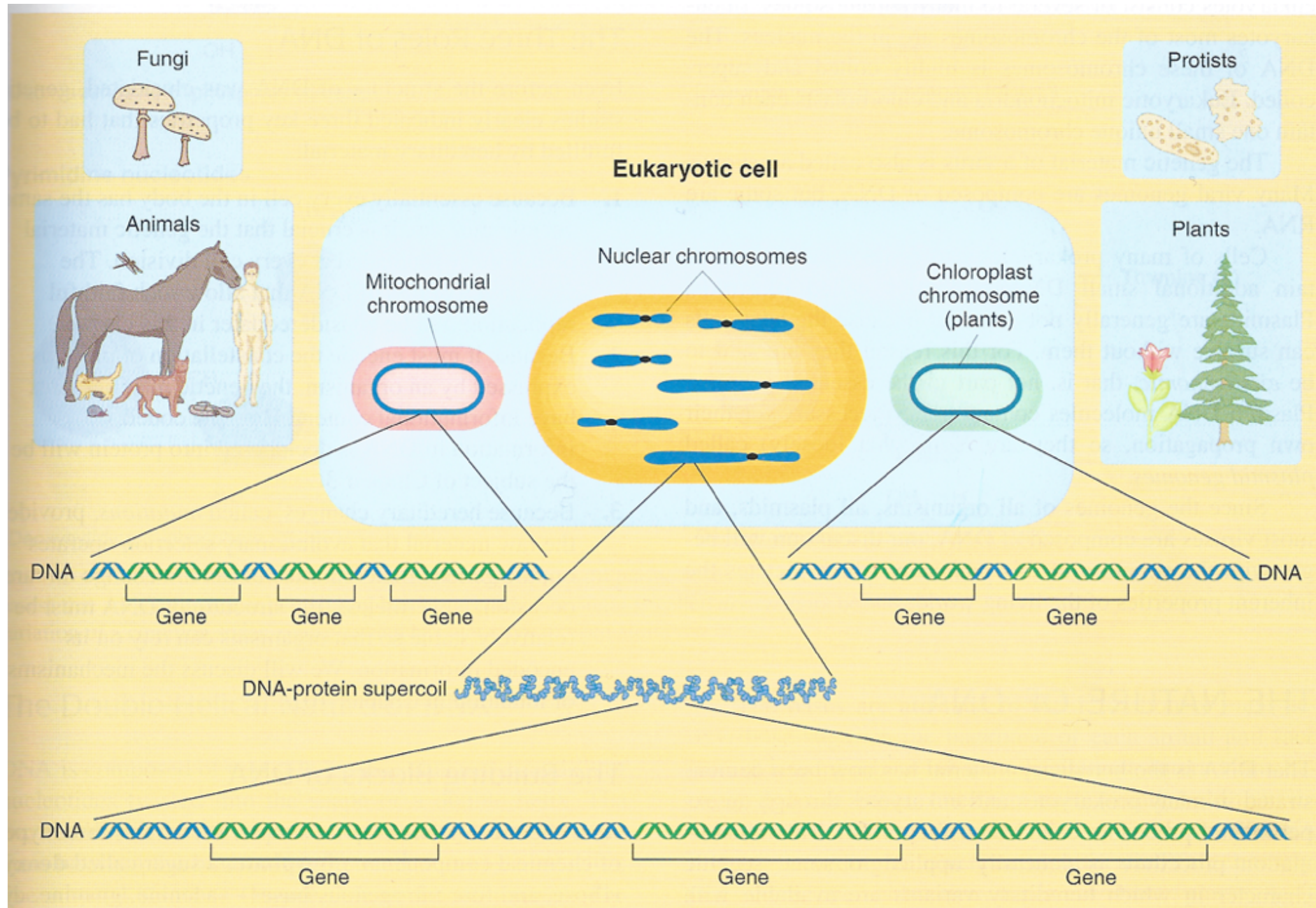
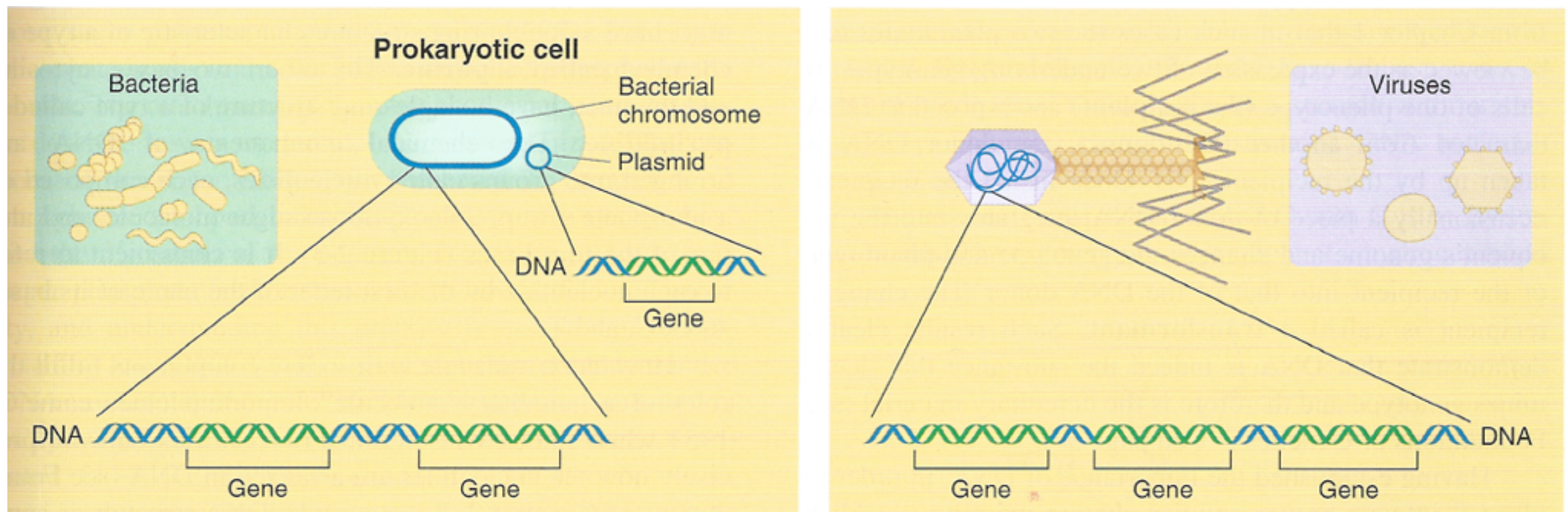


figure: Modern Genetic Analysis Fig 2-2

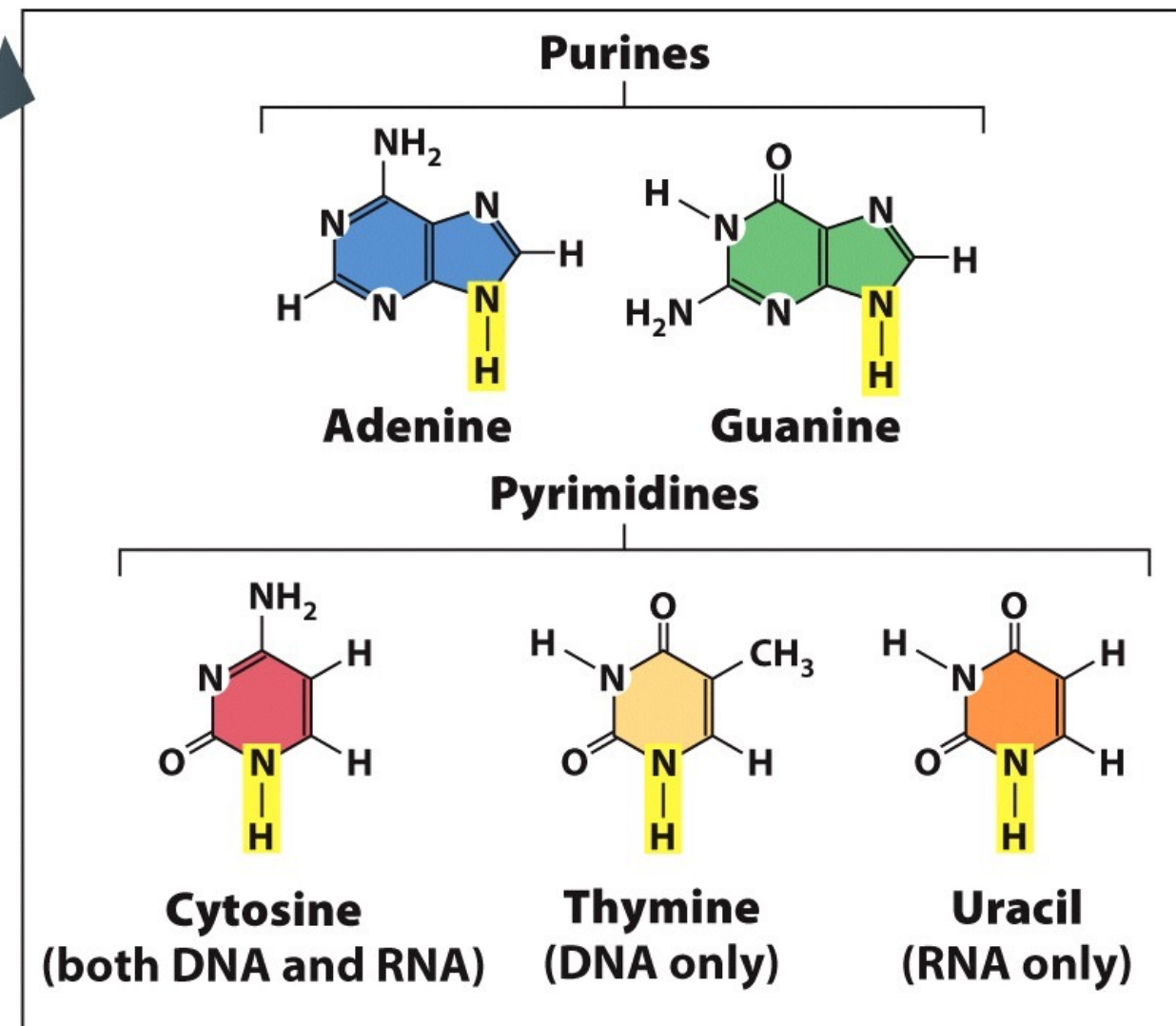
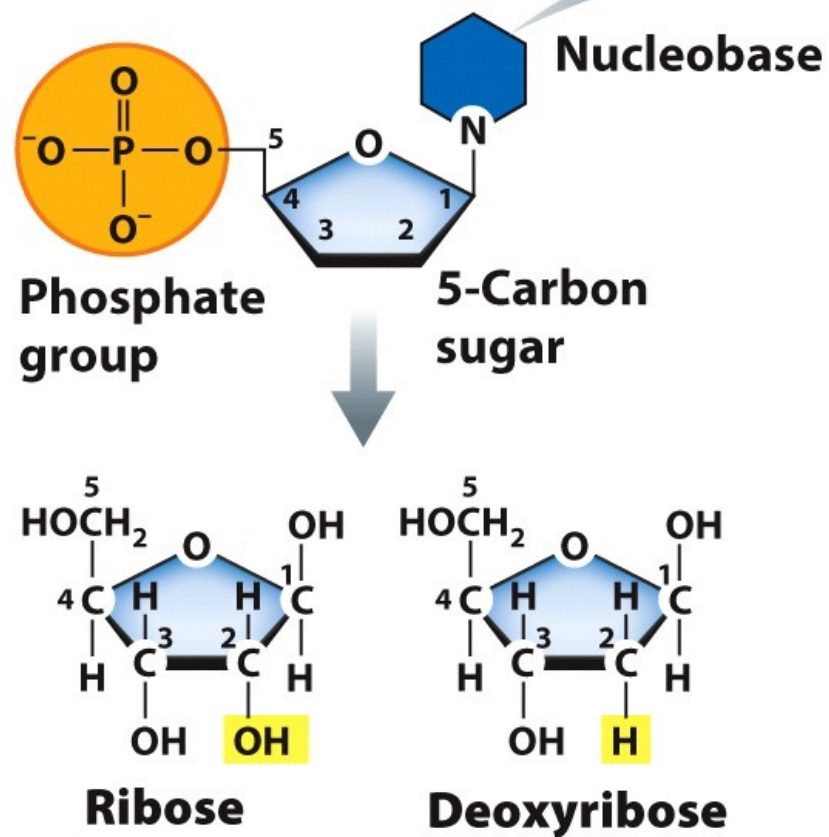
Genetic elements



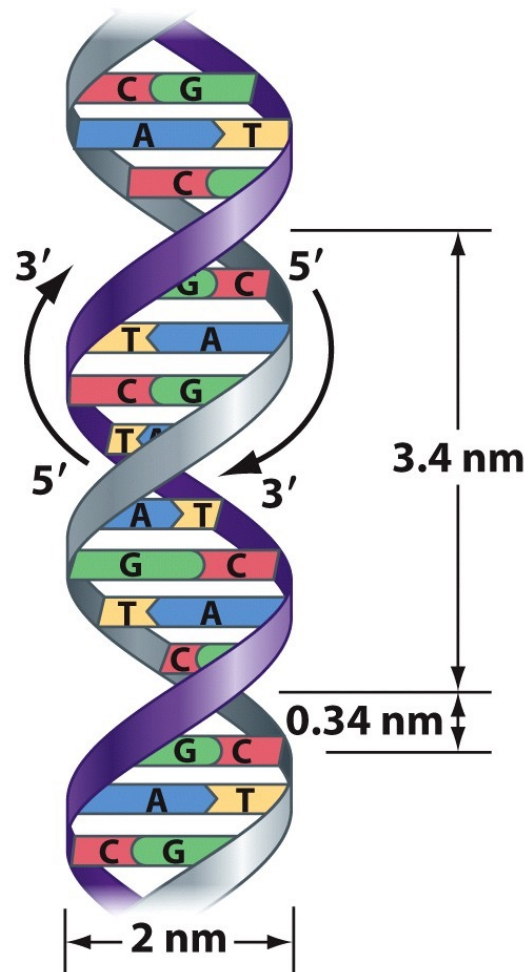
Nucleotide and DNA structure

Nucleotide and DNA structure

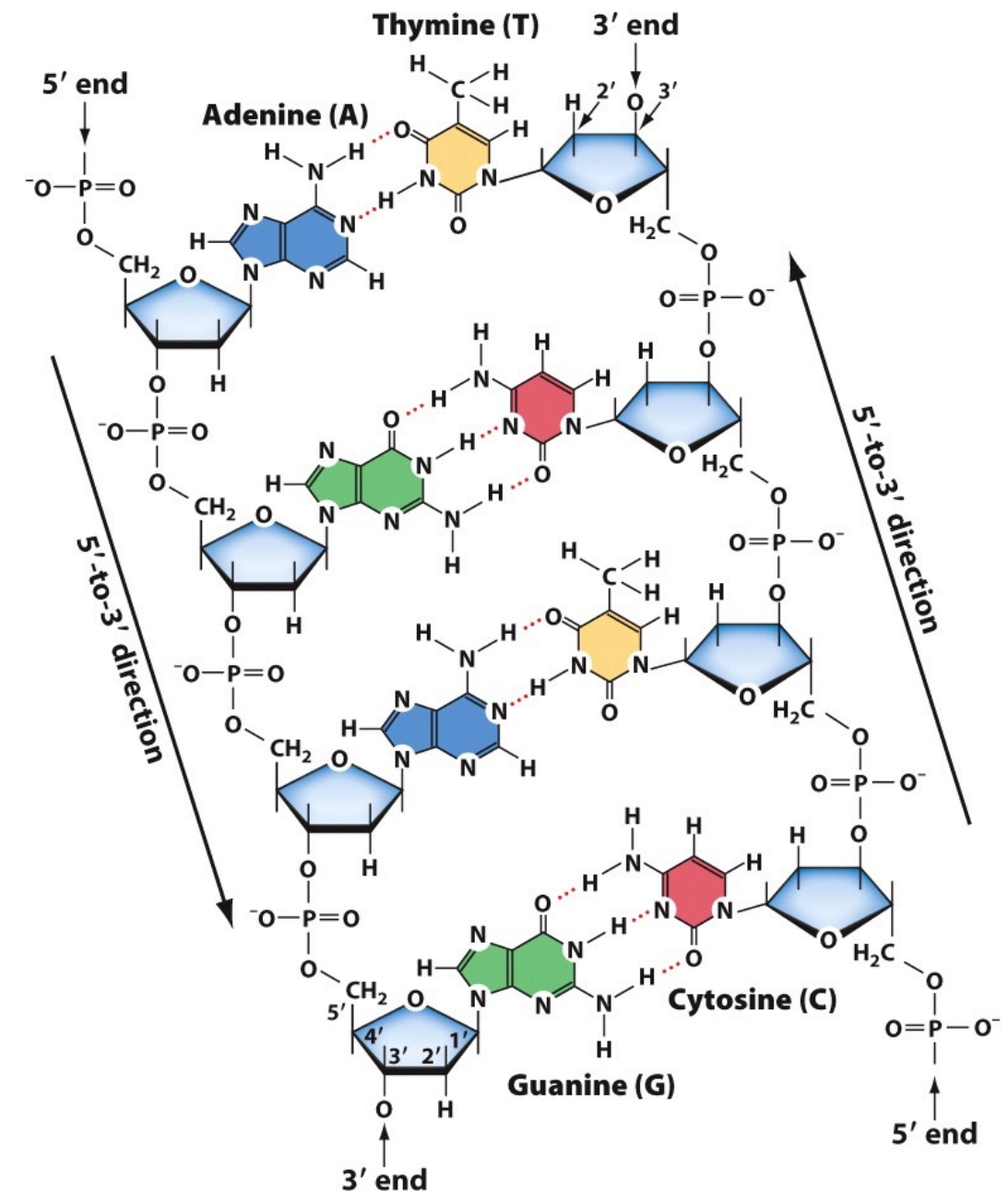
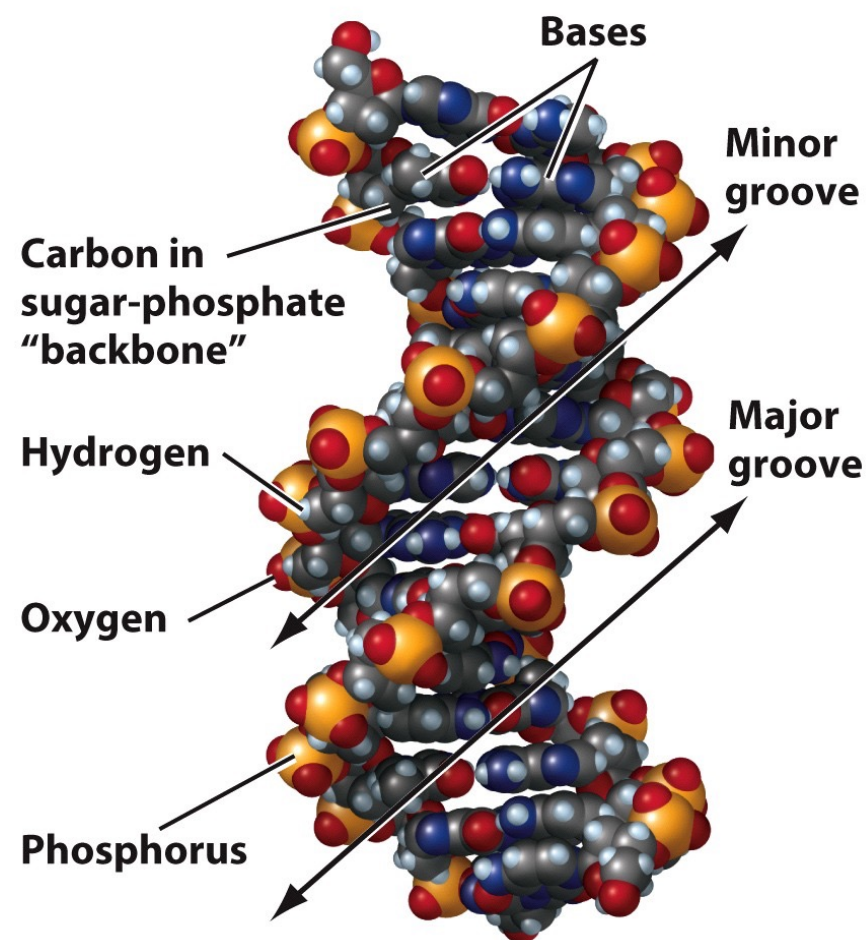
Nucleotide



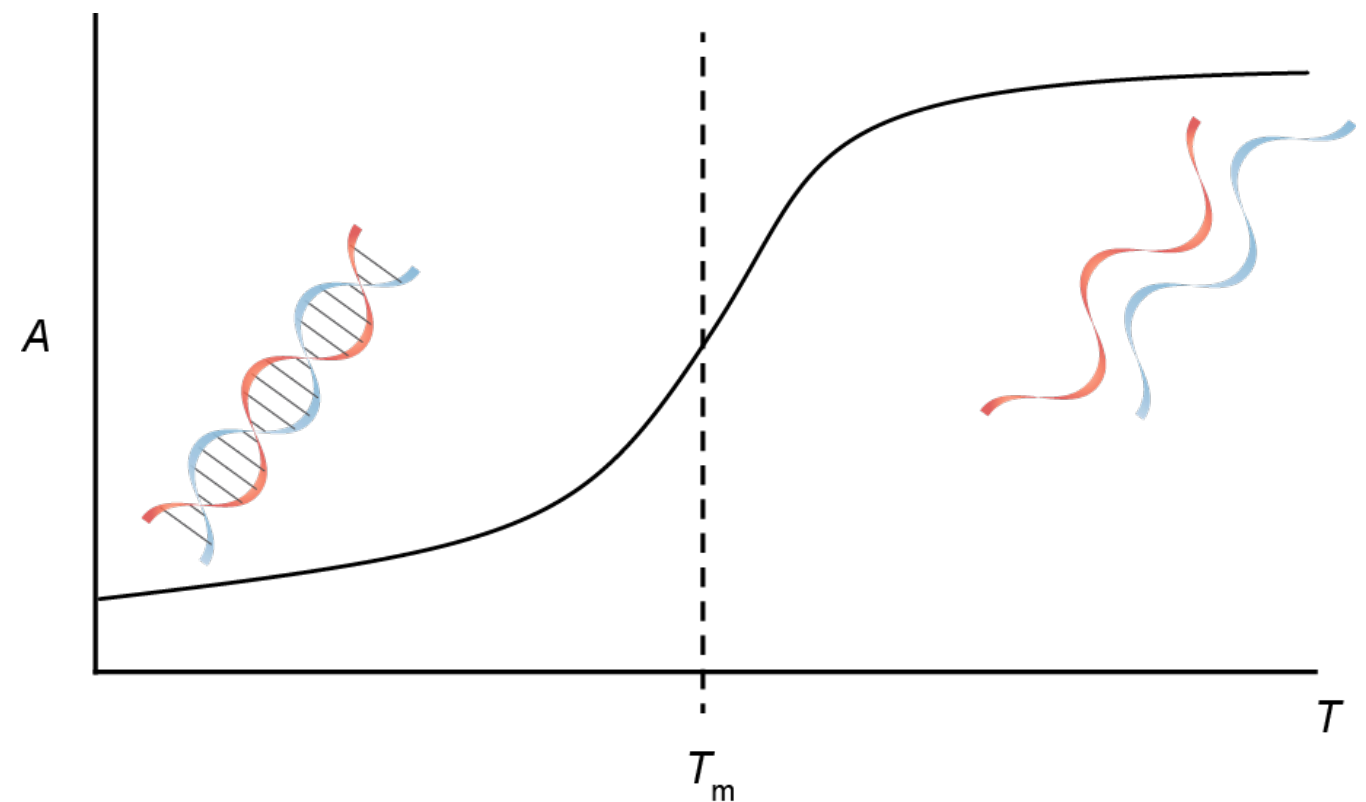
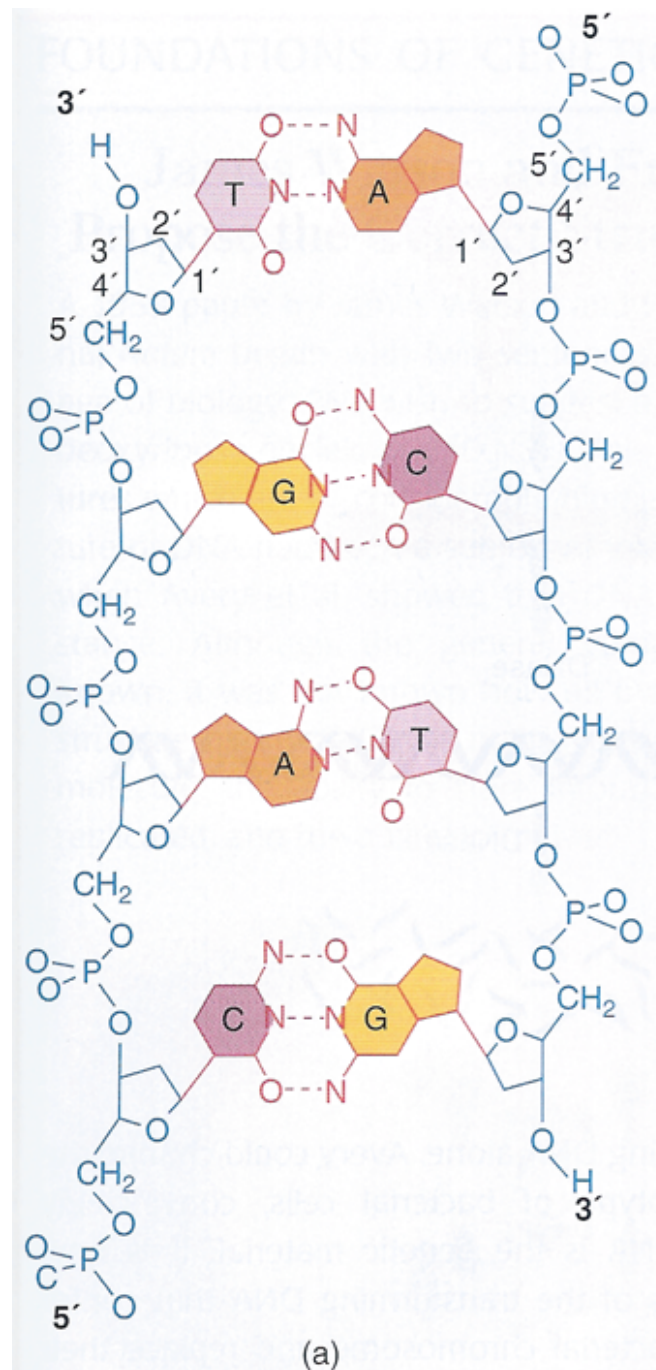
Nucleotide and DNA structure



Microbiology: An Evolving Science, Third Edition Figure A1.16
Copyright © 2014 W. W. Norton & Company, Inc.



Melting of DNA



rule-of-thumb for T_m : 4°C for G/C, 2°C for A/T

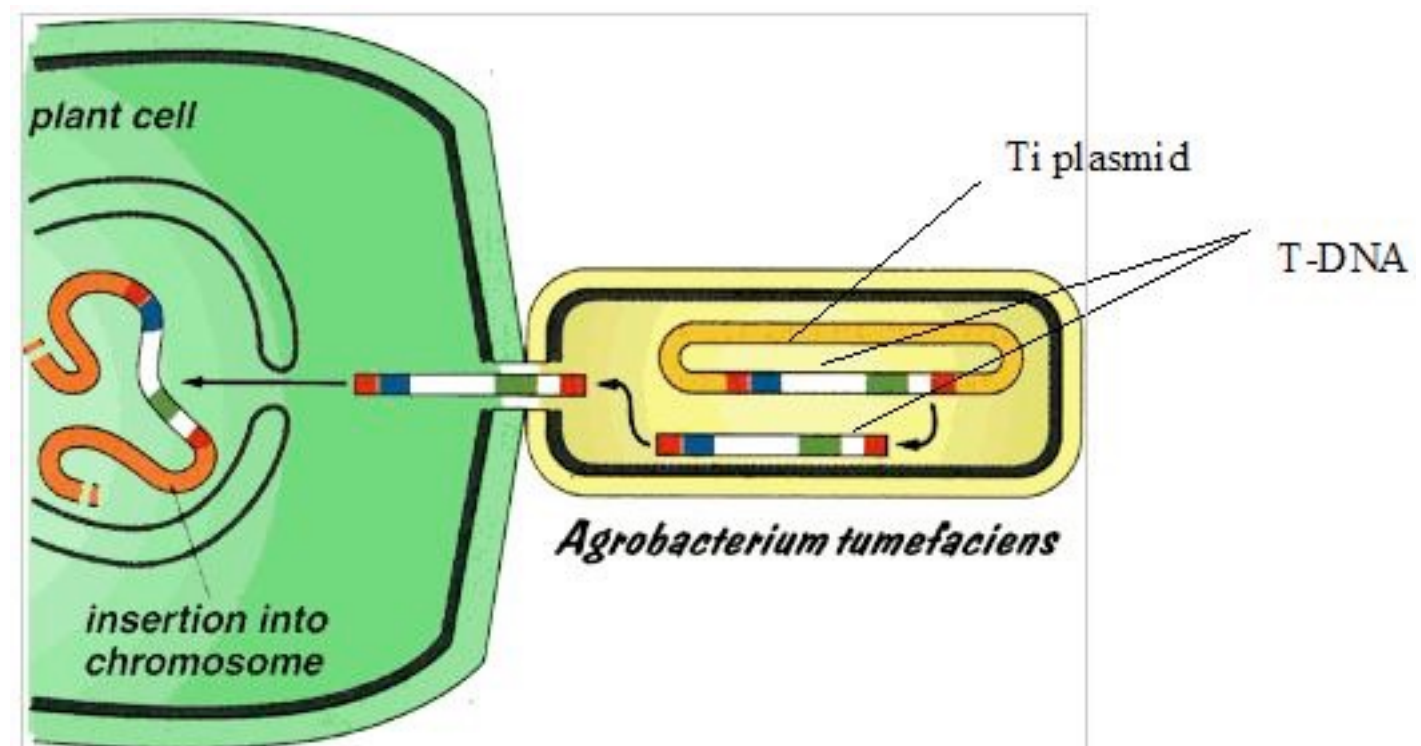


Part II: Genetic manipulation

Natural genetic manipulation



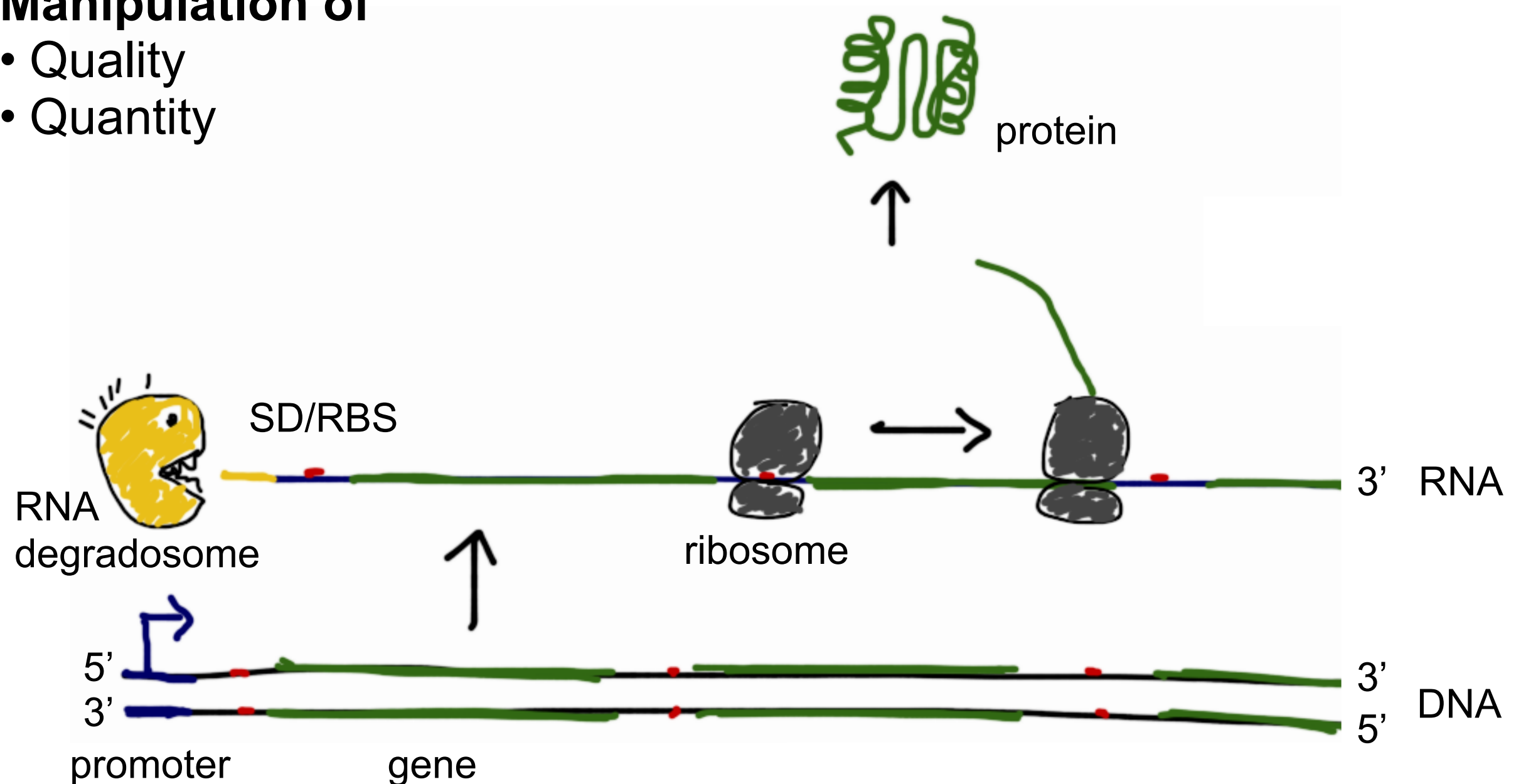
figures: lifesciencefoundation.org



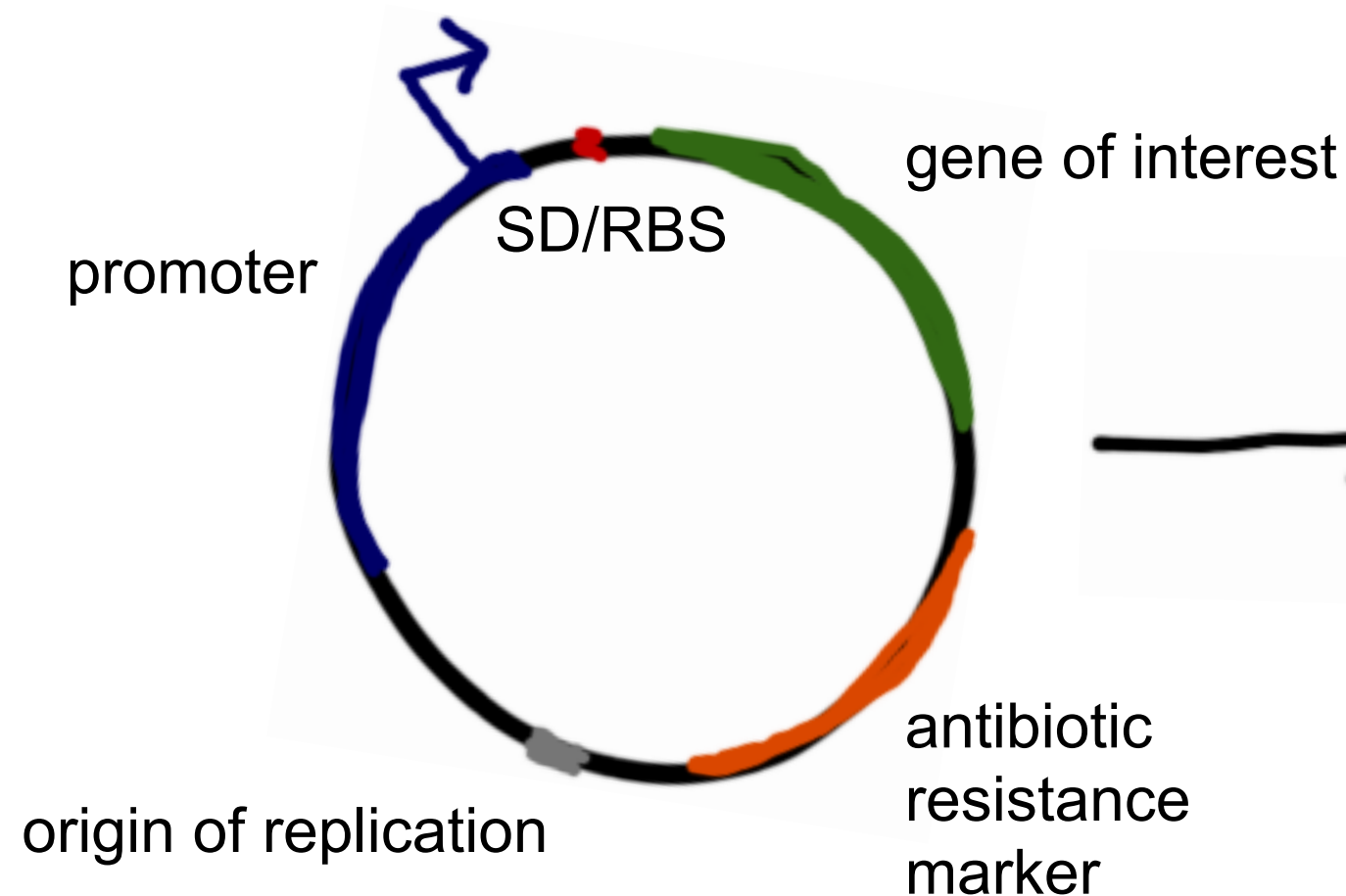
In the end it is all about making proteins...

Manipulation of

- Quality
- Quantity

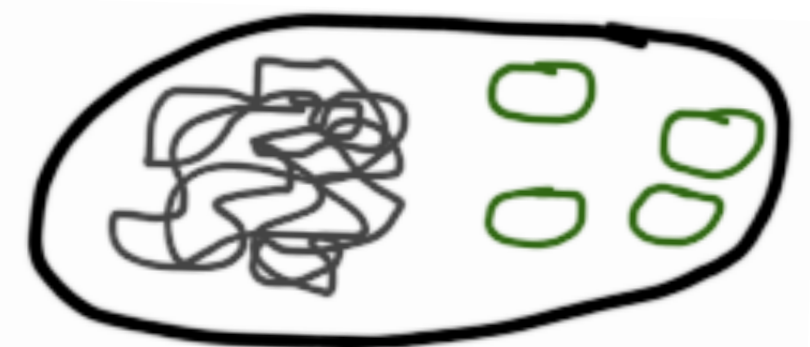


Most simple thing: cloning of gene on plasmid



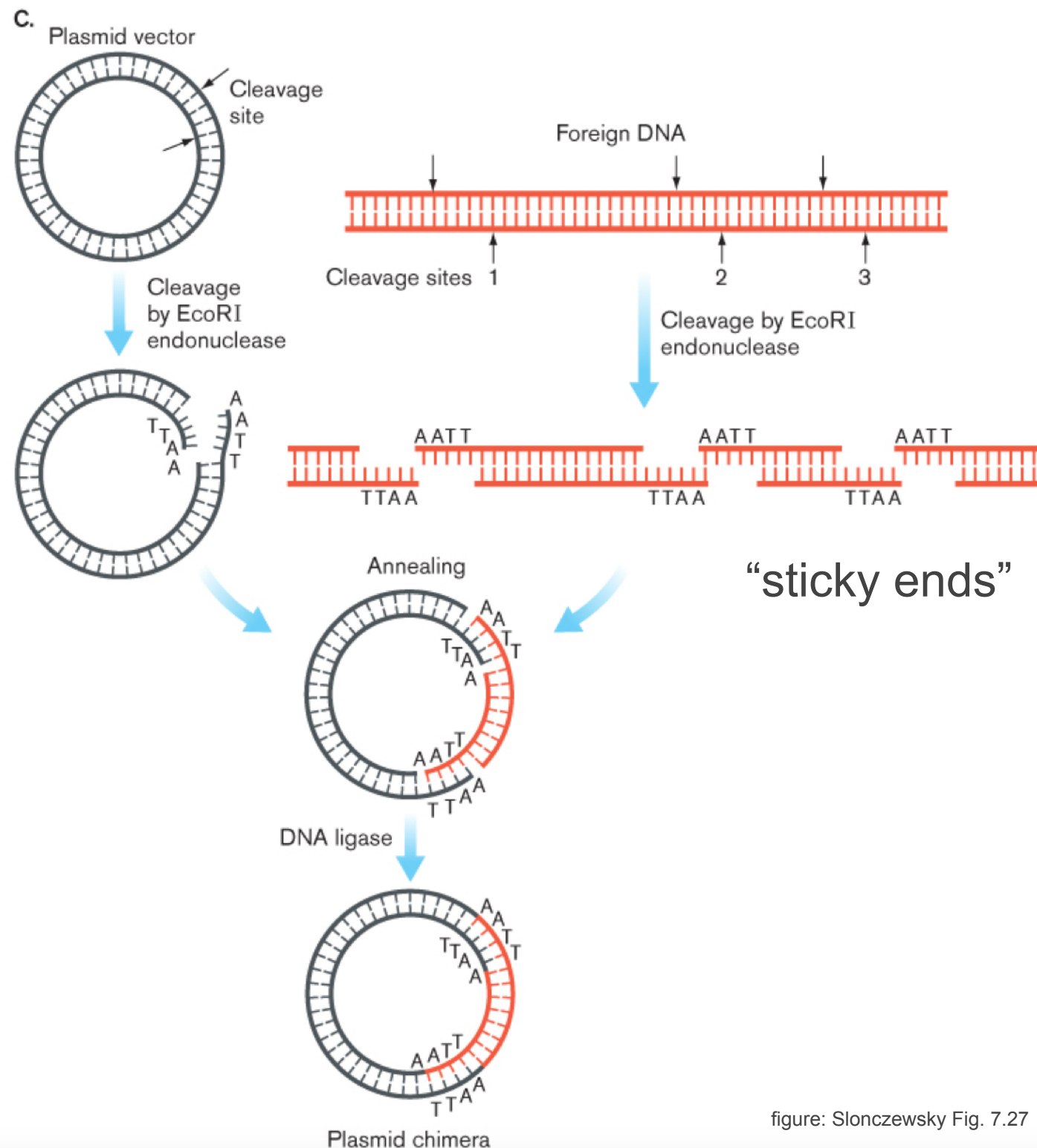
Two options:

- amplification of plasmid
- expression of gene/protein

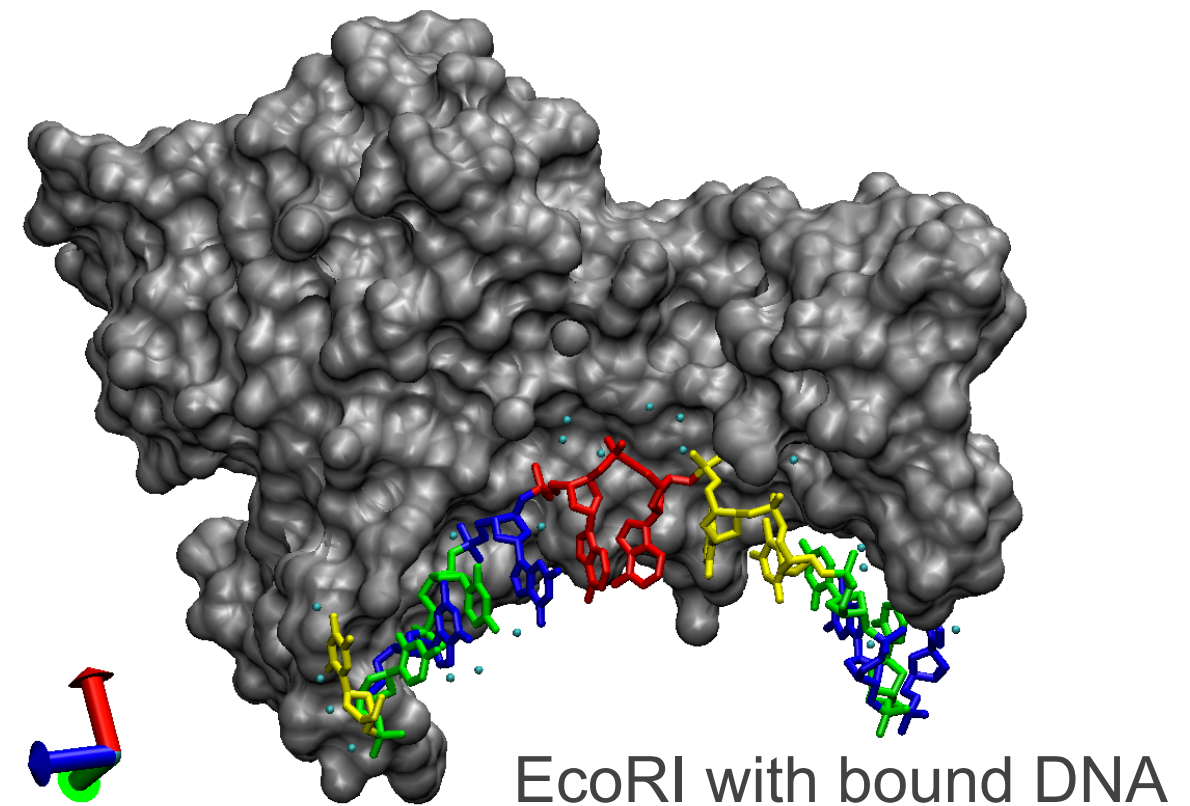


bacterium, often *Escherichia coli*

Cloning by using restriction endonuclease (old school)



cleave palindromic DNA sequence:
EcoRI: GAATTC

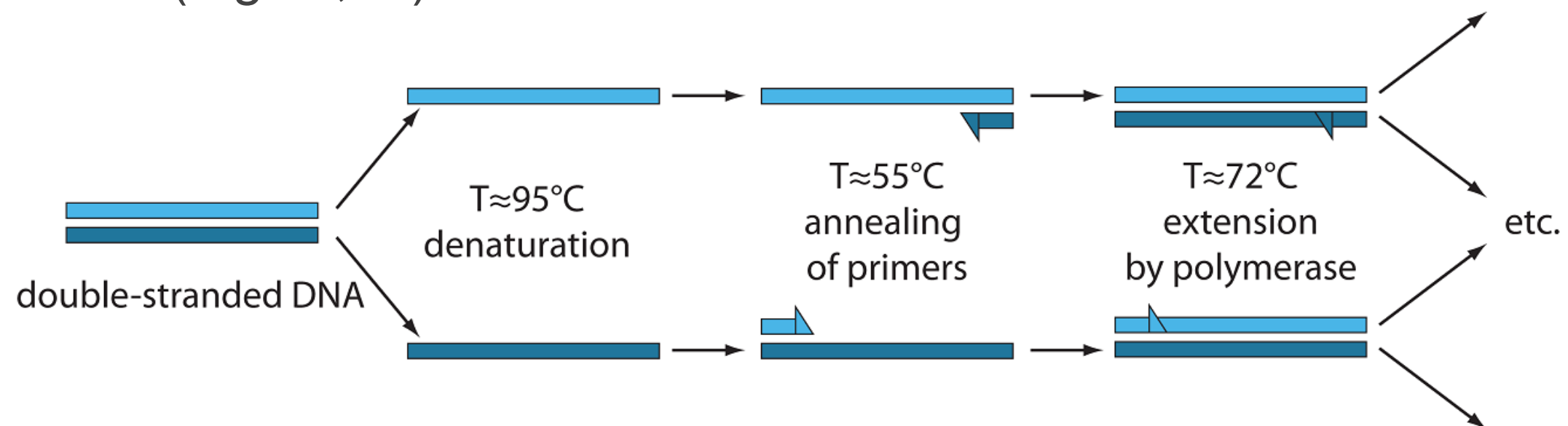


Excursus: Amplification of DNA by PCR

PCR: polymerase chain reaction

What's in the mix:

- DNA template
- DNA primer
- DNA polymerase
- dNTPs
- Buffer (MgCl₂, ...)



35 cycles: $2^{35} = 34$ billion copies



PCR – primer design

ACAGTATATG GACATTGCAA CATGACATCA TTACGAGACG GGATAGTTAA
TGTCATATAC CTCTAACGTT GTACTGTAGT AATGCTCTGC CCTATCAATT

ATGGATGATT TAGTGTTTGC AGG
ATGGATGATT TAGTGTTTGC AGGTAATAAG GCGCTCTATC TTGTTTTGAT
TACCTACTAA ATCACAAACG TCCATTATTC CGCGAGATAG AACAAAACCTA

CCTGTCAGGG TGGCCGACGA TTGTCGCAAC GATTATCGGC CTCCTGGTAG
GGACAGTCCC ACCGGCTGCT AACAGCGTTG CTAATAGCCG GAGGACCATC

GGTTATTCCA GACGGTAACG CAATTACAGG AACAGACGCT GCCTTTTGGC
CCAATAAGGT CTGCCATTGC GTTAATGTCC TTGTCTGCGA CGGAAAACCG

ATTAAATTAC TTGGCGTGTG TTTATGCTTG TTTTACTGT CTGGCTGGTA
TAATTTAATG AACCGCACAC AAATACGAAC AAAAATGACA GACCGACCAT

TGGCGAAGTT TTA CTCTCTT ACGGGCGTCA GGTGATATTC CTGGCGTTGG
ACCGCTTCAA AATGAGAGAA TGCCCGCAGT CCACTATAAG GACCGCAACC
CCGCAACC

CTAAGGGGTA A
GATTCCCAT T
GATTCCCAT T

Forward: 5' – ATGGATGATTTAGTGTTTGCAGG – 3'
Reverse: 5' – TTACCCCTTAGCAGGTGAT – 3'



Cloning a PCR product

ACAGTATATG GACATTGCAA CATGACATCA TTACGAGACG GGATAGTTAA
TGTCATATAC CTCTAACGTT GTACTGTAGT AATGCTCTGC CCTATCAATT

ATGGATGATT TAGTGTTTGC AGG
ATGGATGATT TAGTGTTTGC AGGTAATAAG GCGCTCTATC TTGTTTTGAT
TACCTACTAA ATCACAAACG TCCATTATTC CGCGAGATAG AACAAAACATA

CCTGTCAGGG TGGCCGACGA TTGTCGCAAC GATTATCGGC CTCCTGGTAG
GGACAGTCCC ACCGGCTGCT AACAGCGTTG CTAATAGCCG GAGGACCATC

GGTTATTCCA GACGGTAACG CAATTACAGG AACAGACGCT GCCTTTTGGC
CCAATAAGGT CTGCCATTGC GTTAATGTCC TTGTCTGCGA CGGAAAACCG

ATTAAATTAC TTGGCGTGTG TTTATGCTTG TTTTACTGT CTGGCTGGTA
TAATTTAATG AACCGCACAC AAATACGAAC AAAAATGACA GACCGACCAT

TGGCGAAGTT TTA CTCTCTT ACGGGCGTCA GGTGATATTC CTGGCGTTGG
ACCGCTTCAA AATGAGAGAA TGCCCGCAGT CCACTATAAG GACCGCAACC
CCGCAACC

CTAAGGGGTA A
GATTCCCAT T
GATTCCCAT T

Addition of a restriction site: EcoRI

Forward: 5' – GAATTC ATGGATGATTTAGTGTTTGCAGG – 3'
Reverse: 5' – GAATTC TTACCCCTTAGCAGGTGAT – 3'



Cloning a PCR product

PCR Product:

```
GAATTC ATGGATGATT TAGTGTTTGC AGGTAATAAG GCGCTCTATC ...  
CTTAAG TACCTACTAA ATCACAAACG TCCATTATTC CGCGAGATAG
```

```
ACGGGCGTCA GGTGATATTC CTGGCGTTGG CTAAGGGGTA A GAATTC  
TGCCCGCAGT CCACTATAAG GACCGCAACC GATTCCCAT T CTTAAG
```

After EcoRI digest:

```
AATTC ATGGATGATT TAGTGTTTGC AGGTAATAAG GCGCTCTATC ...  
G TACCTACTAA ATCACAAACG TCCATTATTC CGCGAGATAG
```

```
ACGGGCGTCA GGTGATATTC CTGGCGTTGG CTAAGGGGTA A G  
TGCCCGCAGT CCACTATAAG GACCGCAACC GATTCCCAT T CTTAA
```

=> into EcoRI-cut vector

Gibson Assembly

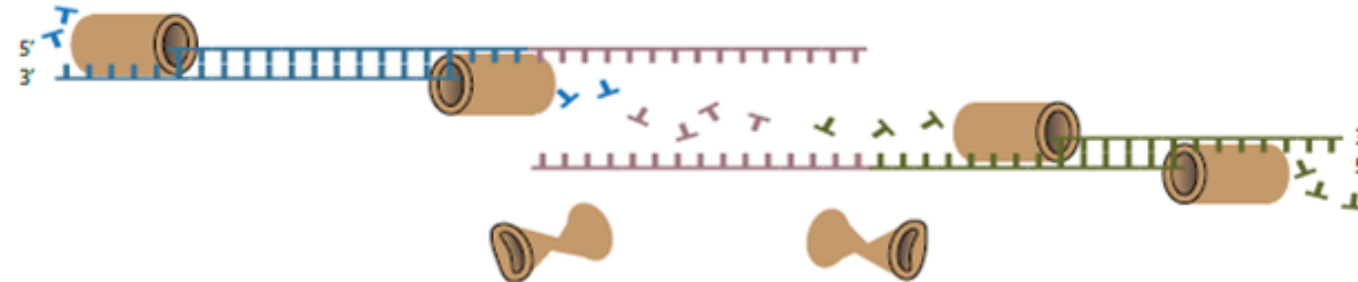
no restriction site but
complementary to the plasmid



Step 1.



Step 2.



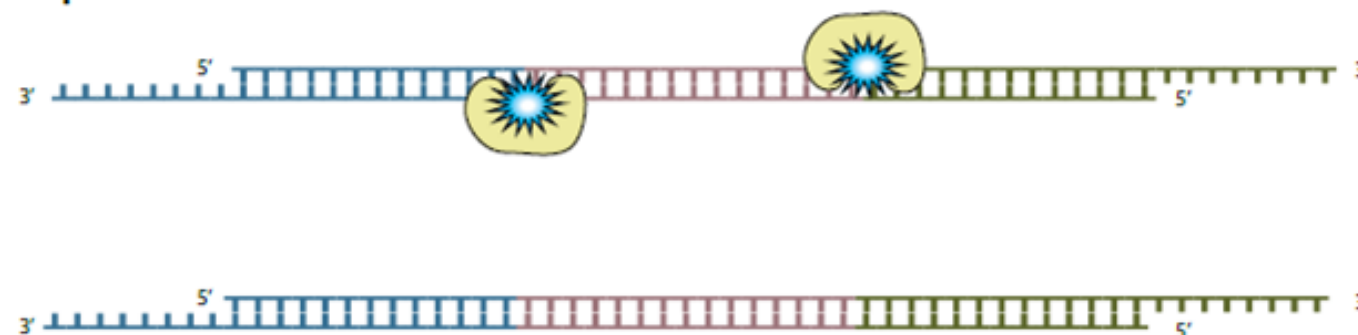
T5 exonuclease

Step 3 and 4.

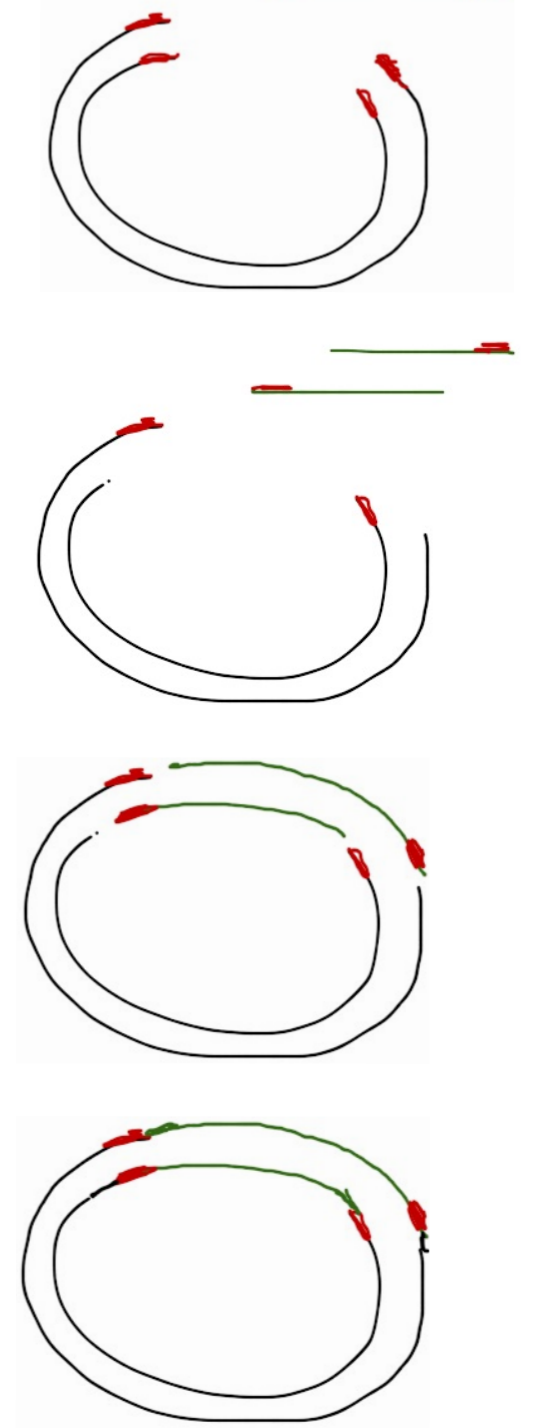


polymerase

Step 5.



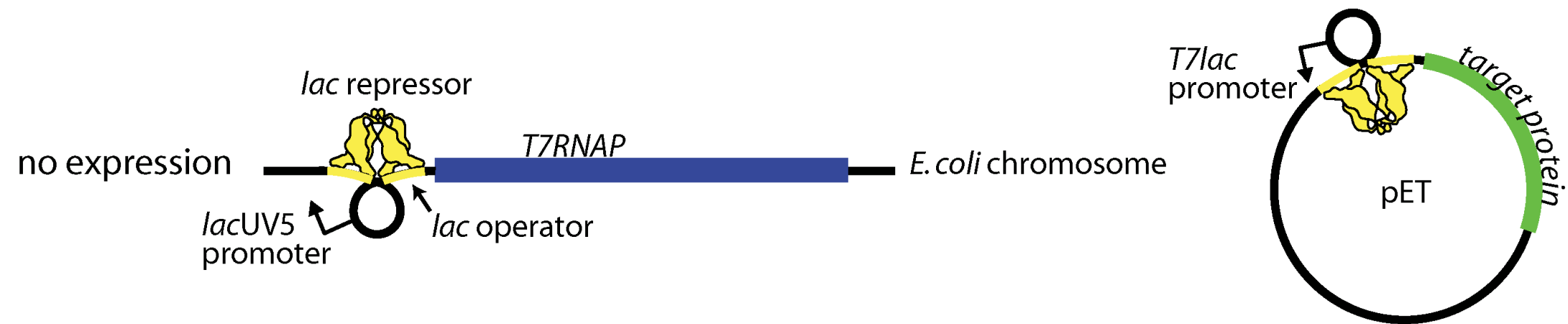
Taq ligase



all at 50°C => isothermal assembly, just 10 - 30 min



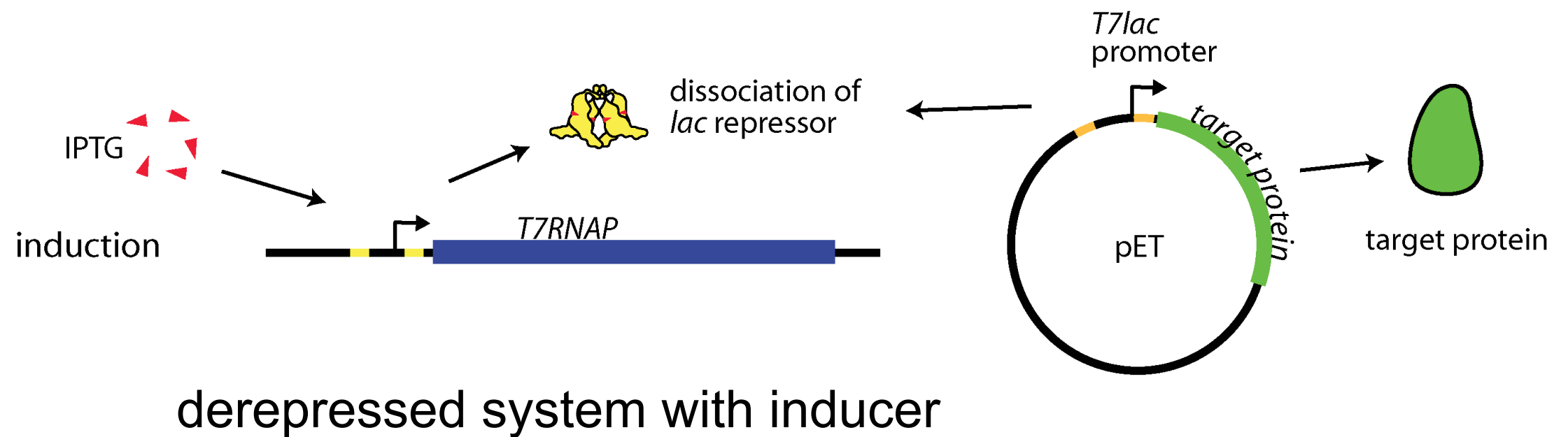
Example: Protein expression system Lemo21



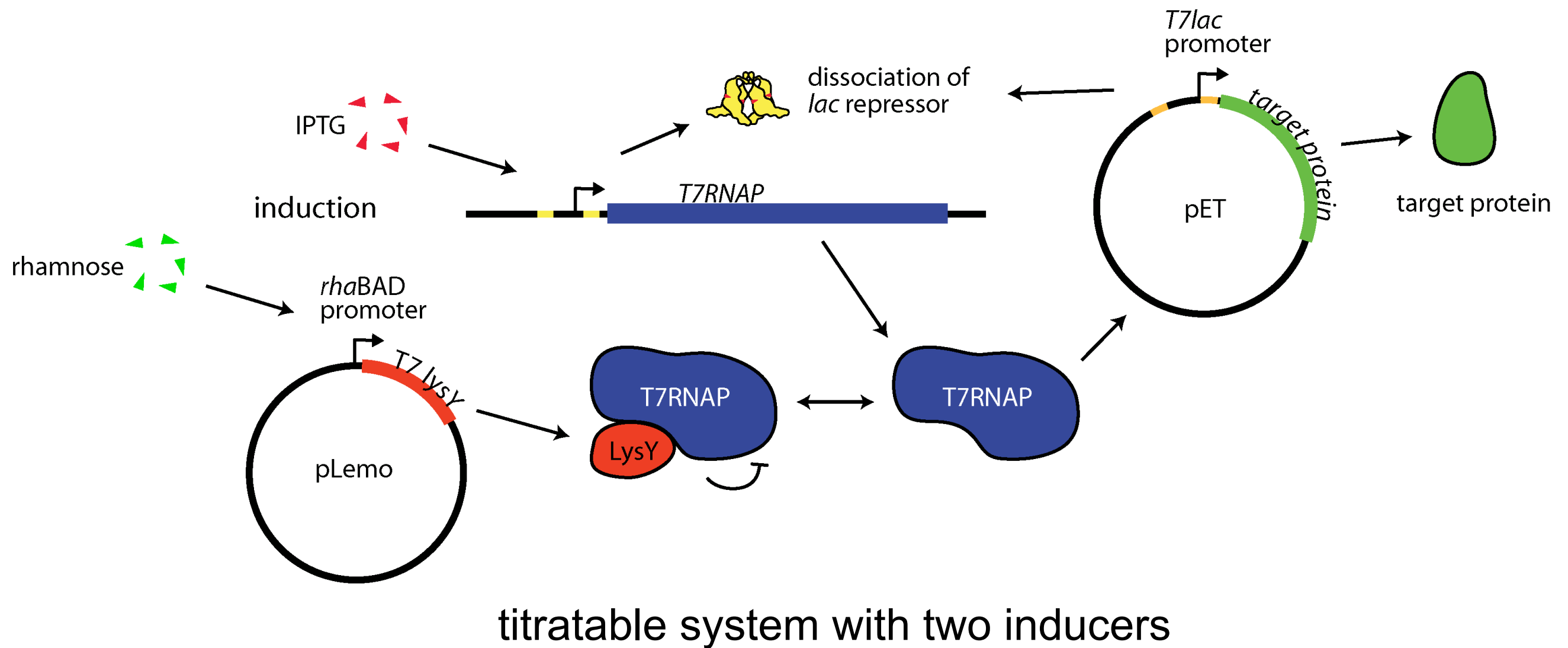
repressed system without inducer



Example: Protein expression system Lemo21



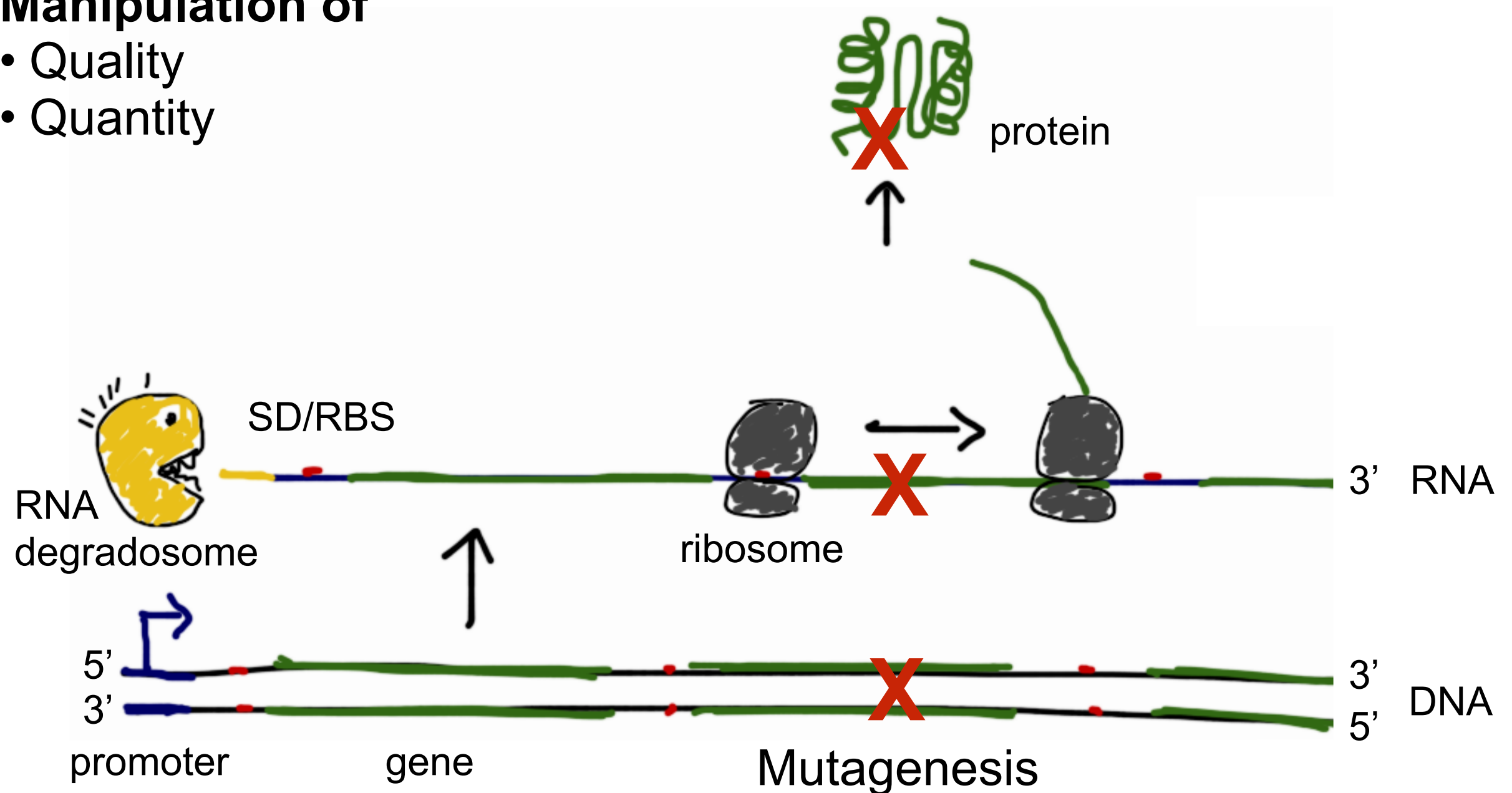
Example: Protein expression system Lemo21



In the end it is all about making proteins...

Manipulation of

- Quality
- Quantity



Mutagenesis

random mutagenesis vs. **site-directed** mutagenesis

random:

- PCR products – error prone PCR, e.g. addition of Mn^{2+} , disbalance of dNTPs
- Plasmids – amplification in strains with impaired DNA repair mechanisms
- Organisms – addition of mutagens, infection with virus

site-directed:

- PCR amplification with primers containing specific mutation

ATG	GAT	GAT	TTA	GTG	TTT	GCA	GGT	AAT	AAG	GCG	CTC	TAT	CTT	GTT
TAC	CTA	CTA	AAT	CAC	AAA	CGT	CCA	TTA	TTC	CGC	GAG	ATA	GAA	CAA
M	D	D	L	V	F	A	G	N	K	A	L	Y	L	V

TTG	ATC	CTG	TCA	GGG	TGG	CCG	ACG	ATT	GTC	GCA	ACG	ATT	ATC	GGC
AAC	TAG	GAC	AGT	CCC	ACC	GGC	TGC	TAA	CAG	CGT	TGC	TAA	TAG	CCG
L	I	L	S	G	W	P	T	I	V	A	T	I	I	G

Mutation at N9

Mutagenesis

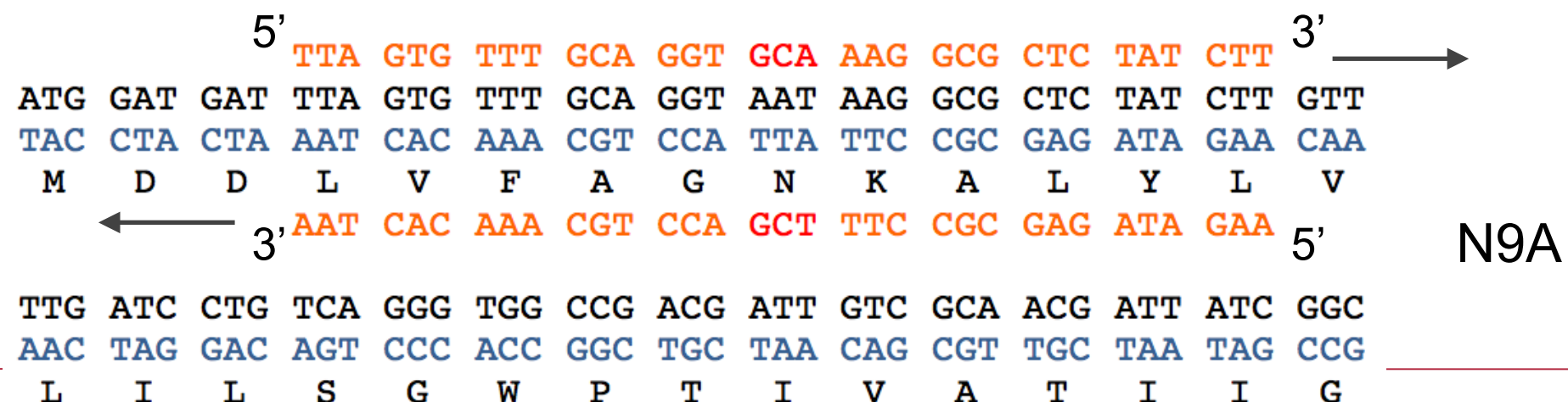
random mutagenesis vs. **site-directed** mutagenesis

random:

- PCR products – error prone PCR, e.g. addition of Mn^{2+} , disbalance of dNTPs
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site-directed:

- PCR amplification with primers containing specific mutation



Mutagenesis

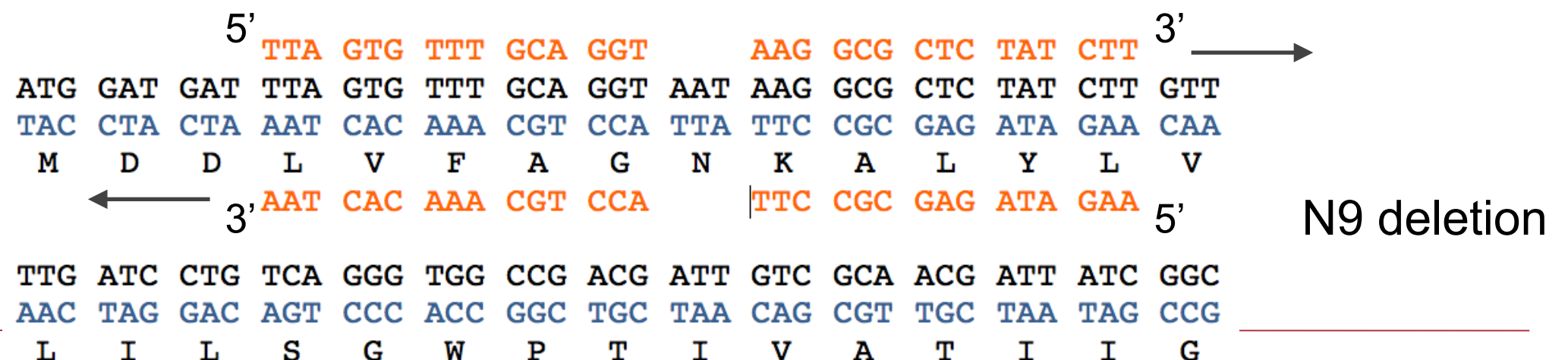
random mutagenesis vs. site-directed mutagenesis

random:

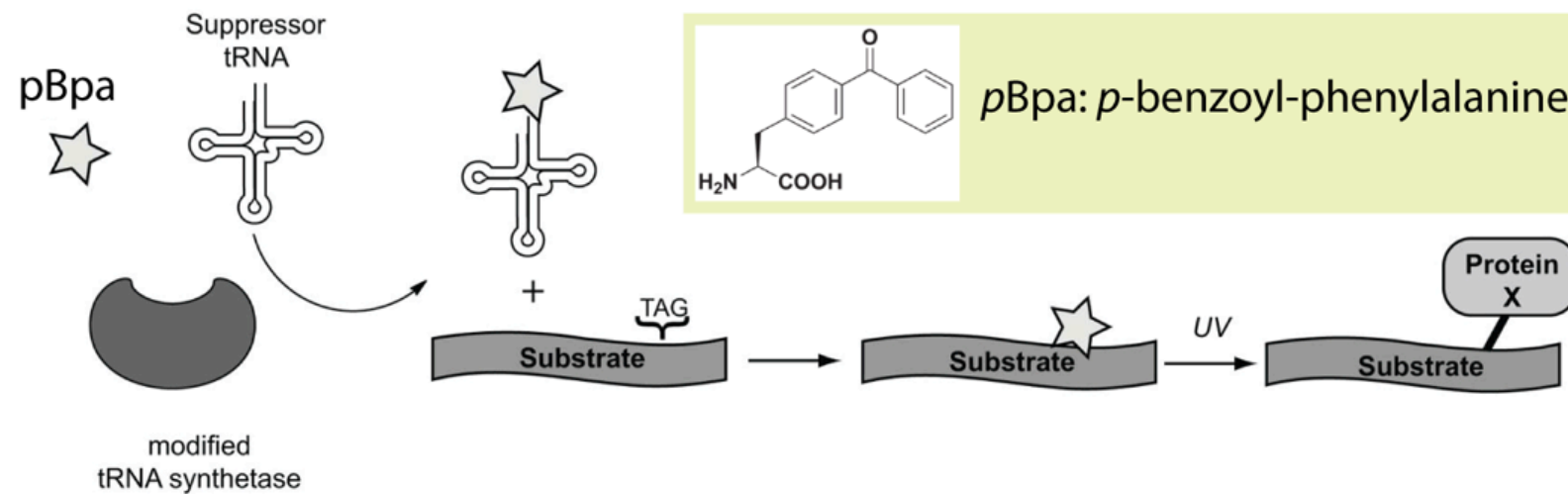
- PCR products – error prone PCR, e.g. addition of Mn^{2+} , disbalance of dNTPs
- Plasmids – amplification in strains with impaired DNA repair mechanisms
- Organisms – addition of mutagens, infection with virus

site-directed:

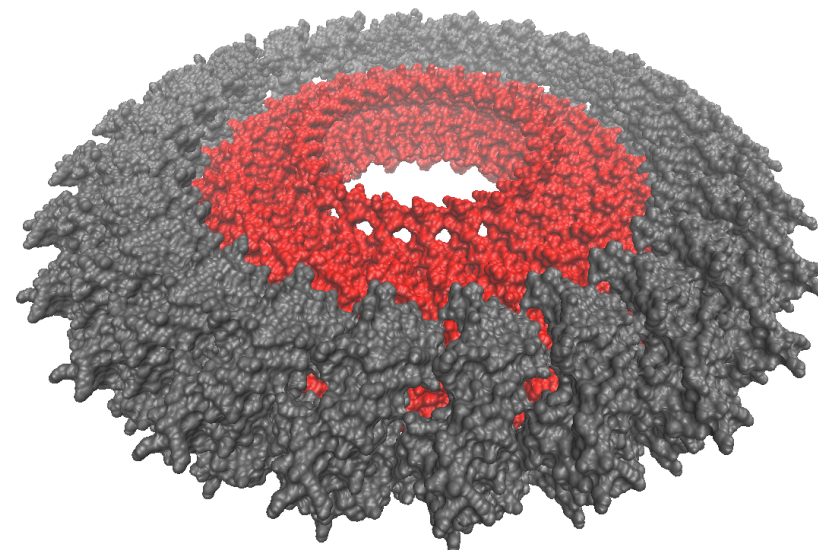
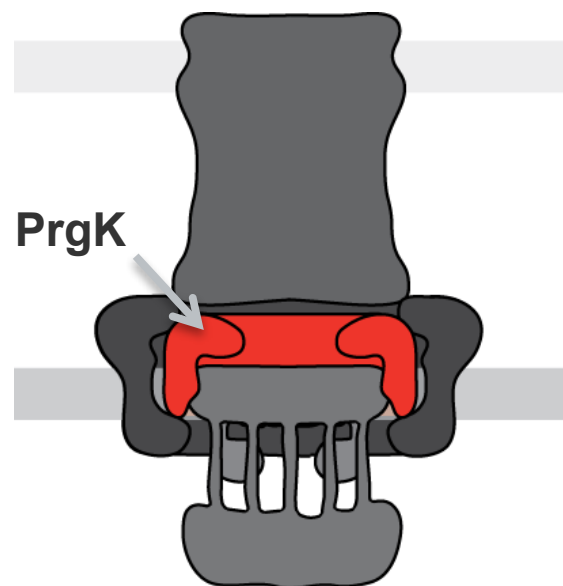
- PCR amplification with primers containing specific mutation



Example: Site-specific *in vivo* photocrosslinking

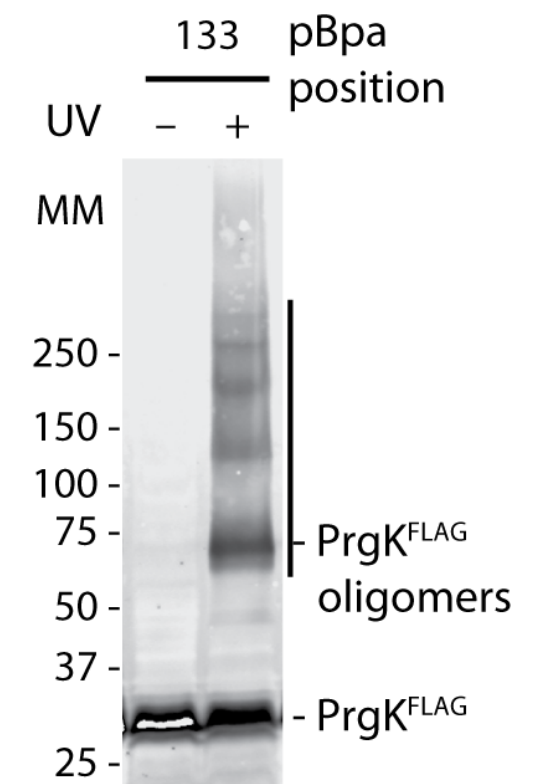
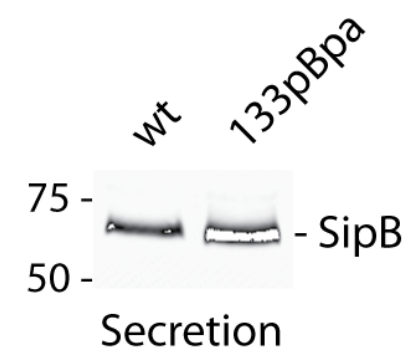
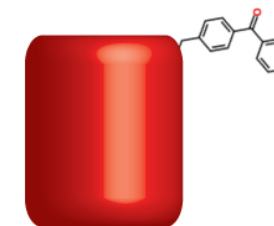


Adapted from Stanley et al. 2011. FEBS Lett



Schraidt, Marlovits. 2011. Science

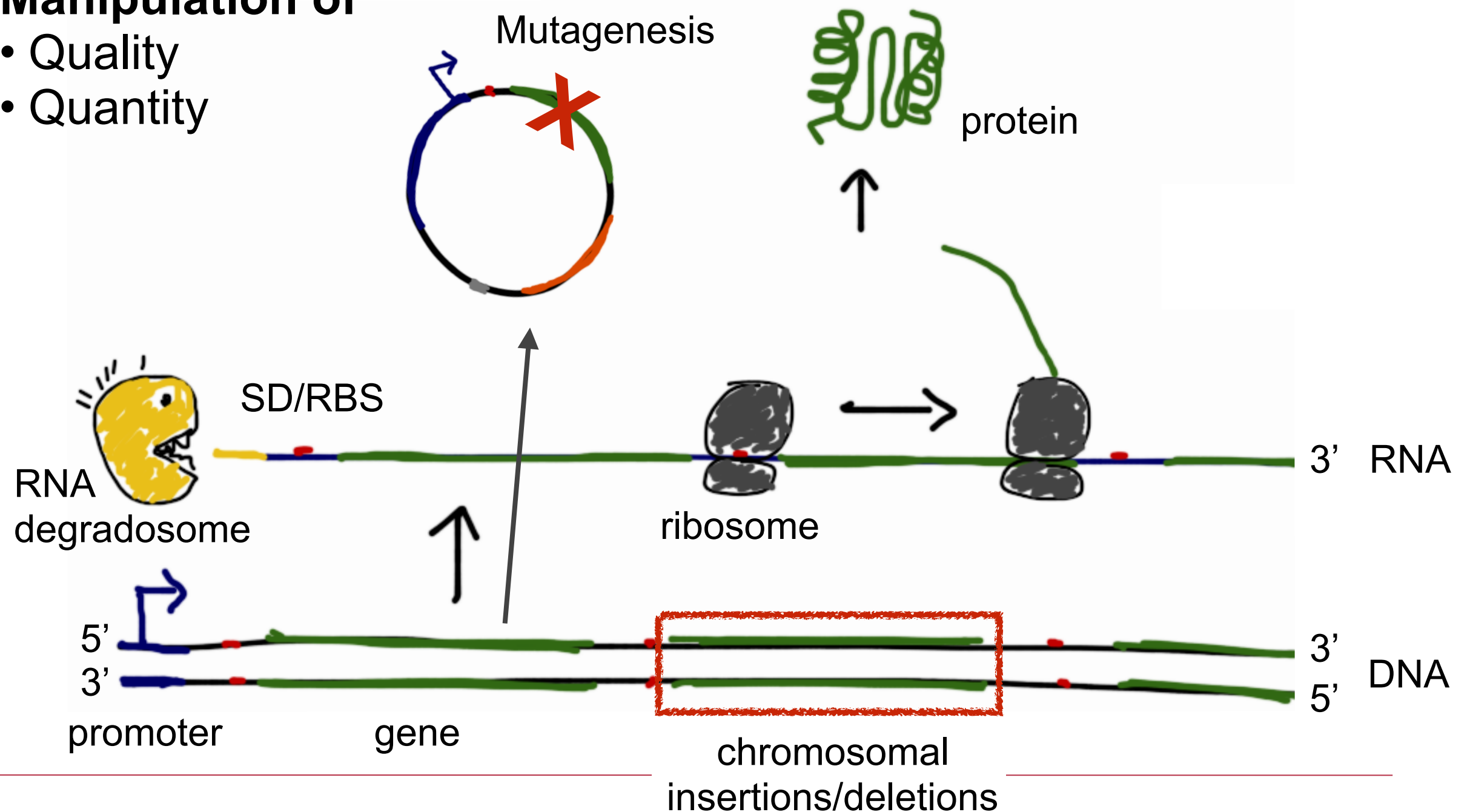
PrgK^{FLAG}



In the end it is all about making proteins... or not making

Manipulation of

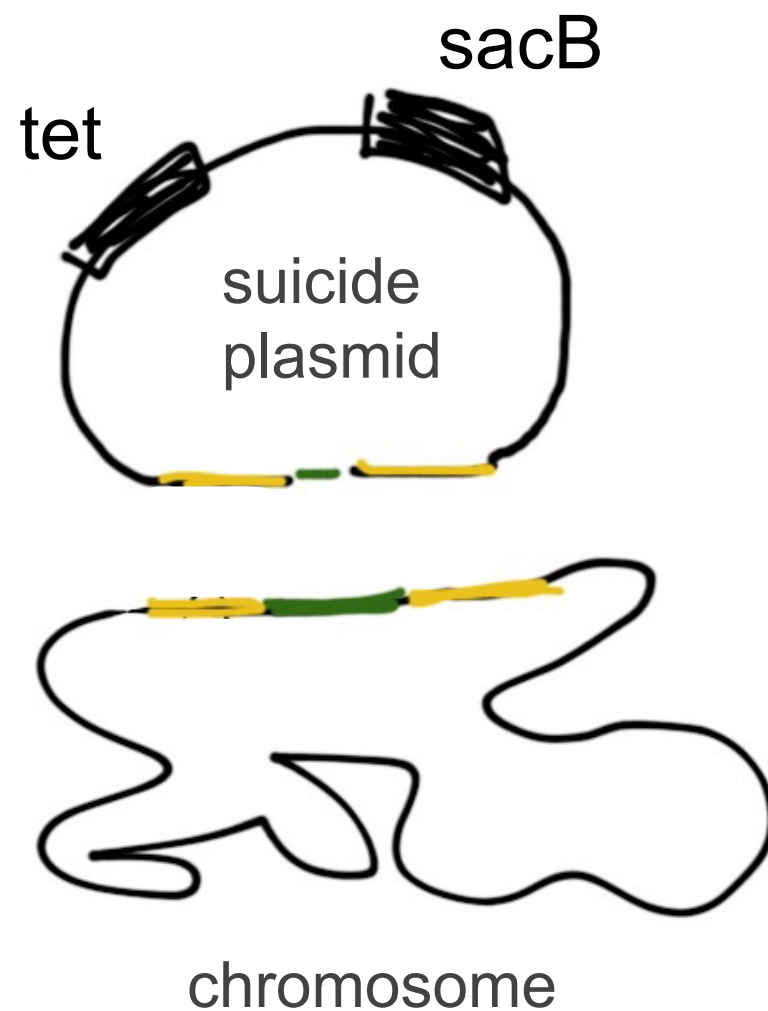
- Quality
- Quantity



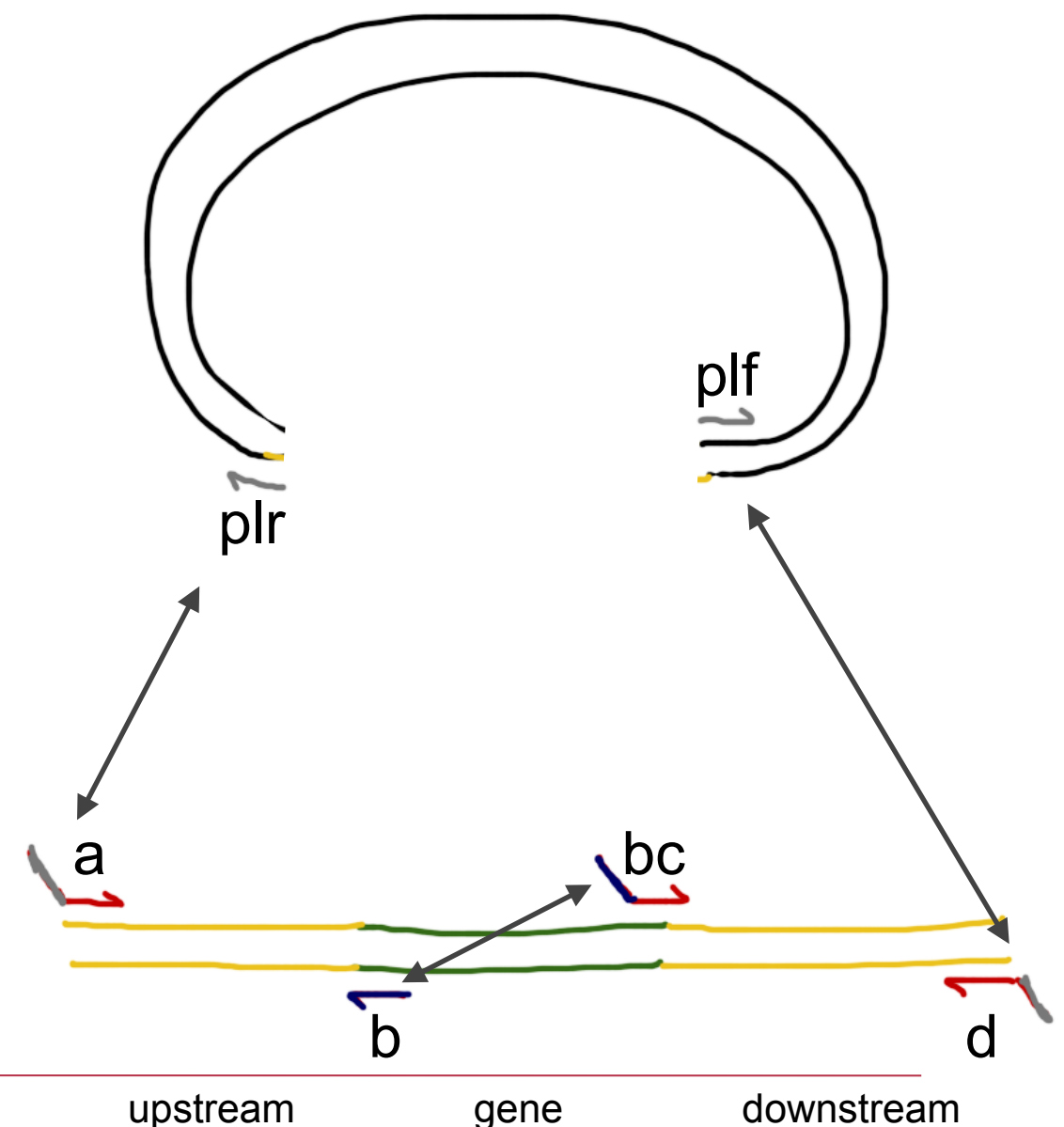
Modifying bacterial chromosomes – Allelic exchange

Using inherent homologues recombination to seamlessly exchange alleles

Making the suicide plasmid

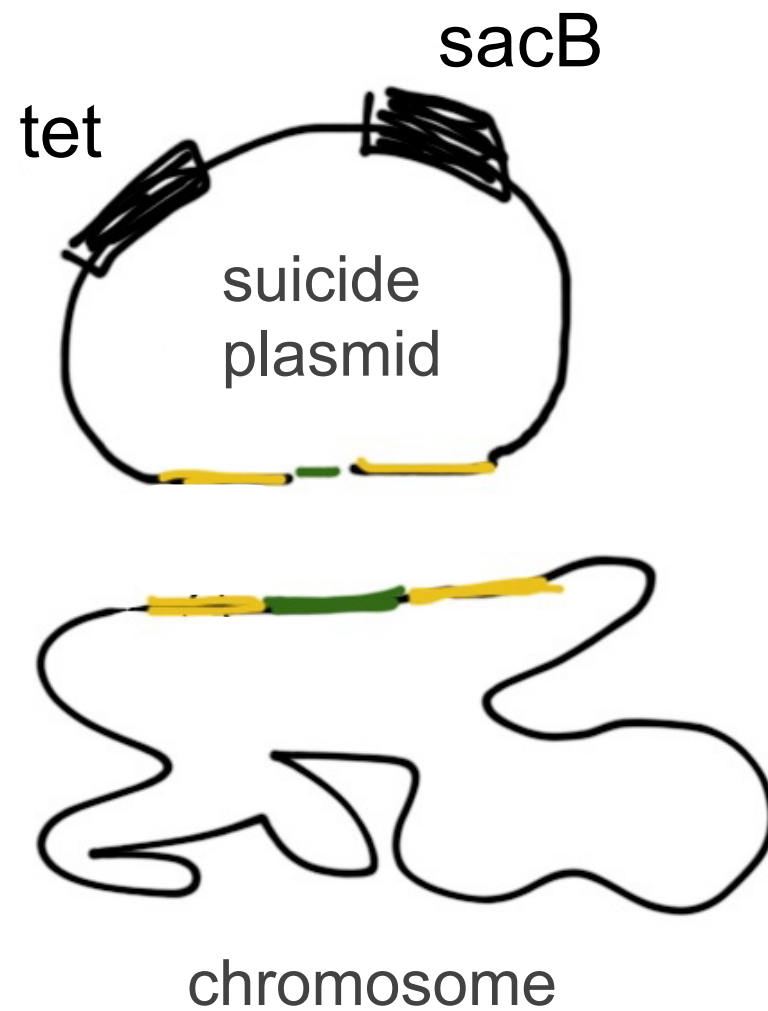


1. PCR



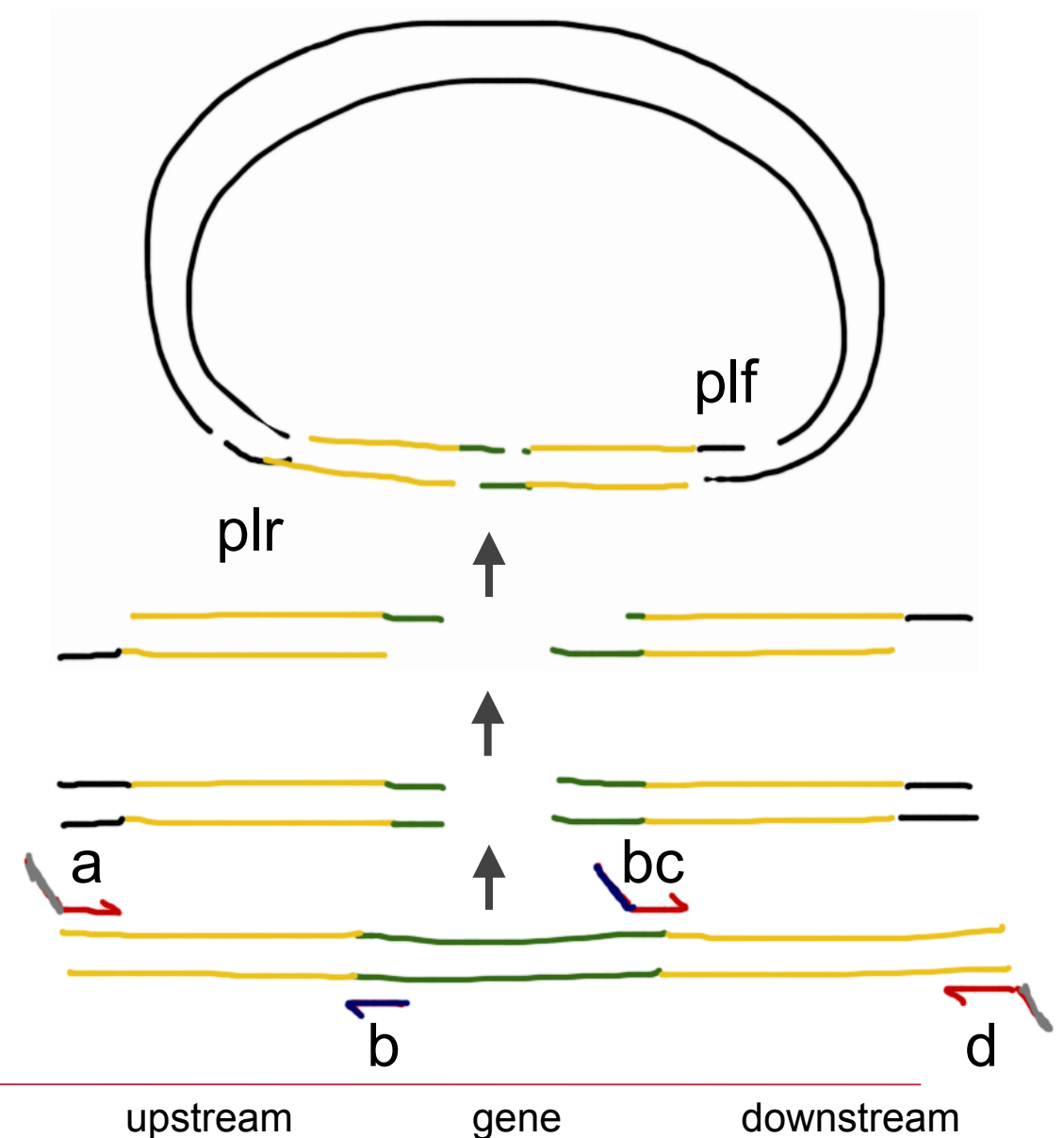
Modifying bacterial chromosomes – Allelic exchange

Making the suicide plasmid



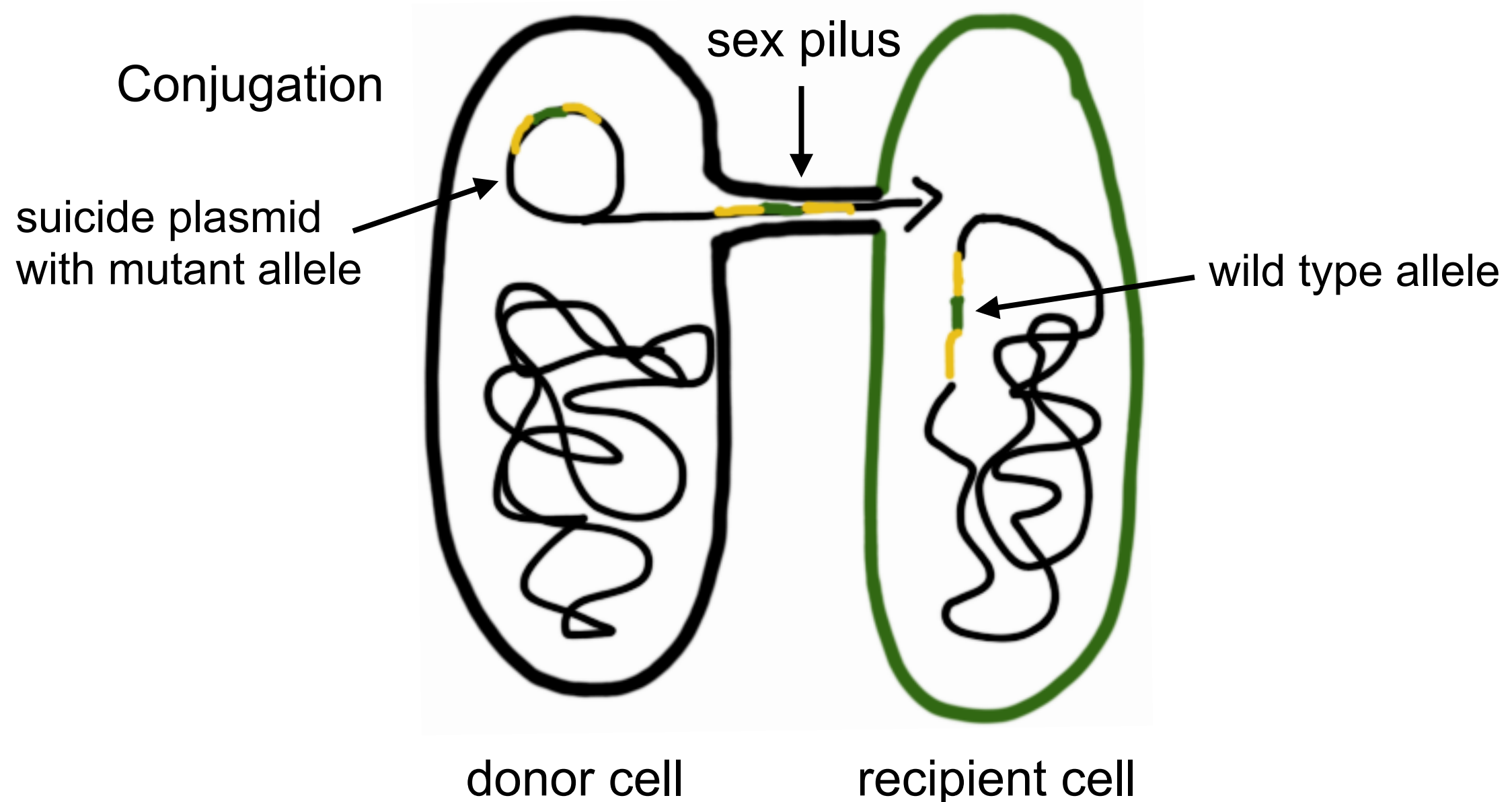
2. Gibson assembly

1. PCR

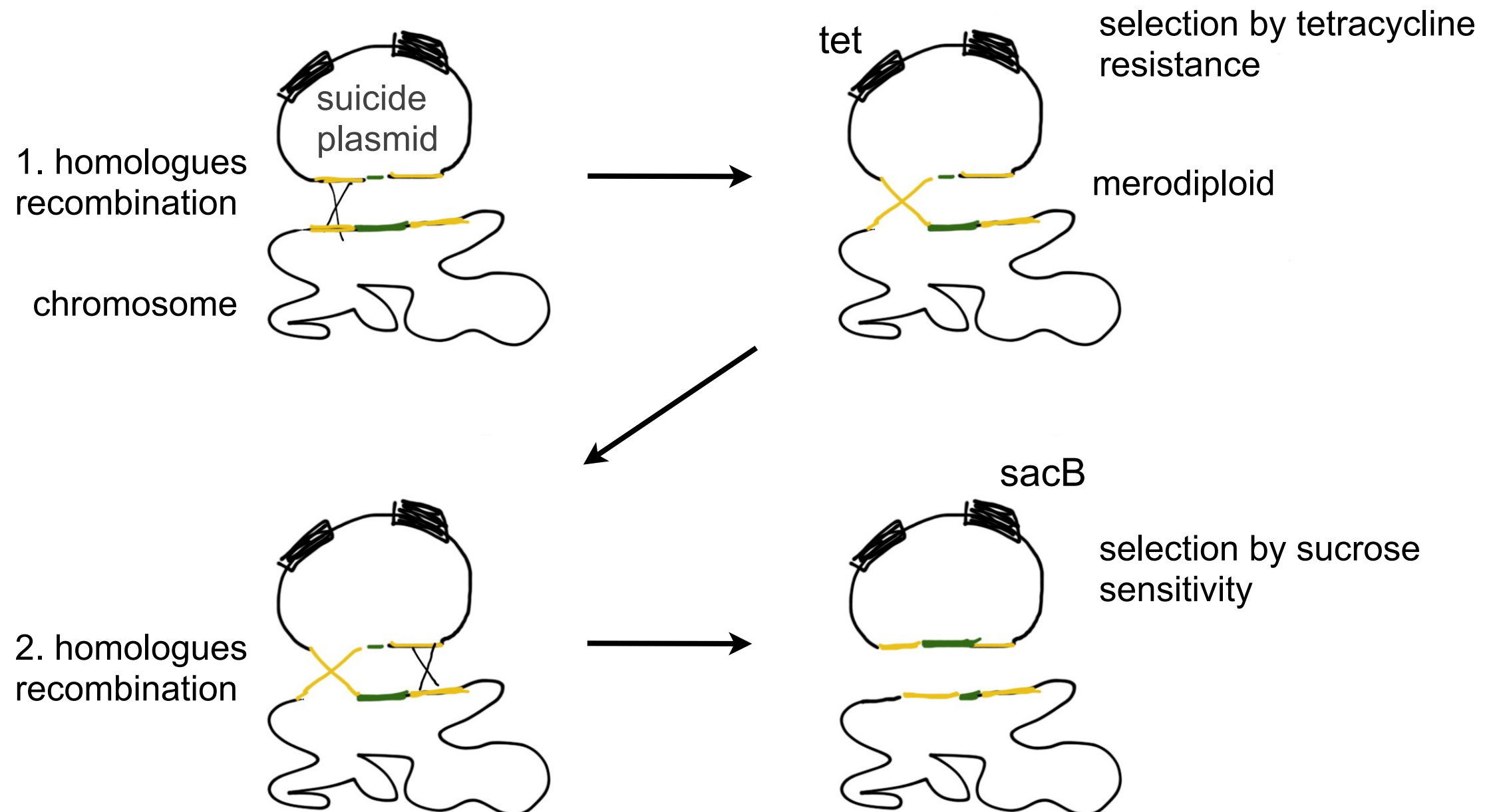


Modifying bacterial chromosomes – Allelic exchange

Mating bacteria



Modifying bacterial chromosomes – Allelic exchange



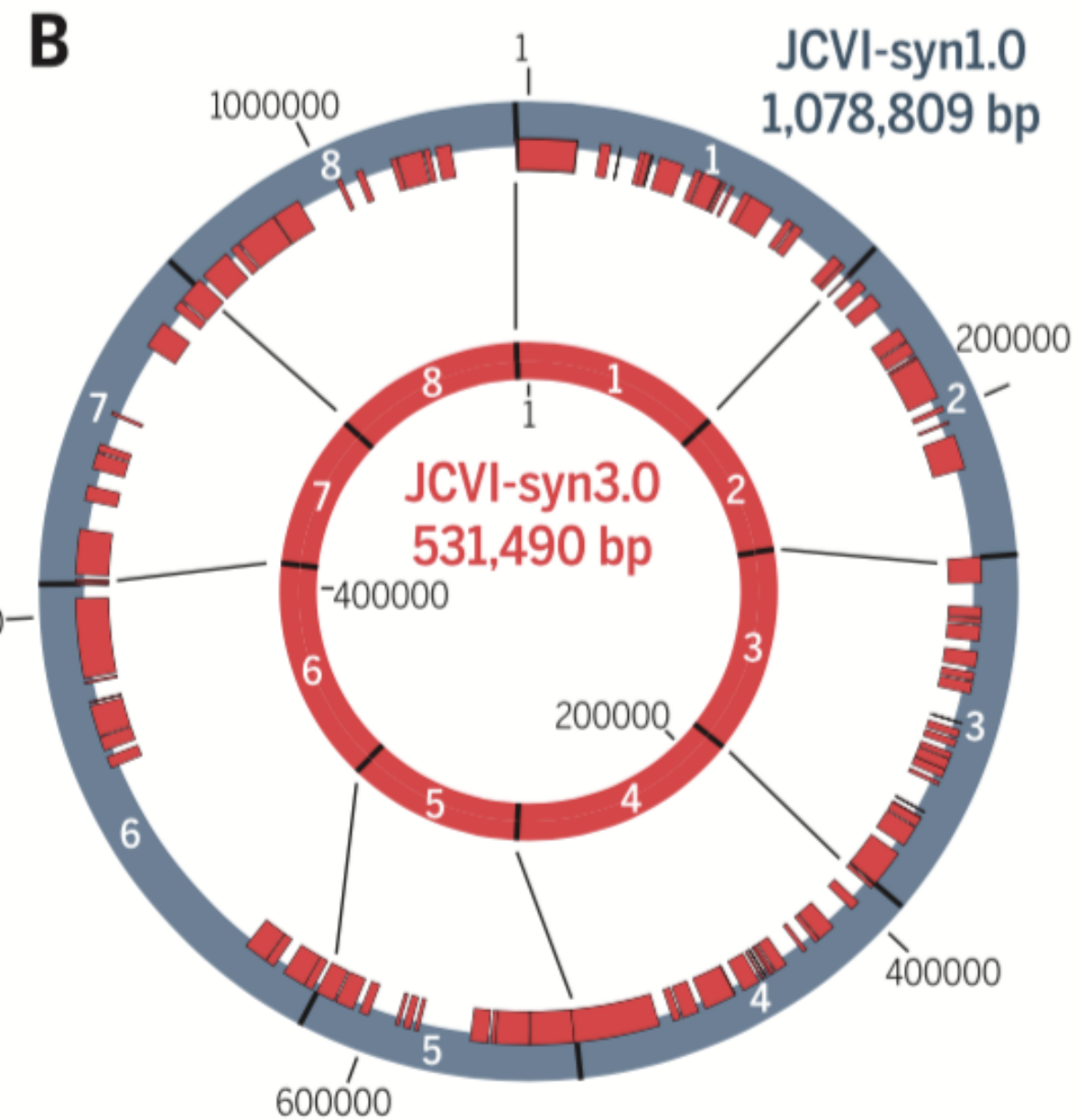
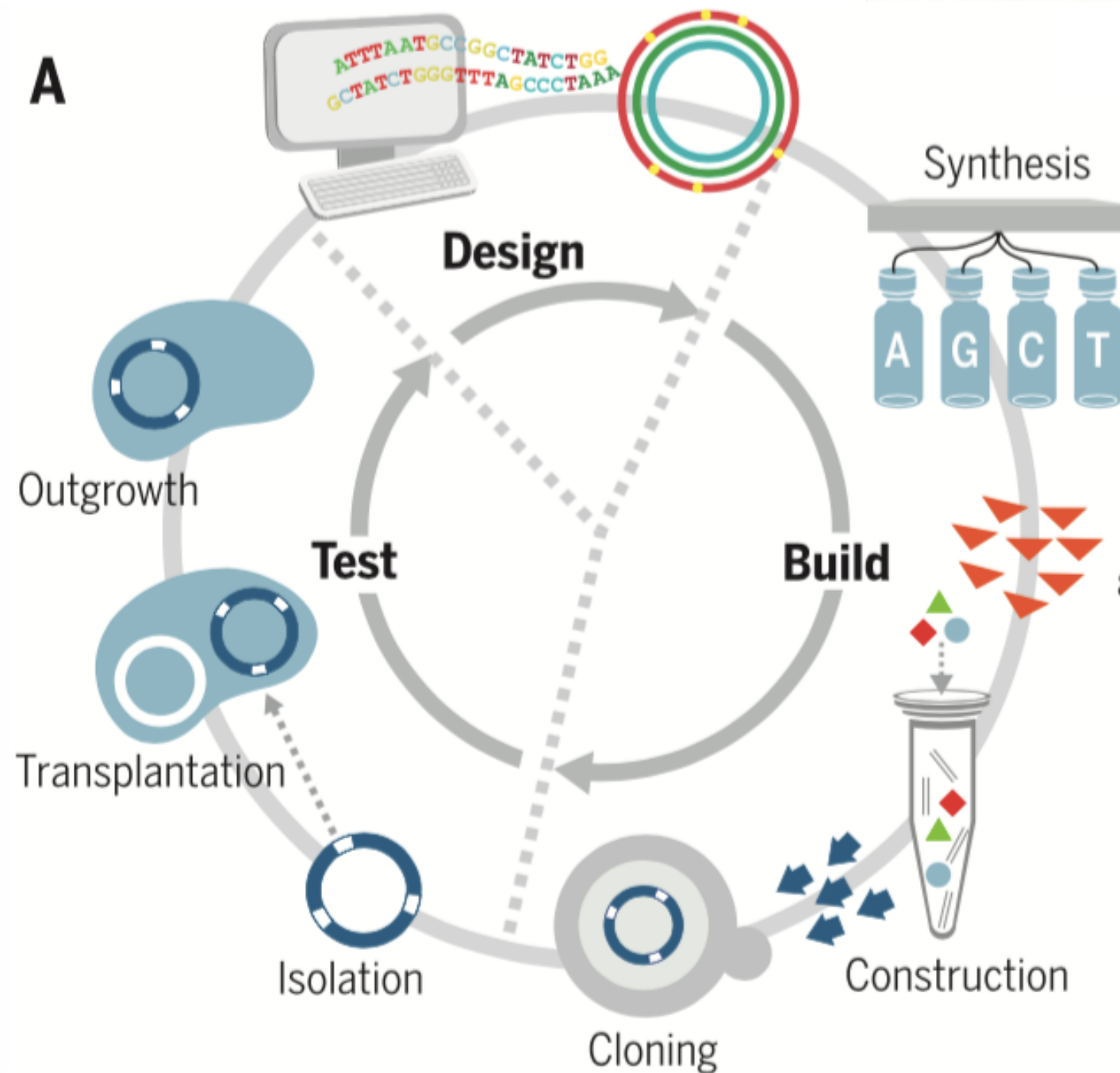
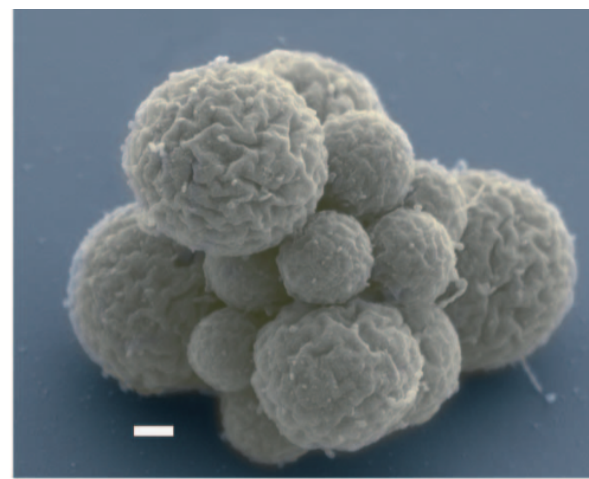


RESEARCH ARTICLE SUMMARY

SYNTHETIC BIOLOGY

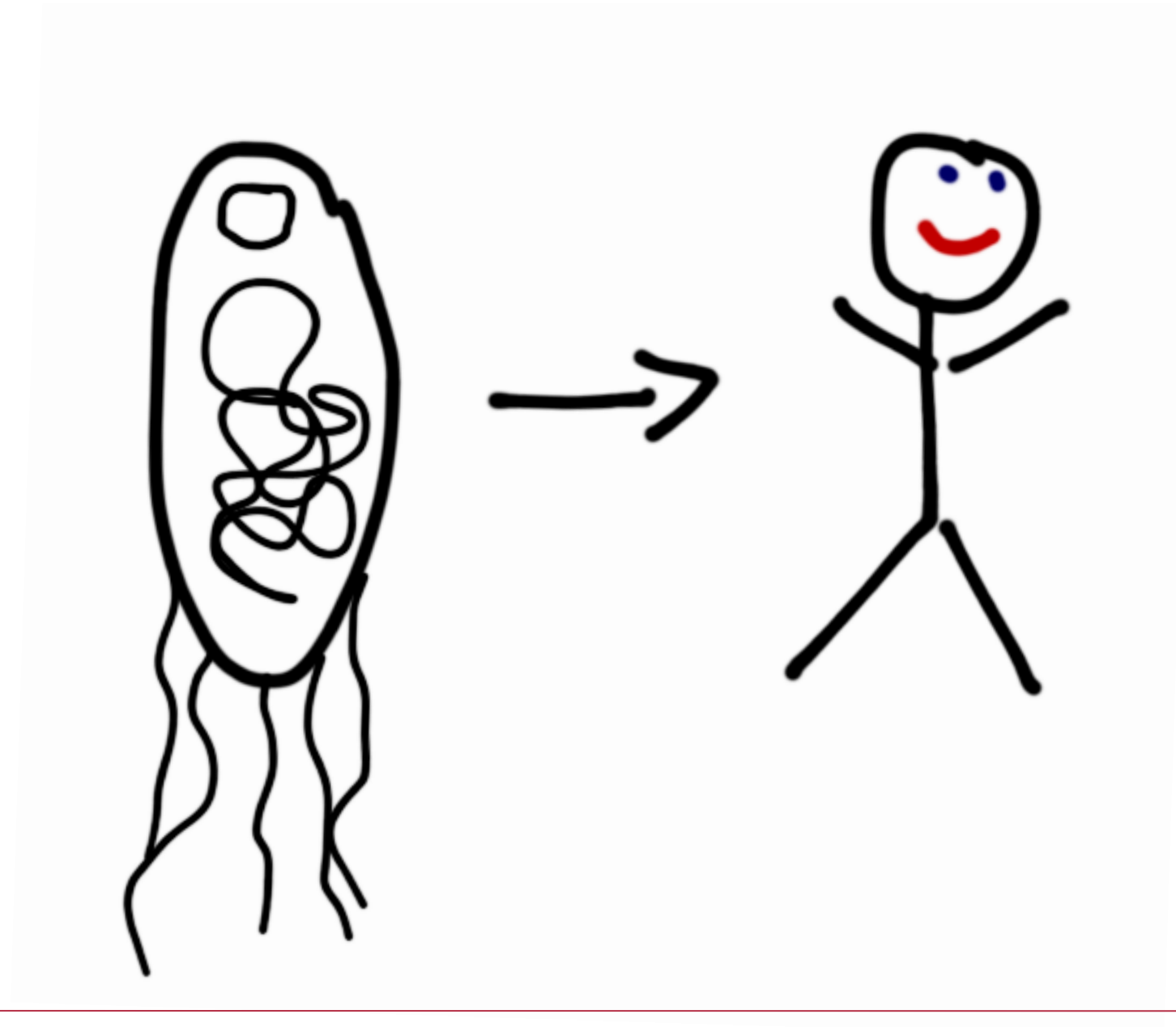
Design and synthesis of a minimal bacterial genome

Clyde A. Hutchison III,^{*†} Ray-Yuan Chuang,[†] Vladimir N. Noskov, Nacyra Assad-Garcia, Thomas J. Deerinck, Mark H. Ellisman, John Gill, Krishna Kannan, Bogumil J. Karas, Li Ma, James F. Pelletier, Zhi-Qing Qi, R. Alexander Richter, Elizabeth A. Strychalski, Lijie Sun, Yo Suzuki, Billyana Tsvetanova, Kim S. Wise, Hamilton O. Smith, John I. Glass, Chuck Merryman, Daniel G. Gibson, J. Craig Venter^{*}



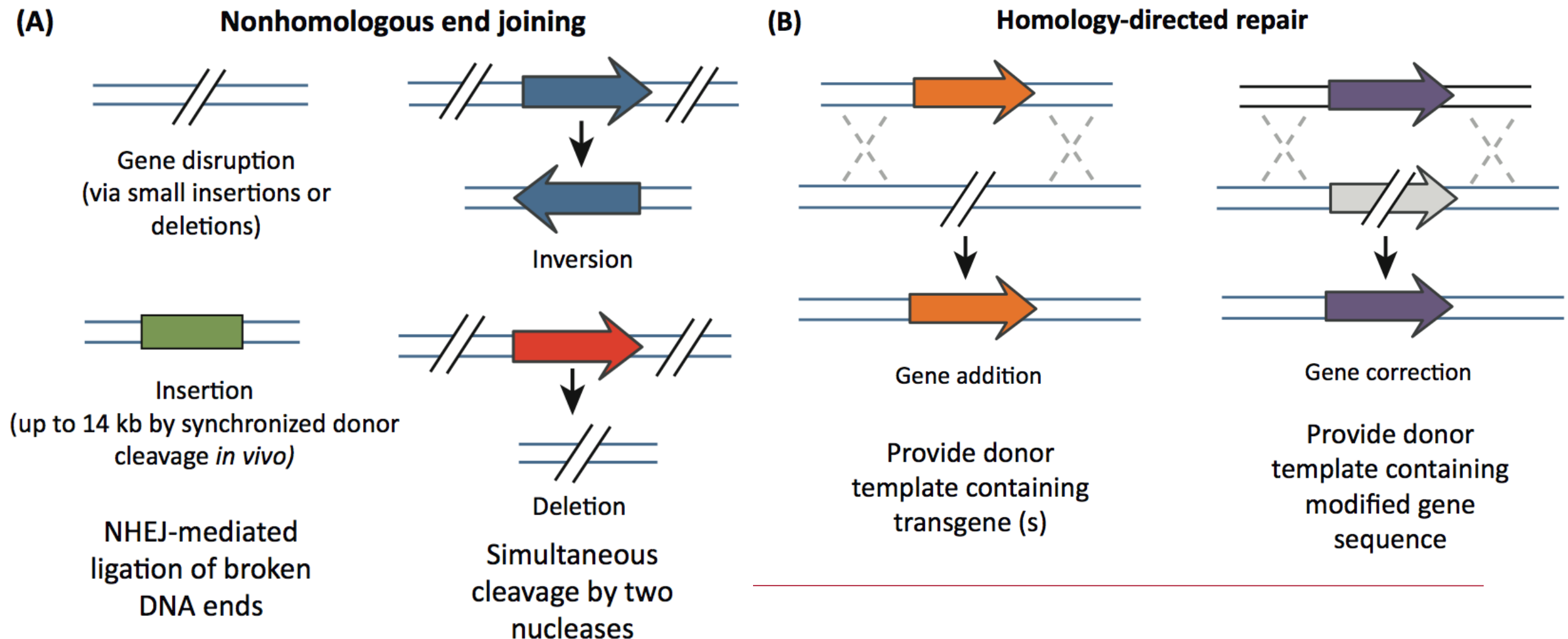


‘almost’ leaving bacteria and focusing on eukaryotes



Editing eukaryotic genomes

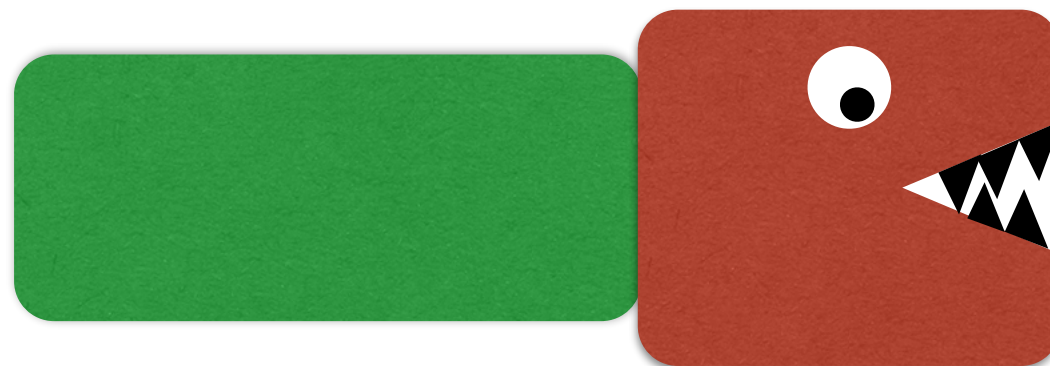
- homologues recombination is very inefficient in many eukaryotes
- introduction of **double strand breaks** activates DNA repair by either
 - **non-homologous end joining**
 - **homology-directed repair**





Engineered sequence-specific nucleases to induce targeted double-strand breaks

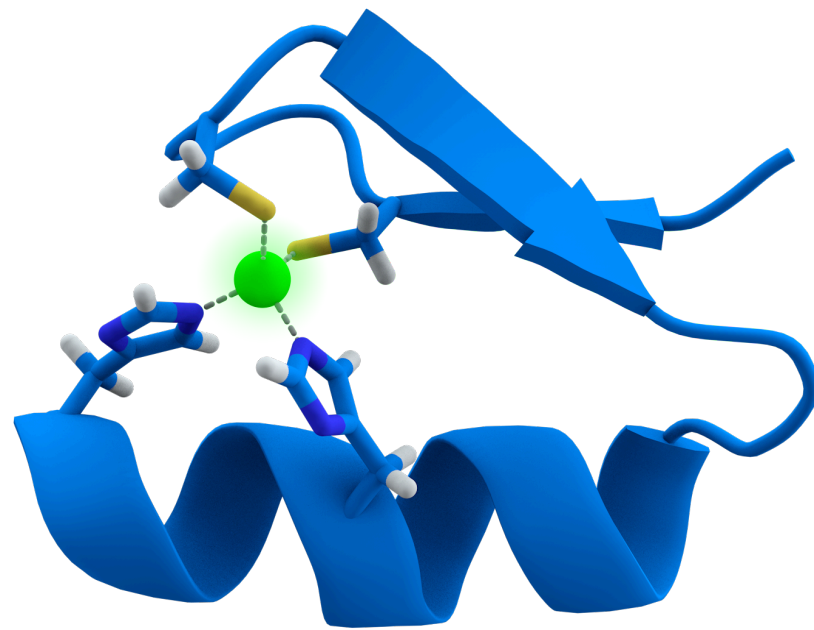
nonspecific DNA
cleavage module



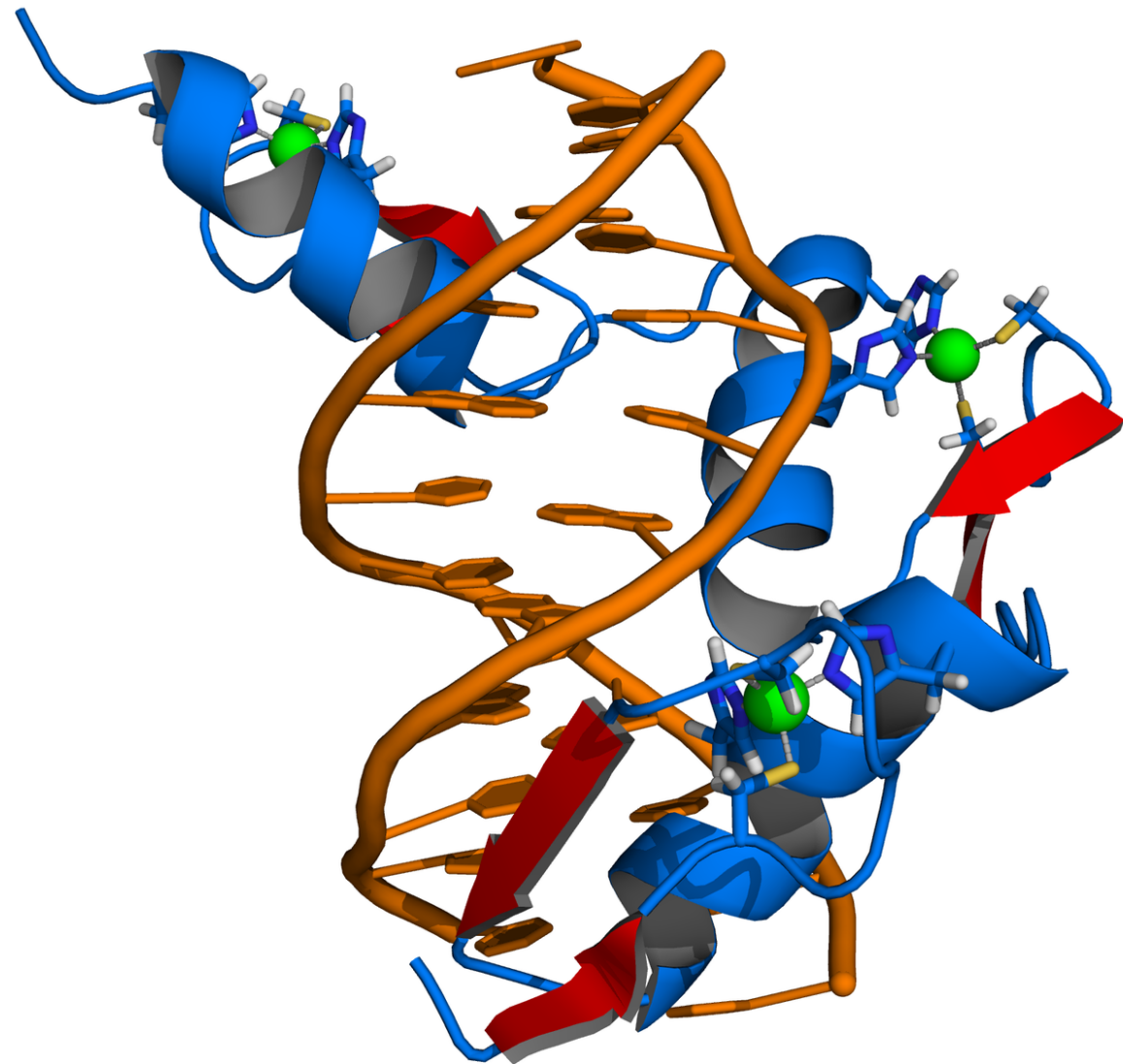
sequence-specific
DNA-binding domain

How to target a specific gene/DNA sequence?

Zinc fingers

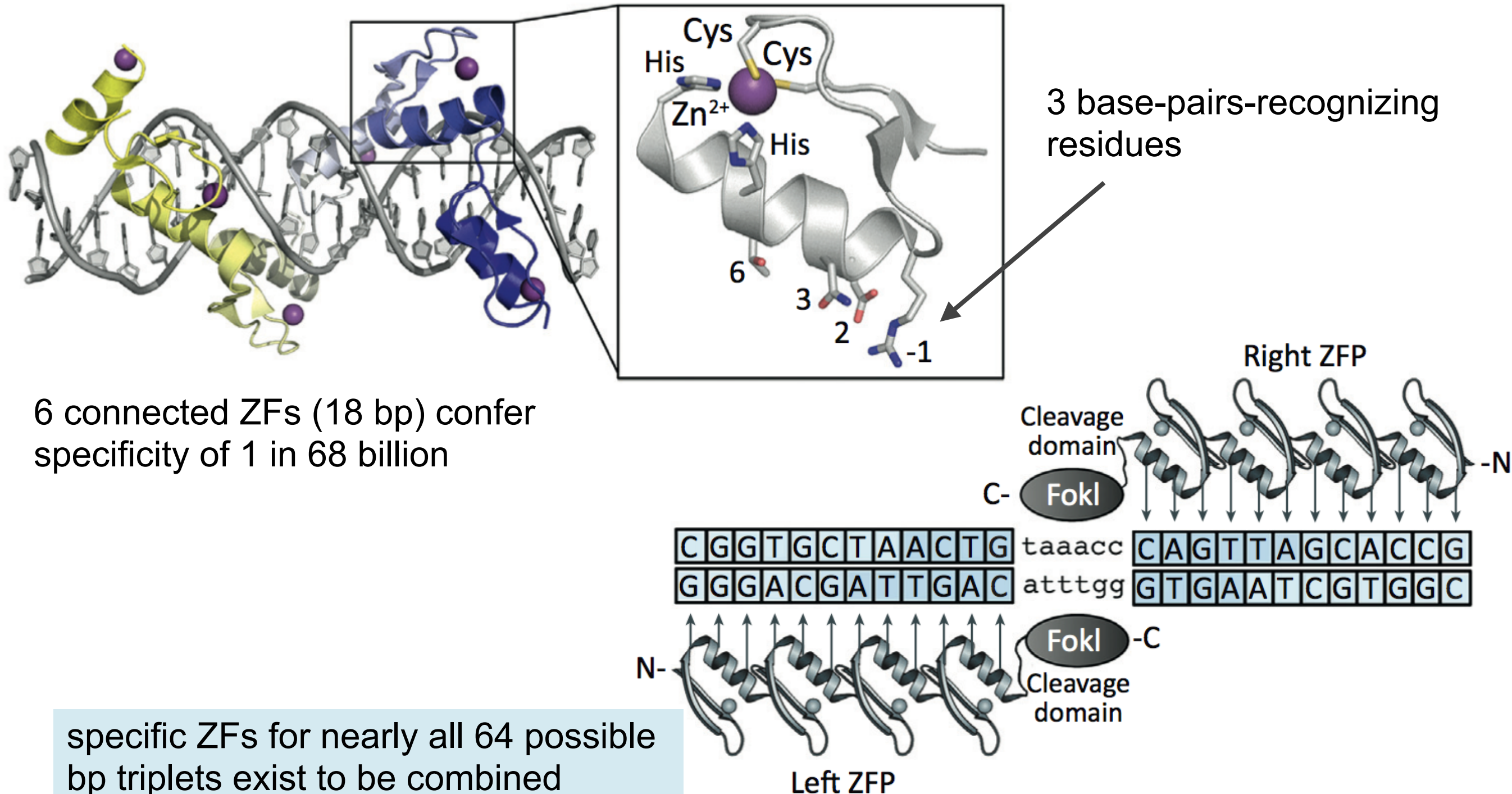


Cys₂His₂
zinc coordination



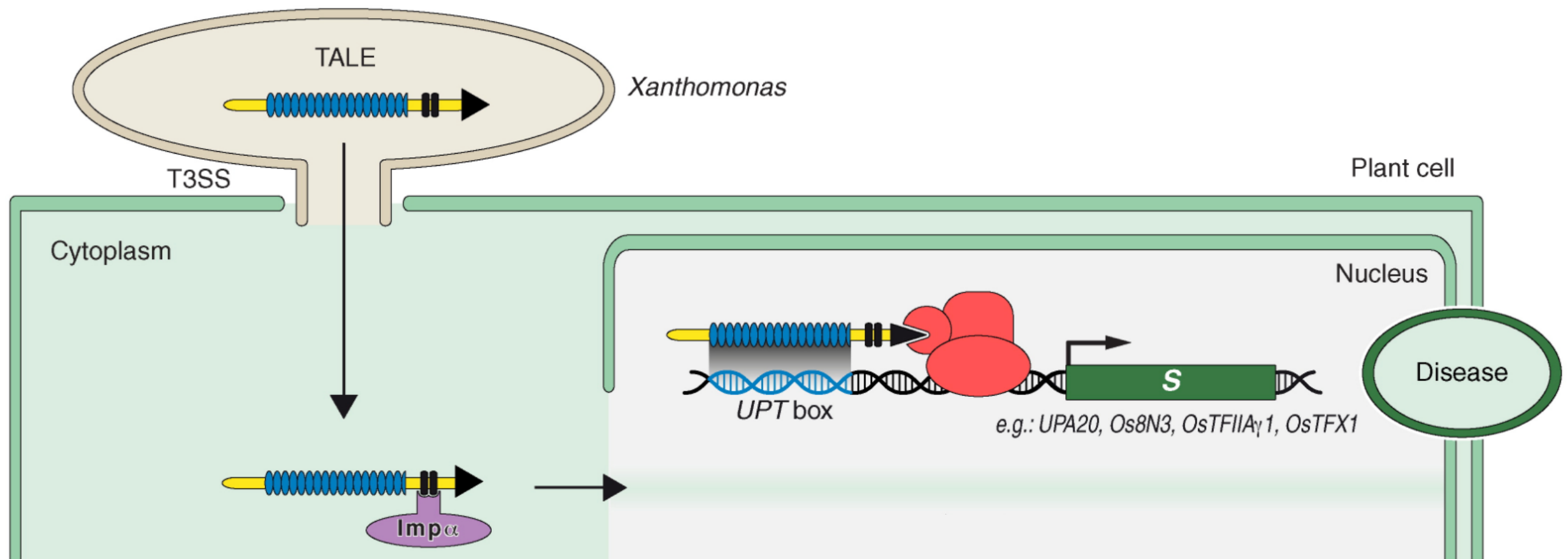
How to target a specific gene/DNA sequence?

ZFN: Zinc finger nuclea



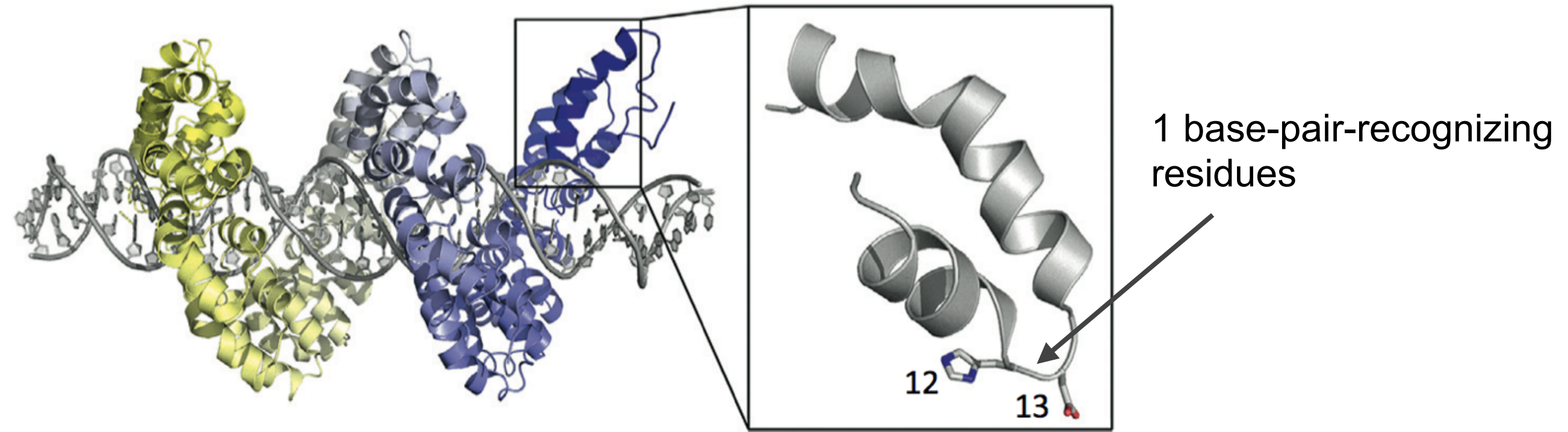
How to target a specific gene/DNA sequence?

TALE: transcription activator-like effectors

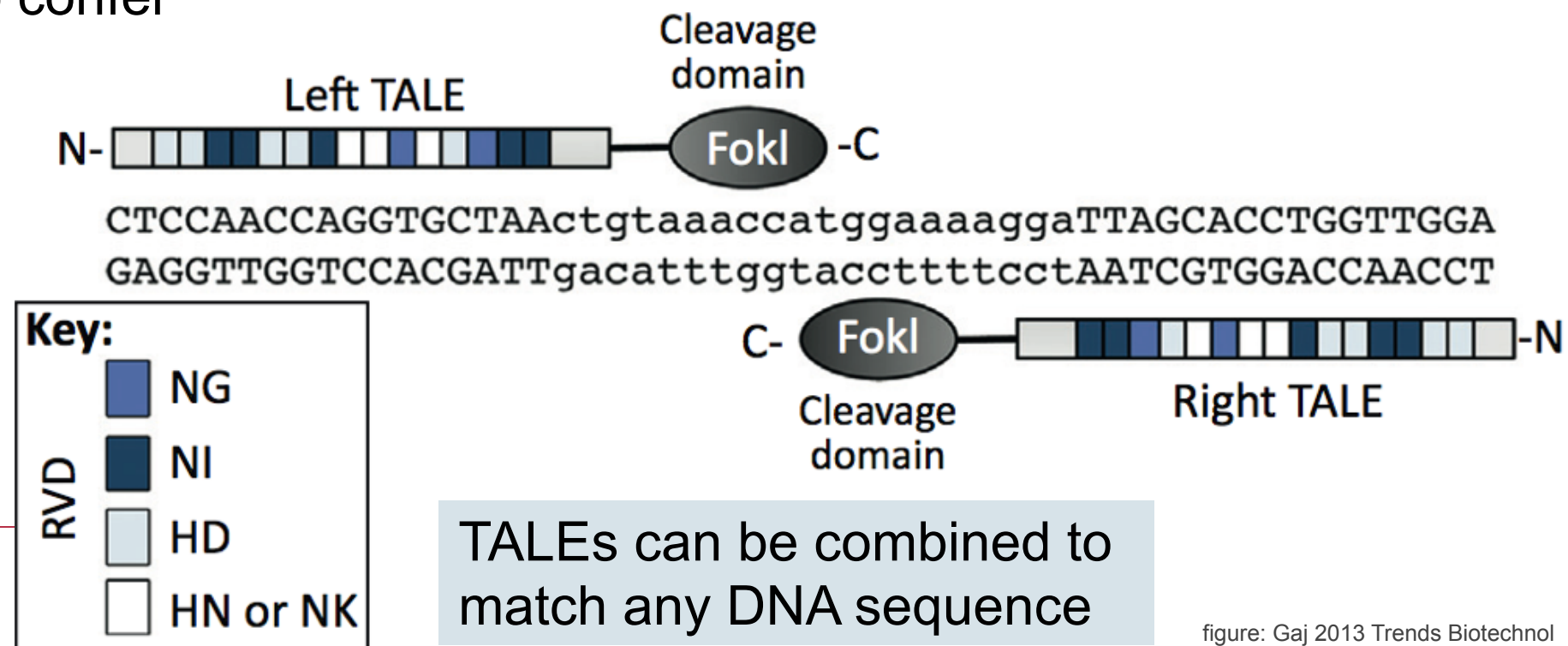


How to target a specific gene/DNA sequence?

TALEN: transcription activator-like effector nucleases



18 connected TALEs (18 bp) confer specificity of 1 in 68 billion





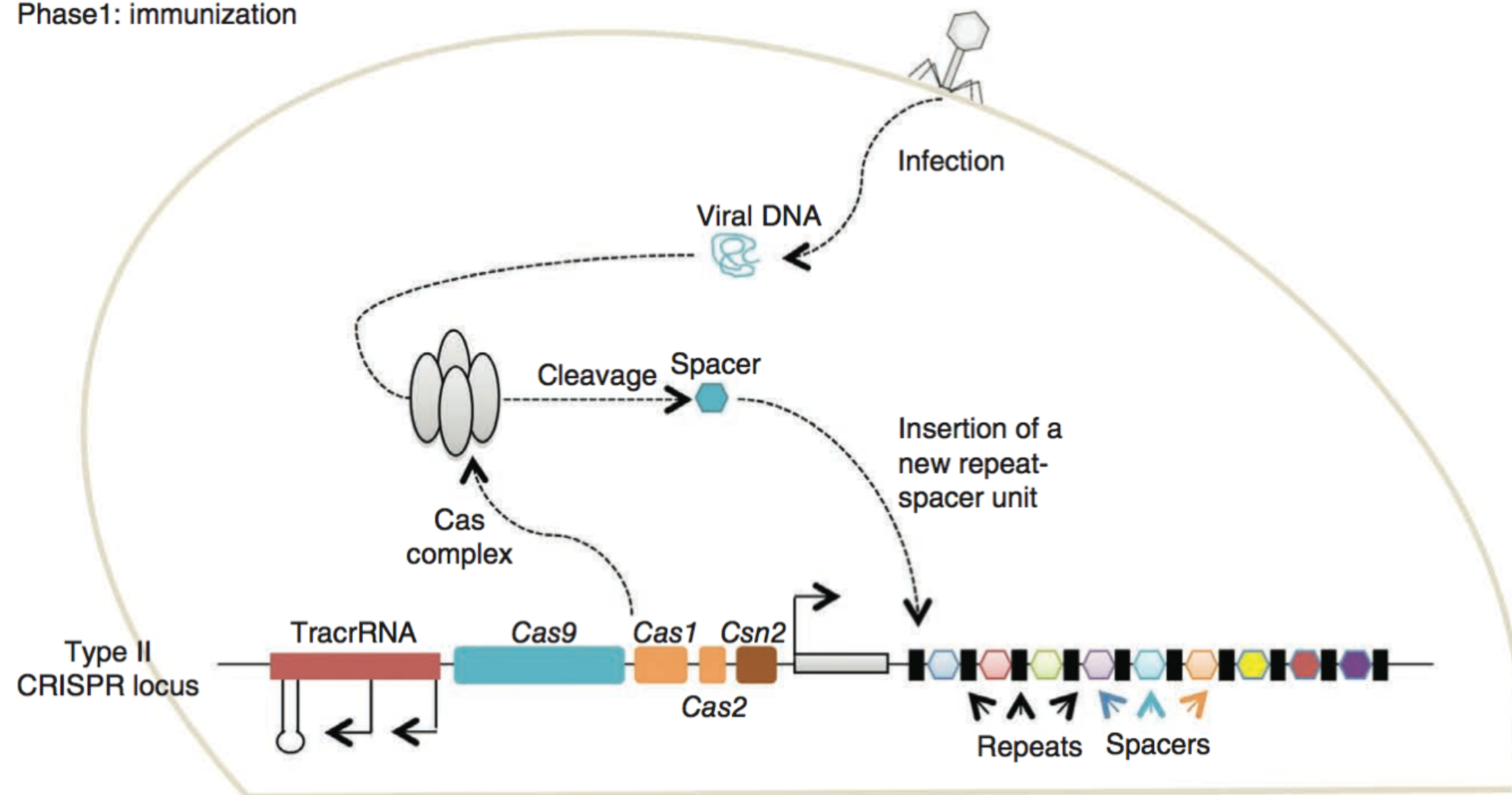
How to target a specific gene/DNA sequence?

CRISPR-Cas: clustered regularly interspaced short palindromic repeats/
CRISPR-associated

RNA-mediated DNA-targeting system that confers immunity in bacteria

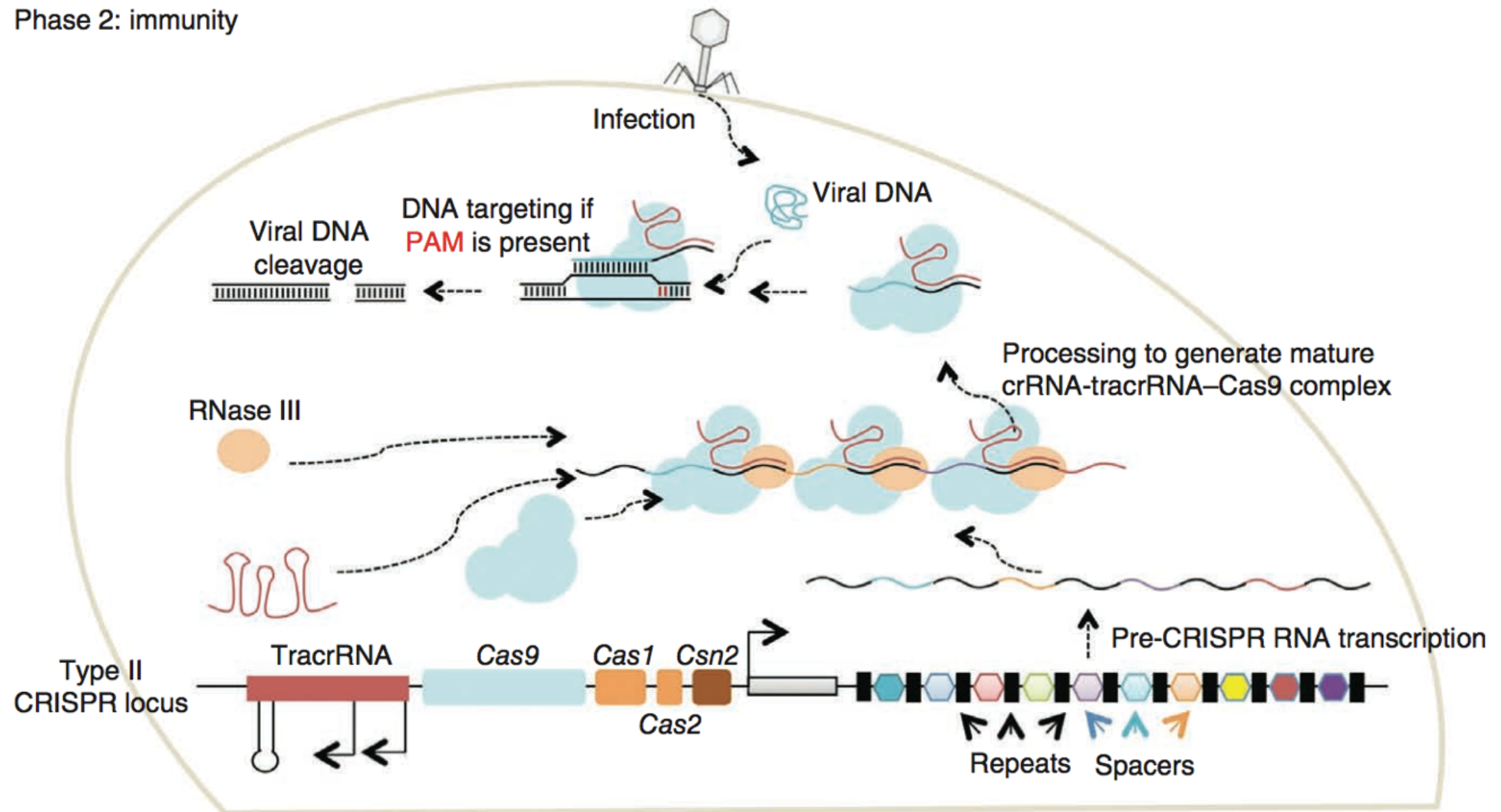
CRISPR-Cas - basics: immunization

Phase1: immunization

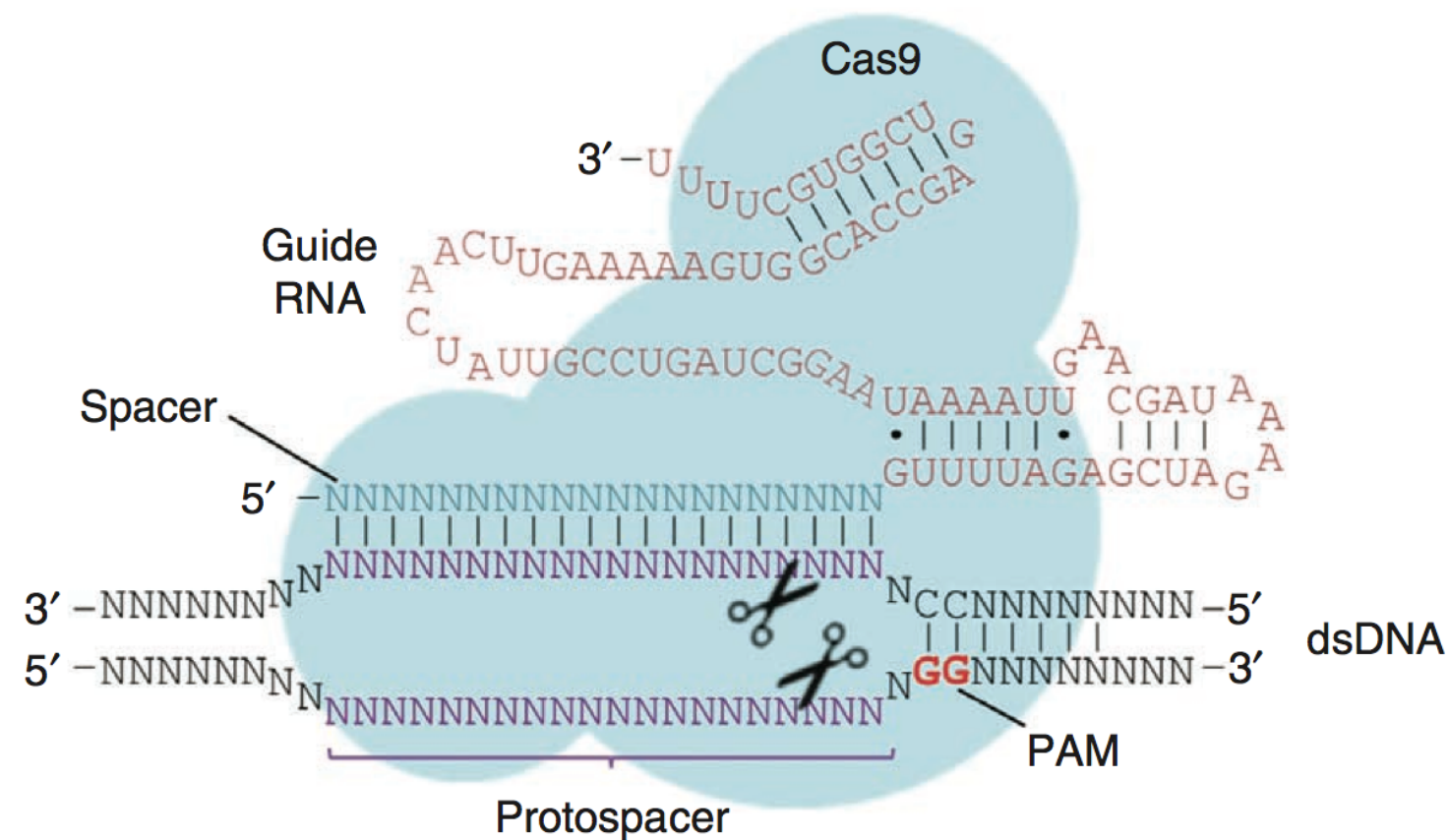


CRISPR-Cas - basics: immunity

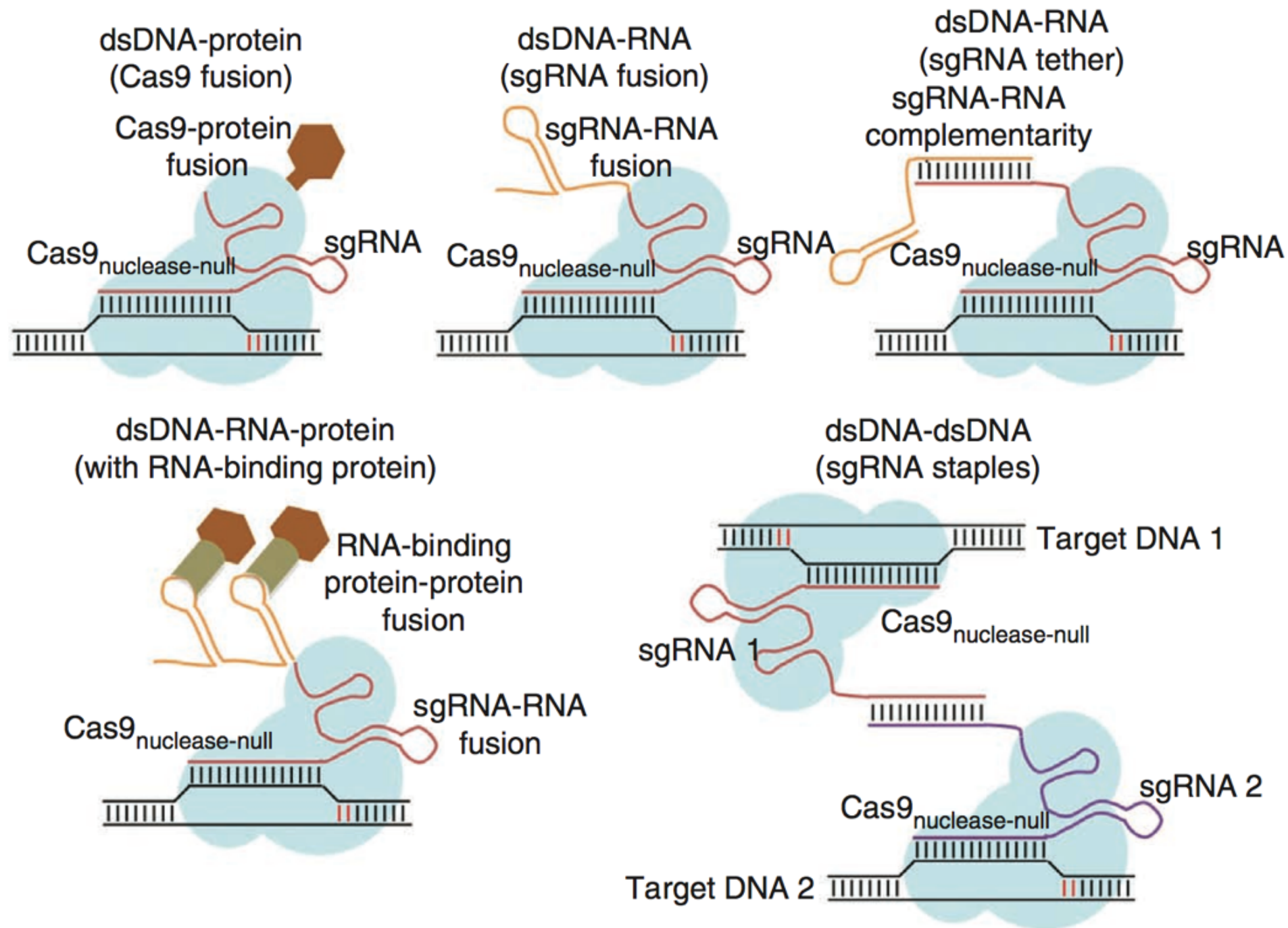
Phase 2: immunity



CRISPR-Cas - targeted application using a Guide RNA (gRNA)



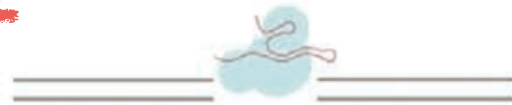
CRISPR - basics and potential



CRISPR - potential applications

Cas9 nuclease or nickase cleavage

Cuts



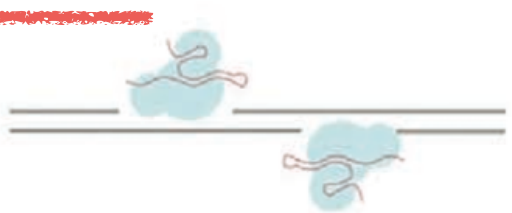
Deletions



Nicks



Offset nicks



Cas9_{nuclease-null} protein fusions

Regulation



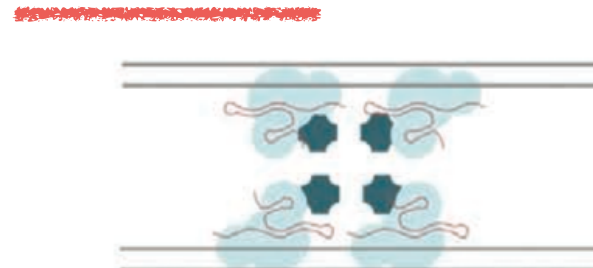
Labeling



Nucleases



Recombinases



Cas9_{nuclease-null} nucleic acid tethers

Regulation



Scaffold



Competition



Aggregation





CRISPR-Cas genome-wide libraries

Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells

Ophir Shalem,^{1,2*} Neville E. Sanjana,^{1,2*} Ella Hartenian,¹ Xi Shi,^{1,3}
David A. Scott,^{1,2} Tarjei S. Mikkelsen,¹ Dirk Heckl,⁴ Benjamin L. Ebert,⁴ David E. Root,¹
John G. Doench,¹ Feng Zhang^{1,2†}

Science 2014

Resource

Cell

Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation

Luke A. Gilbert,^{1,2,3,4,8} Max A. Horlbeck,^{1,2,3,4,8} Britt Adamson,^{1,2,3,4} Jacqueline E. Villalta,^{1,2,3,4}
Yuwen Chen,^{1,2,3,4} Evan H. Whitehead,^{1,3,5,10} Carla Guimaraes,⁶ Barbara Panning,⁷ Hidde L. Ploegh,⁶
Michael C. Bassik,^{1,2,3,4,9} Lei S. Qi,^{1,3,5,10} Martin Kampmann,^{1,2,3,4,*} and Jonathan S. Weissman^{1,2,3,4,*}

Cell 2014



USDA will not regulate gene-edited CRISPR mushroom that resists browning

Emily Waltz | April 15, 2016 | Nature



PRINTER FRIENDLY 

The GLP aggregated and excerpted this blog/article to reflect the diversity of news, opinion and analysis.

The US Department of Agriculture (USDA) will not regulate a mushroom genetically modified with the gene-editing tool **CRISPR-Cas9**.

The long-awaited decision means that the mushroom can be cultivated and sold without passing through the agency's regulatory process — making it the first CRISPR-edited organism to receive a green light from the US government.



White button mushrooms, Photo by Darkone

1 of 6 polyphenol oxidase deleted by CRISPR CAS



Concepts

Restriction:

- site-specific cleavage of palindromic DNA by restriction enzymes

Assembly:

- isothermal fusion of dsDNA fragments using exonucleases, polymerases, and ligases

Plasmids:

- mobile genetic elements used for cloning and expression of genes

Homologous Recombination:

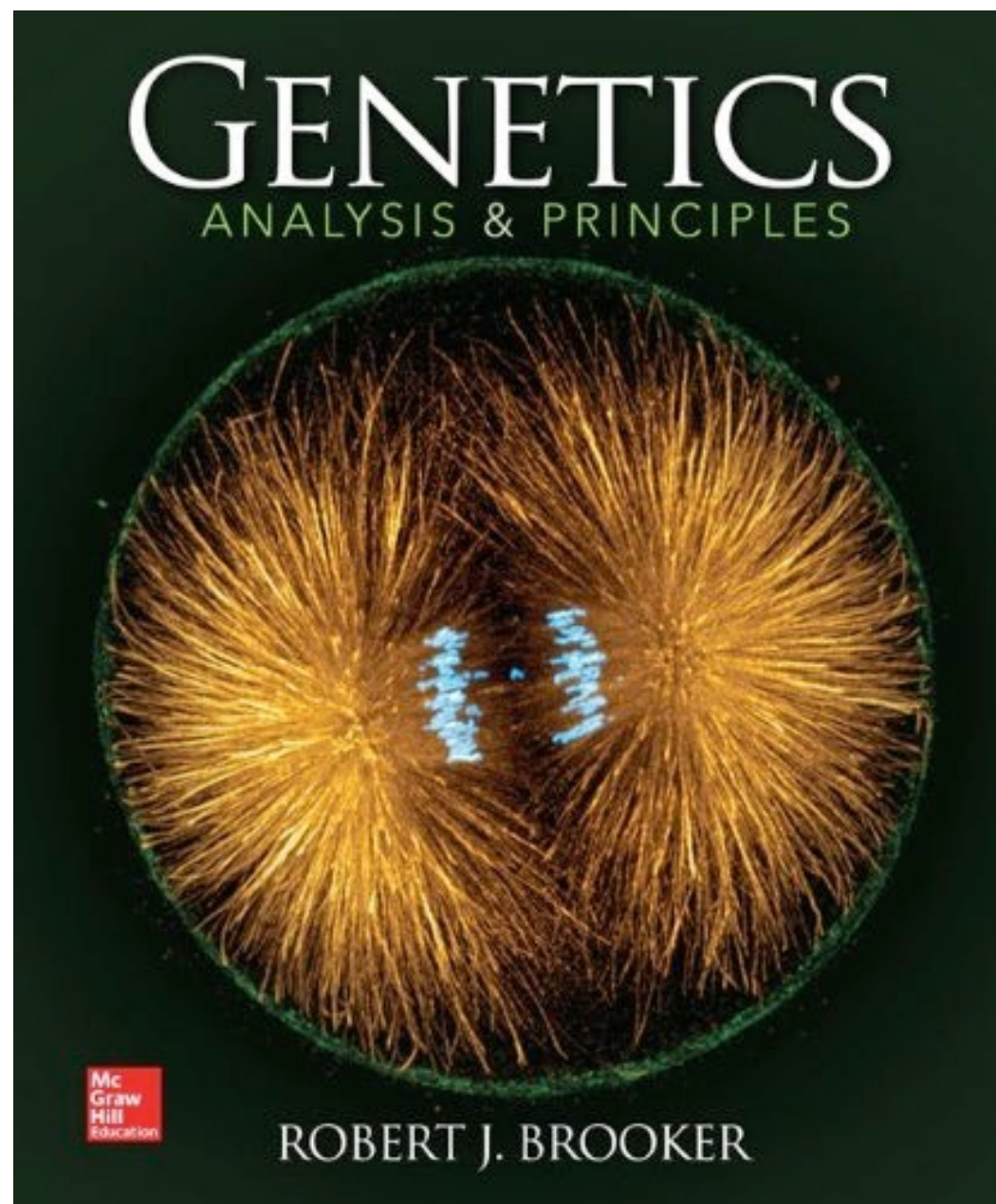
- cross-over (exchange) of homologues DNA fragments

Site-specific DNA editing: requires double strand breaks

- protein-guided nucleases: ZFN, TALEN
 - RNA-guided nucleases: CRISPR-Cas
-



Resources



5th edition 2014

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Questions?



Contact:

Samuel Wagner, Tel: 84238, samuel.wagner@med.uni-tuebingen.de
E.-Aulhorn-Str. 6, Room 1.1.05
