Proteins (& nucleic acids) for manipulating DNA

1) Enzymes and other proteins require appropriate buffers and solution conditions for proper function

2) Specific tools and their uses
   - *a)* *Nucleic acid polymerases*: make and repair DNA
   - *b)* *Nucleases*: break down DNA or RNA
   - *c)* *Restriction endonuclease*: cut the DNA backbone at a specific site
   - *d)* *Ligase*: fix gaps in DNA backbone
   - e) The importance of hybridization/base pairing in putting DNA together/finding targets
   - *f)* *CRISPR-Cas9*: a genetic homing device that uses an RNA to find specific DNA targets

Guide to readings:

1) *13 MC4 Buffers and Reagents.* Tris, Good, and phosphate buffers, buffer recipes for various enzymes/protocols.

2) *14 MC4 Enzymes.* Activity and uses for DNA polymerases, single subunit RNA polymerase, alkaline phosphatase

3) 14.5 MC4 Cut and paste. Restriction enzymes, ligases, and other information about this cloning technique

4) Cas9: The new frontier (2014)

5) Berg First rDNA (1972)

Enzyme “ reaction buffers” : typical components

- **Buffer:** Tris or other buffer, maintain constant pH

- **Salt:** NaCl, KCl, PO_4^-, etc. – maintains protein structure, and facilitates protein-DNA interactions

- **Divalent metal ions**: Mg^{2+}, Ca^{2+}, Zn^{2+}, etc. – protein structure, enzyme activity

- **Glycerol:** (for storage) – stabilizes protein structure

Enzyme “ reaction buffers” : typical components

• **EDTA:** chelates (removes) divalent cations – important especially for storage, if your enzyme is especially sensitive to metal ion-dependent proteases

• **Beta mercaptoethanol or dithiothreitol:** reducing agents that prevent illegitimate disulfide bond formation

• **Non-specific protein:** Bovine serum albumin (BSA)

• **Other cofactors**, eg. ATP, NADH: some enzymes need these for function

✓ “ 10X” reaction buffer is ten times too concentrated. Make a 1/10 dilution for “ 1X” , the working concentration

Enzyme structure/activity is pH sensitive: buffer is essential

Ideal biochemical buffers:

- pKa ($\log_{10}$ of the acid dissociation constant) between 6 and 8 (buffering capacity is greatest when pH = pKa
- Chemically unreactive
- Polar (soluble, not membrane permeable)
- Non-toxic
- Inexpensive
- Buffering minimally influenced by temperature or salt

Tris: widely used but not perfect

- Tris: pKa is 8.0, so buffering is weak below pH 7.5 and above pH 9
- Tris is toxic to many types of mammalian cell cultures

- *Tris solution pH changes with temperature.* pH falls by 0.03 units for each degree C increase (pH 8.0 at 25°C becomes pH 6.5 at 75°C)
- *Tris solution pH changes with concentration*
  Example: 100mM Tris, pH 8.0 → dilute to 10mM Tris, pH is now 7.9

Other buffers, e.g. Good's buffers

- *N*-substituted aminosulfonic acids: HEPES, Tricine, BES, MOPS, MES
- Useful at pH below 7.5

Proteins for manipulating DNA

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DNA polymerases: making copies, adding labels, or fixing DNA

Klenow fragment of DNA polymerase – the C-terminal 70% of E. coli DNA polymerase I

- Lacks a 5’ →3' exonuclease activity
- Uses include:
  - Synthesis of DNA from a ‘primer’
  - Label DNA termini by filling in ends
  - Repair of ragged DNA ends
  - DNA sequencing

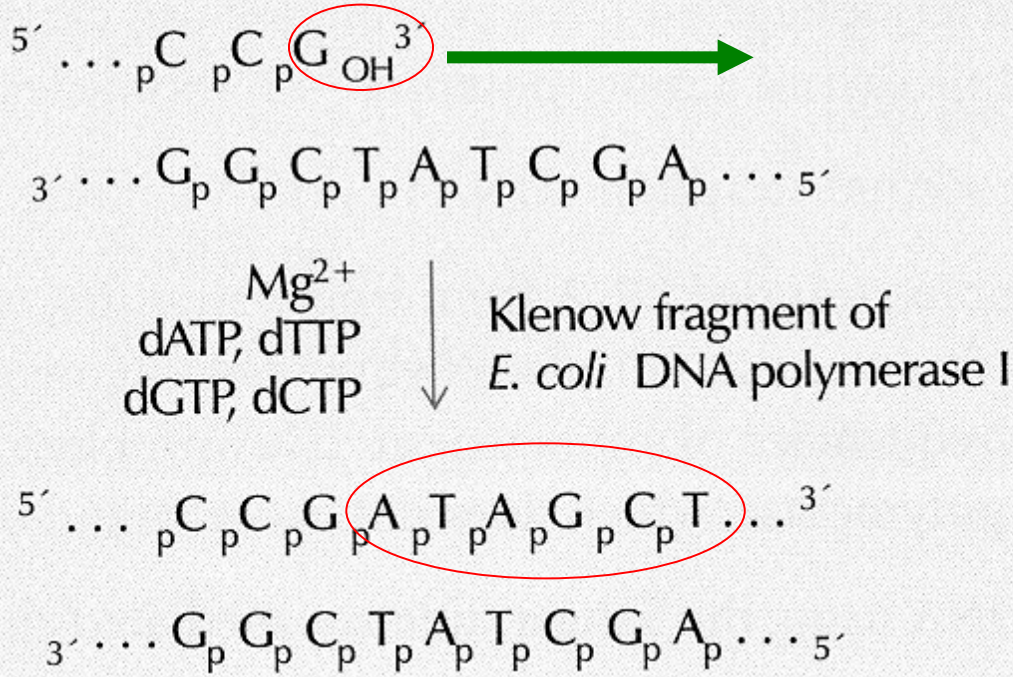
Activity: 5′→3′ DNA polymerase

Substrate: Single-stranded DNA template with a primer containing a free 3′-hydroxyl group.

Reaction:

$$DNA_{OH} \xrightarrow[\text{dATP, dTTP, dGTP, dCTP}]{\substack{\text{Klenow fragment of } E.\ coli \text{ DNA polymerase I} \\ Mg^{2+}}} DNA - ({}_pdN)_n + nPP_i$$

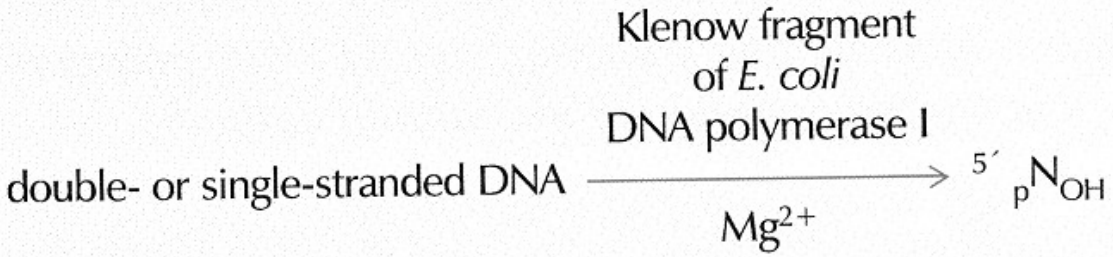
For example:



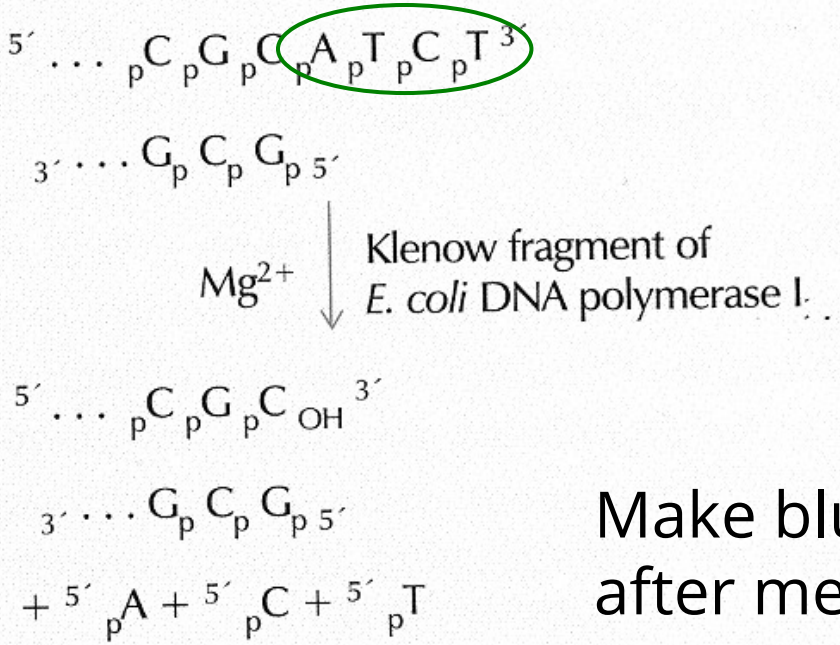
Activity: 3′→5′ Exonuclease

Substrate: Double-stranded or single-stranded DNA degrades from free 3′-hydroxyl termini; exonuclease activity on double-stranded DNAs is blocked by 5′→3′ polymerase activity.

Reaction:



For example:



Make blunt-ended DNA (repair after mechanical fragmentation)

DNA polymerases: for DNA sequencing

- **T7 DNA polymerase** (native) – highly processive, with highly active 3’ →5' exonuclease

- **T7 polymerase** (modified) --lack of both 3’ →5' exonuclease and 5’ →3' exonuclease
  - Ideal for sequencing, due to high processivity

DNA polymerases for DNA amplification

Thermostable DNA polymerases

- Taq: bacterial, high activity, higher mutation rate
- Archaeal DNA pols: lower activity, lower mutation rate

- PCR to amplify specific DNA sequences

- ‘Cycle’ sequencing: DNA sequencing with temperature cycles

DNA polymerases for isothermal DNA amplification

Phi29 DNA polymerase

- Highly processive
- Low mutation rate
- Strand displacement activity (no 5’ to 3’ exonuclease)

- Useful in “ WGA” (whole genome amplification)

Special DNA polymerases

Reverse transcriptase: a retroviral protein

- Makes DNA from an RNA template
- Used for making cDNA copies of RNA transcripts

- Detect, quantify RNA

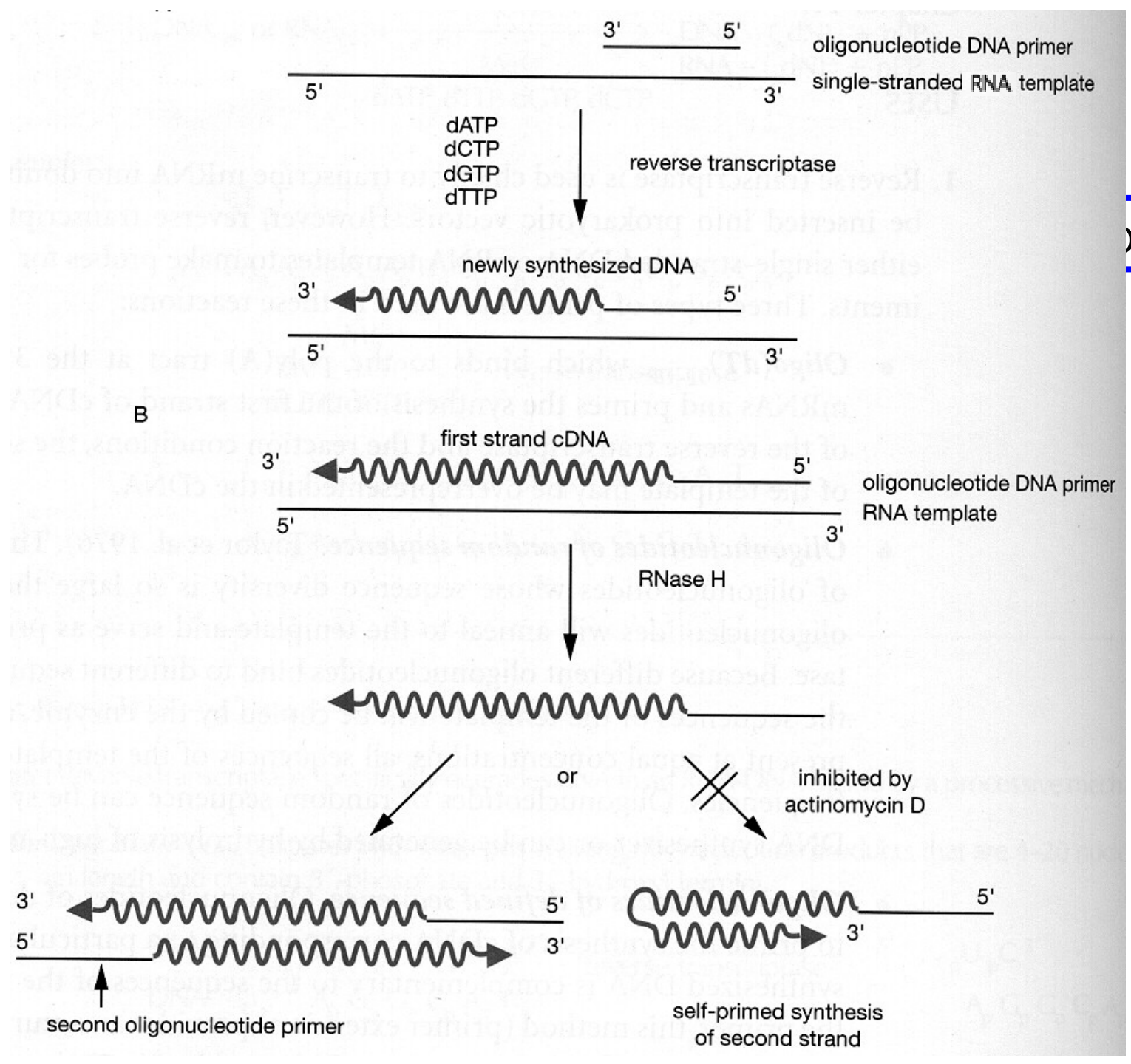
Reverse transcriptase: has some issues

- The Km for dNTPs is very high (relatively non-processive, not good for long RNAs)

- Can make a DNA copy of RNA or DNA

- Can make a double stranded DNA by itself, but inefficiently

- To get clonable, double stranded DNA from RNA, the" second-strand" synthesis is usually done with DNA polymerase and a primer


3'
5'
oligonucleotide DNA primer
single-stranded RNA template
5'
3'
dATP
dCTP
dGTP
dTTP
reverse transcriptase
newly synthesized DNA
3'
5'
5'
3'
B
first strand cDNA
3'
5'
oligonucleotide DNA primer
RNA template
5'
3'
RNase H
or
inhibited by
actinomycin D
3'
5'
5'
3'
5'
3'
second oligonucleotide primer
self-primed synthesis
of second strand

ow RT works

Special DNA polymerases

Terminal transferase: makes new DNA without a template

- template-independent DNA polymerase
- Incorporates dNTPs onto the 3' ends of DNA chains

- Used for adding homopolymer tails to the 3' ends of DNA strands (makes DNA fragments more easily clonable)

RNA polymerase: T7

- Single-subunit RNA polymerase (from bacteriophage), no transcription factor required
- Highly specific promoter sequence determinants, and no cross-promoter recognition by cellular RNA polymerases
- Control transgene expression in a bacterial or eukaryotic host (place transgene under the control of a T7 RNAP promoter, and control expression of the T7 RNAP gene)
- Very active in vitro (makes lots of RNA easily)

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   - *e)* *CRISPR-Cas9*: a genomic homing device

Nucleases

- Exonucleases
  - Remove nucleotides one at a time from a DNA molecule
- Endonucleases
  - Break phosphodiester bonds within a DNA molecule
  - Include restriction enzymes

Applications of exo- and endonucleases

Application	Recommended Enzyme(s)
Removal of 3´ overhangs	T4 DNA Polymerase* + dNTPs
5´ overhang treatment Fill in Cleavage	T4 DNA Polymerase* + dNTPs Klenow + dNTPs Mung Bean Nuclease
Removal of oligonucleotides post PCR	Exonuclease I
Removal of Chromosomal DNA in plasmid preparations	Lambda Exonuclease (Exonuclease I can be added to remove ssDNA generated by Lambda Exonuclease)
Removal of DNA in RNA preparations	DNase I
Chromatin Immunoprecipitation (ChIP) analysis	Micrococcal Nuclease
Generating ssDNA from linear dsDNA If 5´ → 3´ polarity required If 3´ → 5´ polarity required Best general choice	 Lambda Exonuclease Exonuclease III Lambda Exonuclease

* T4 DNA Polymerase has a strong exo- activity.

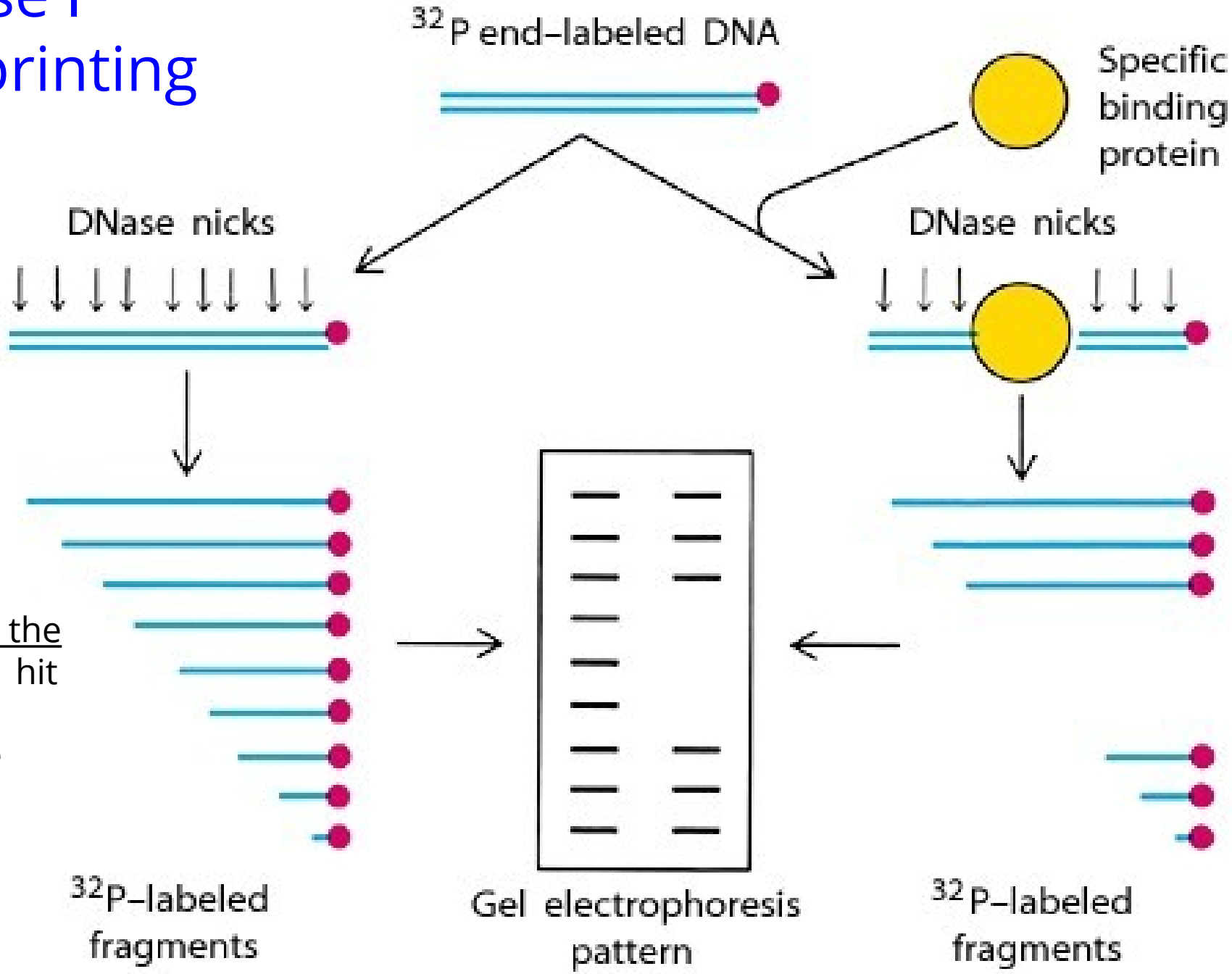
http://www.neb.com/nebecomm/tech_reference/modifying_enzymes/common_app.asp

Endonucleases

Dnase I (deoxyribonuclease I)

- Cleaves double-stranded DNA randomly (also cleaves single-stranded DNA)
- Gets rid of double stranded DNA when only RNA or proteins are desired
- Reduces viscosity of cell lysates
- Useful in defining binding sites for DNA binding proteins

DNAse I footprinting

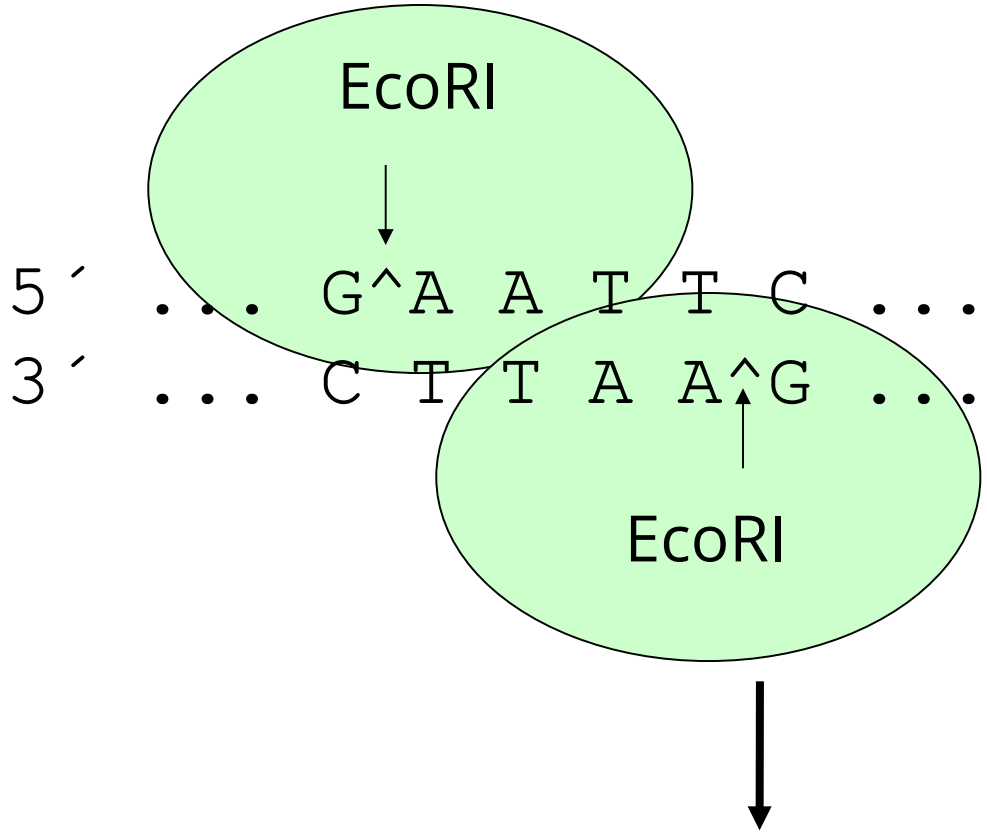


Calibrate the nicking: 1 hit per DNA molecule

Type II endonucleases

- Target a specific, short DNA sequence
- Cut DNA at (or close to) that sequence
- DNA ends have 5’ -phosphates, 3’ -hydroxyls
- Useful for cloning purposes

A type II restriction enzyme: EcoRI



5´ ... G^ 3′ 5′ A A T T C ... 3´

3´ ... C T T A A 5′ 3′ ^G ... 5´

Many type II enzymes, with unique target sequences

4-base recognition site:

AluI	5′ ... AG^CT ... 3′	blunt ends
MspI	5′ ... C^CGG ... 3′	5′ overhang (2 bp)

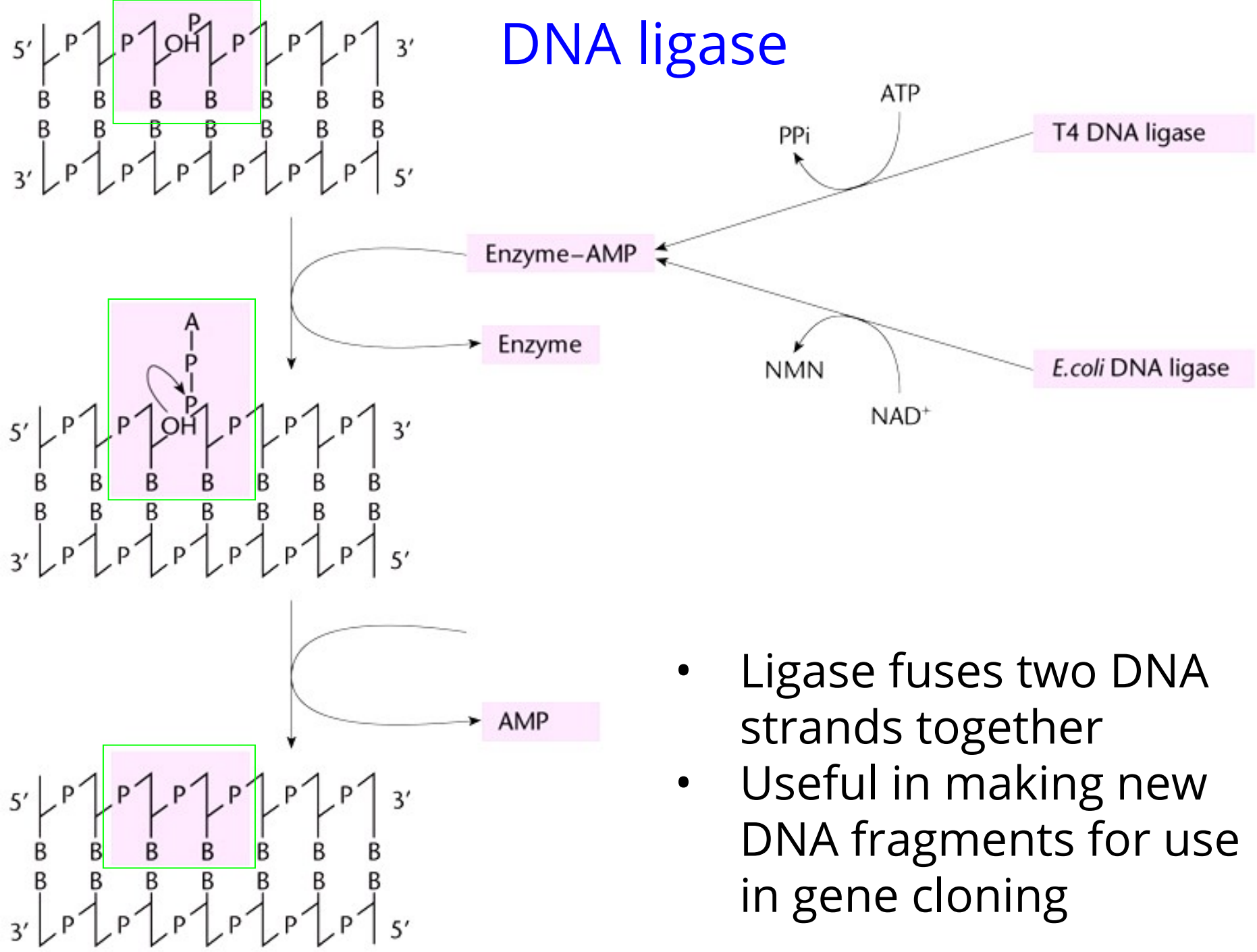
6-bases

PvuII	5′ ... CAG^CTG ... 3′	blunt ends
KpnI	5′ ... GGTAC^C ... 3′	3′ overhang (4 bp)

8-bases

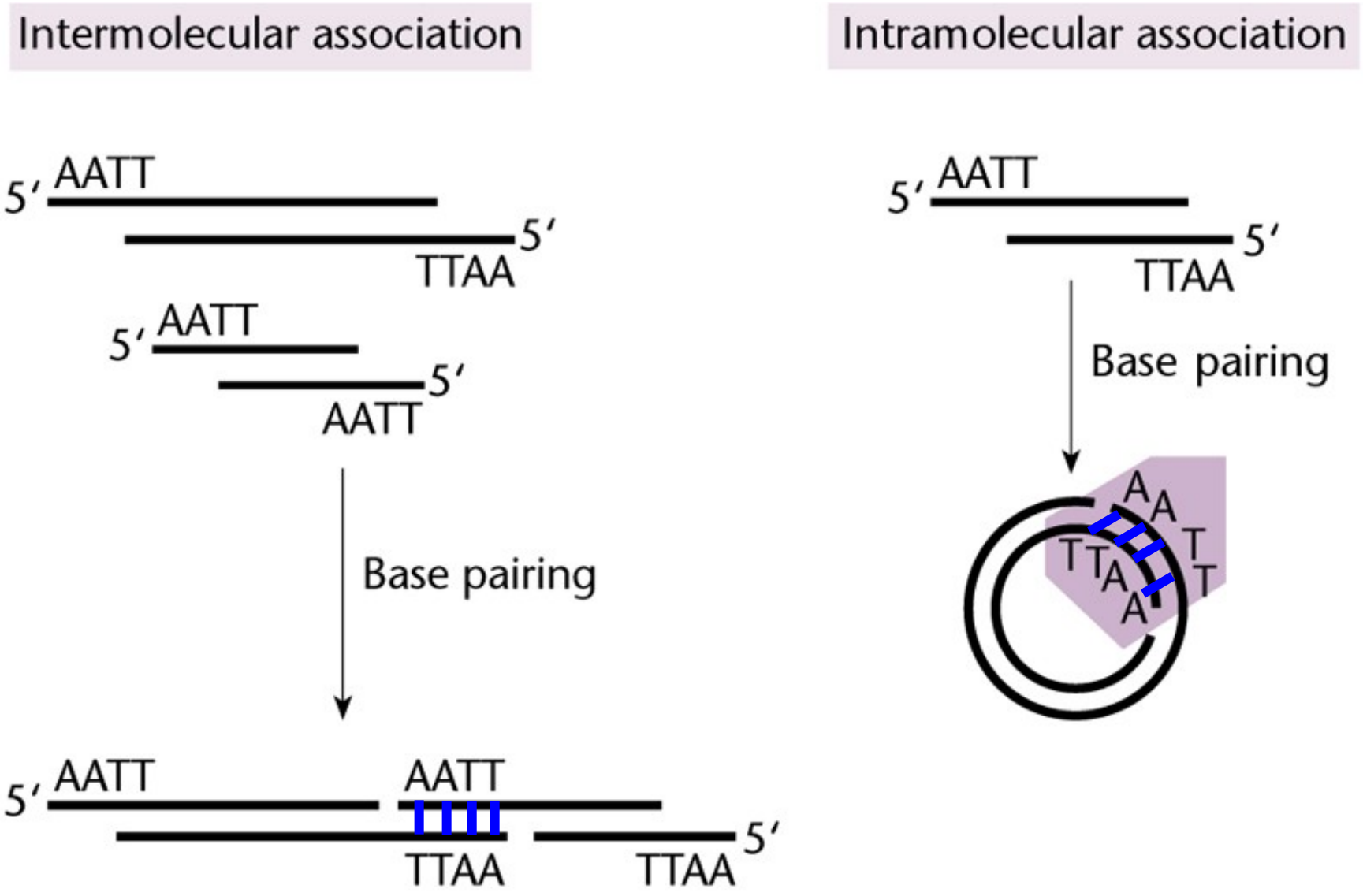
NotI	5′ ... GC^GGCCGC ... 3′	5′ overhang (4 bp)

DNA ligase



- Ligase fuses two DNA strands together
- Useful in making new DNA fragments for use in gene cloning

Ligation of cohesive ends (overhangs)



Base pairing helps in ligation reactions

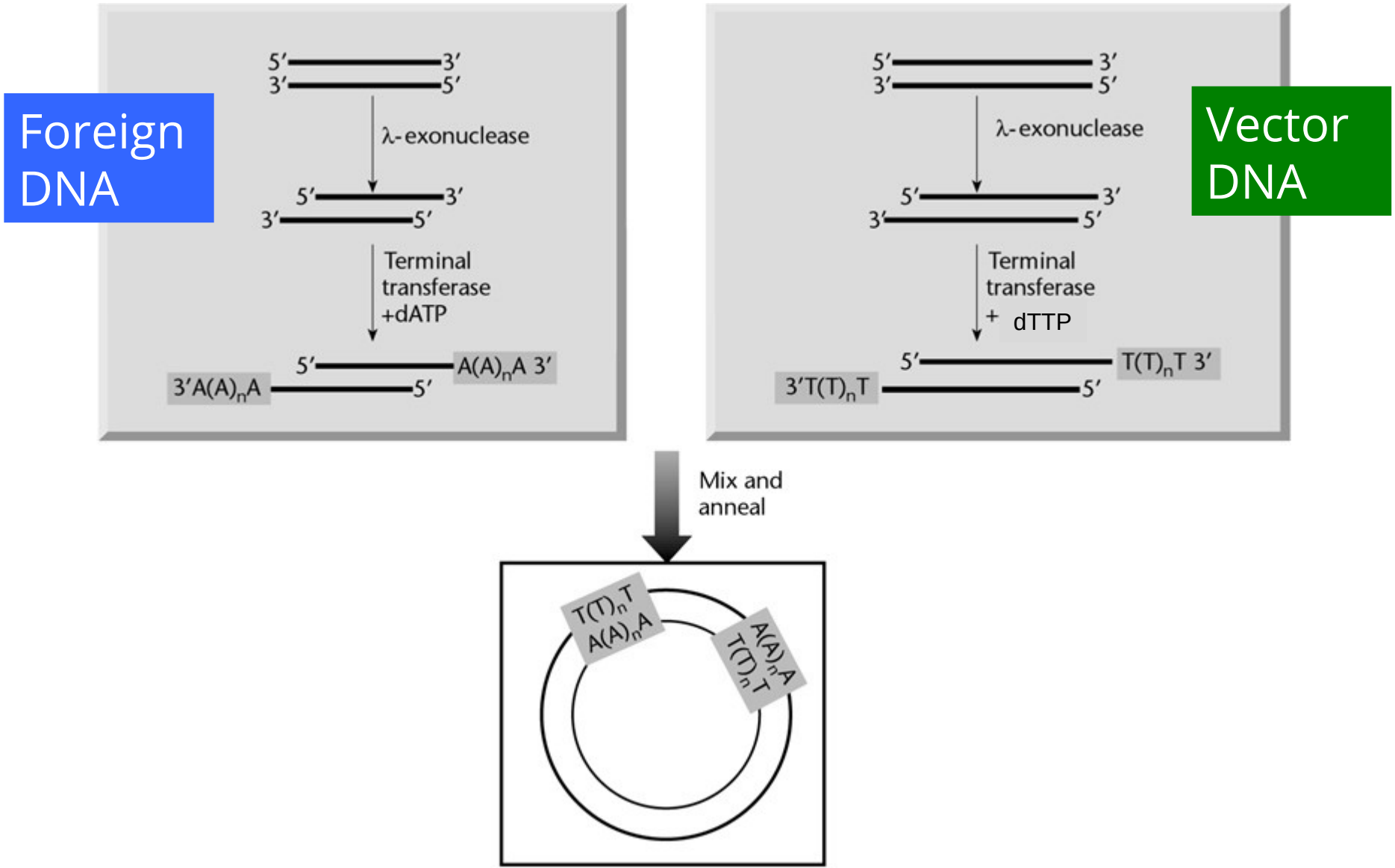
Cloning techniques

A) The importance of the ends of the DNAs – make foreign DNA sequences more ligate-able

B) Directional cloning – generate easily cloned PCR fragments

C) Cloning by hybridization – new developments

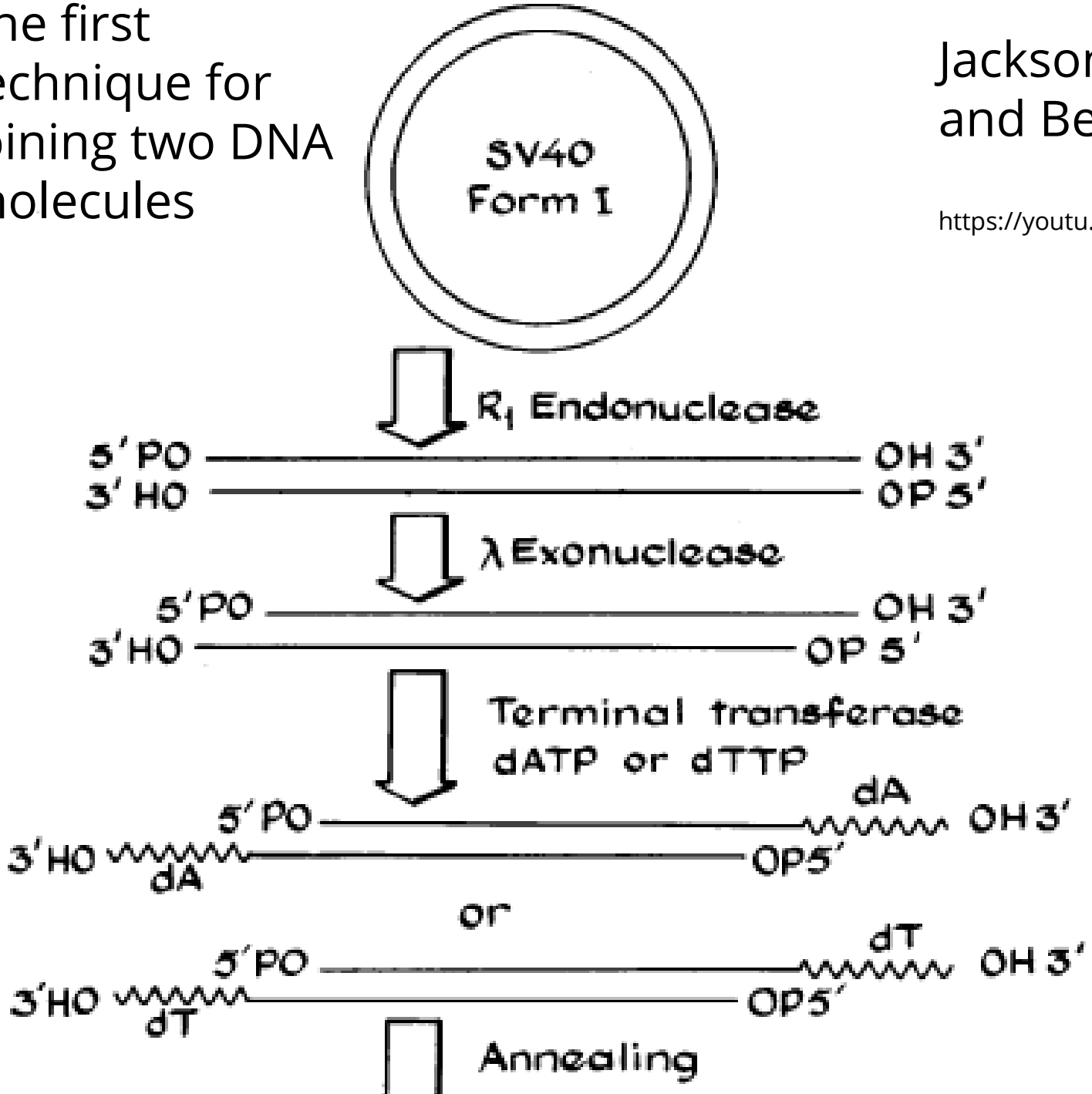
Terminal transferase: add polynucleotide tails to foreign DNA and vector DNA



The first technique for joining two DNA molecules

Jackson, Symons and Berg, 1972

https://youtu.be/u-_10gnpdxI



EcoRI to open DNA

Create 3’ overhang

Add poly-nucleotide tail

The first technique for joining two DNA molecules

Jackson, Symons and Berg, 1972

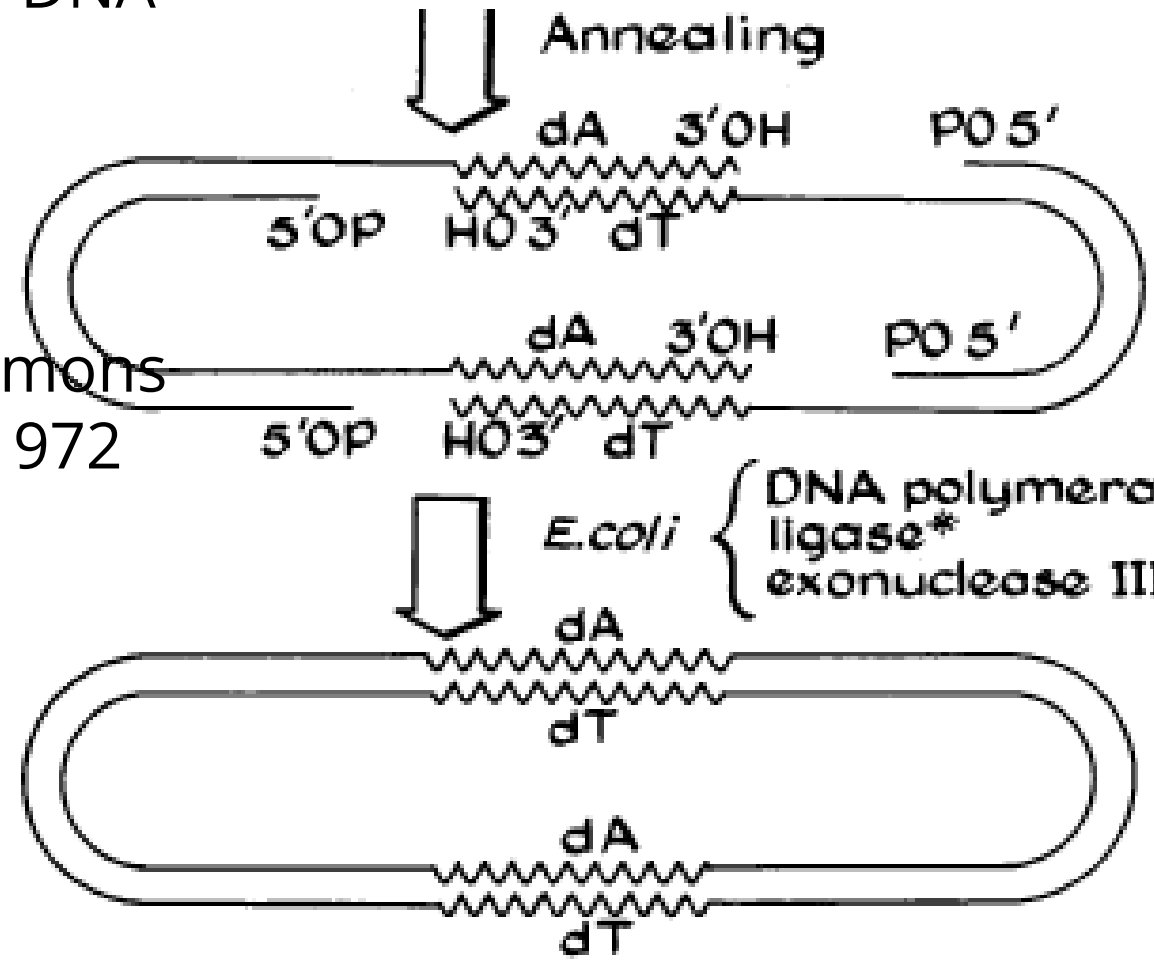


FIG. 1. General protocol for producing covalently closed SV40 dimer circles from SV40(I) DNA.

Cloning techniques

A) The importance of the ends of the DNAs – make foreign DNA sequences more ligate-able

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Directional cloning

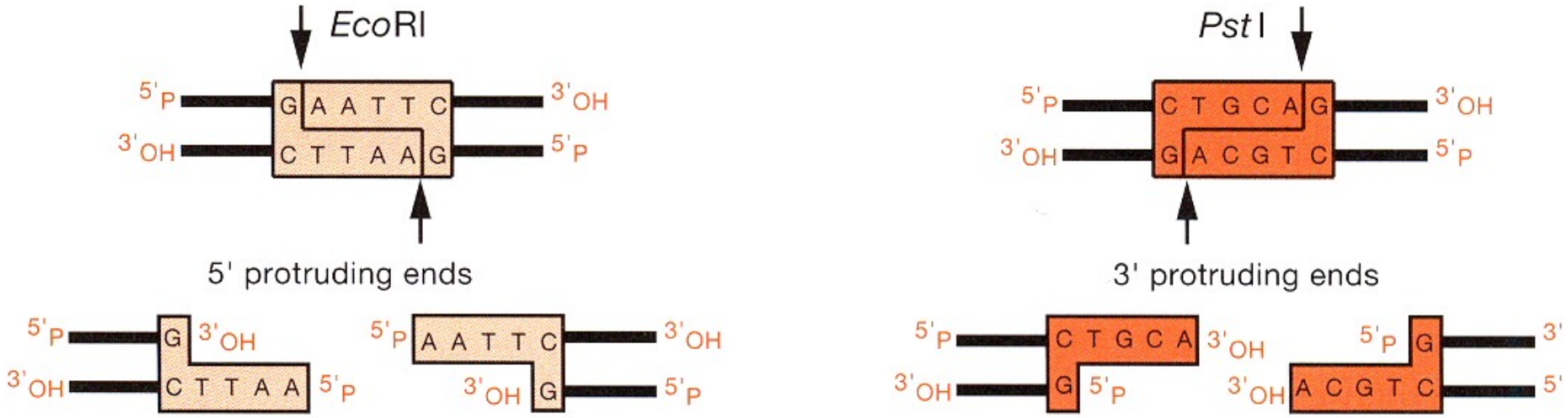
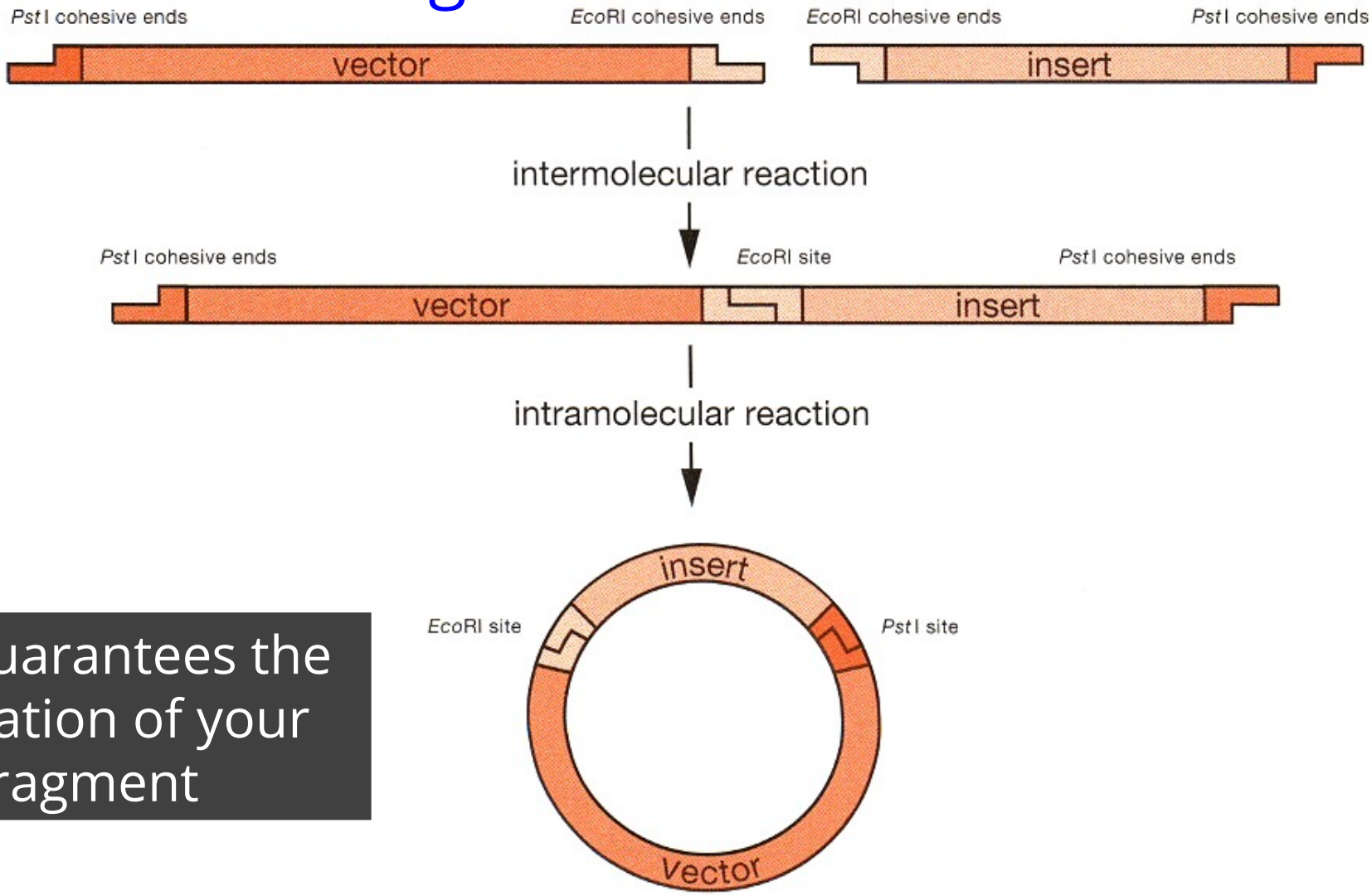


FIGURE 1-2 Cloning 5′ and 3′ Protruding Ends

These sticky ends will not base pair with each other

Directional cloning



This guarantees the orientation of your DNA fragment

FIGURE 1-5 Directional (Forced) Cloning in Plasmid Vectors

Vector sequences are represented by darker shading, and insert sequences by lighter shading.

Cloning by hybridization

• PCR product made using primers with 5’ sequences matching plasmid cloning site

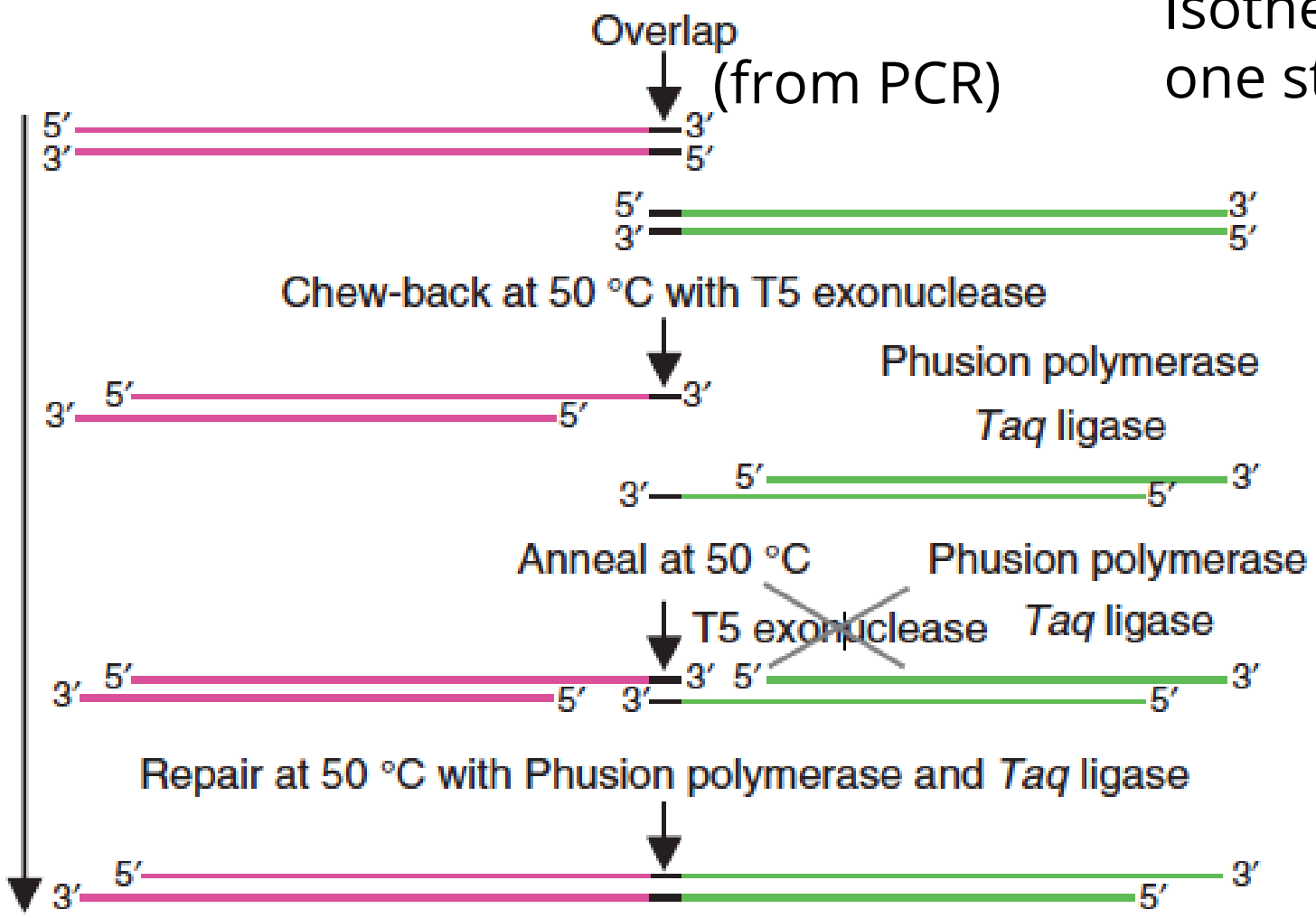
• DNA insert and plasmid are treated with nuclease to generate 5’ or 3’ overhangs, typically ~25 bases

• Base-pairing (hybridization) between plasmid and DNA insert sequence forces assembly

• Gaps in DNA backbone are corrected by DNA polymerase and DNA ligase (Gibson assembly)

Gibson DNA assembly: make synthetic genes, pathways, or *entire genomes*.

Isothermal: one step at 50°C



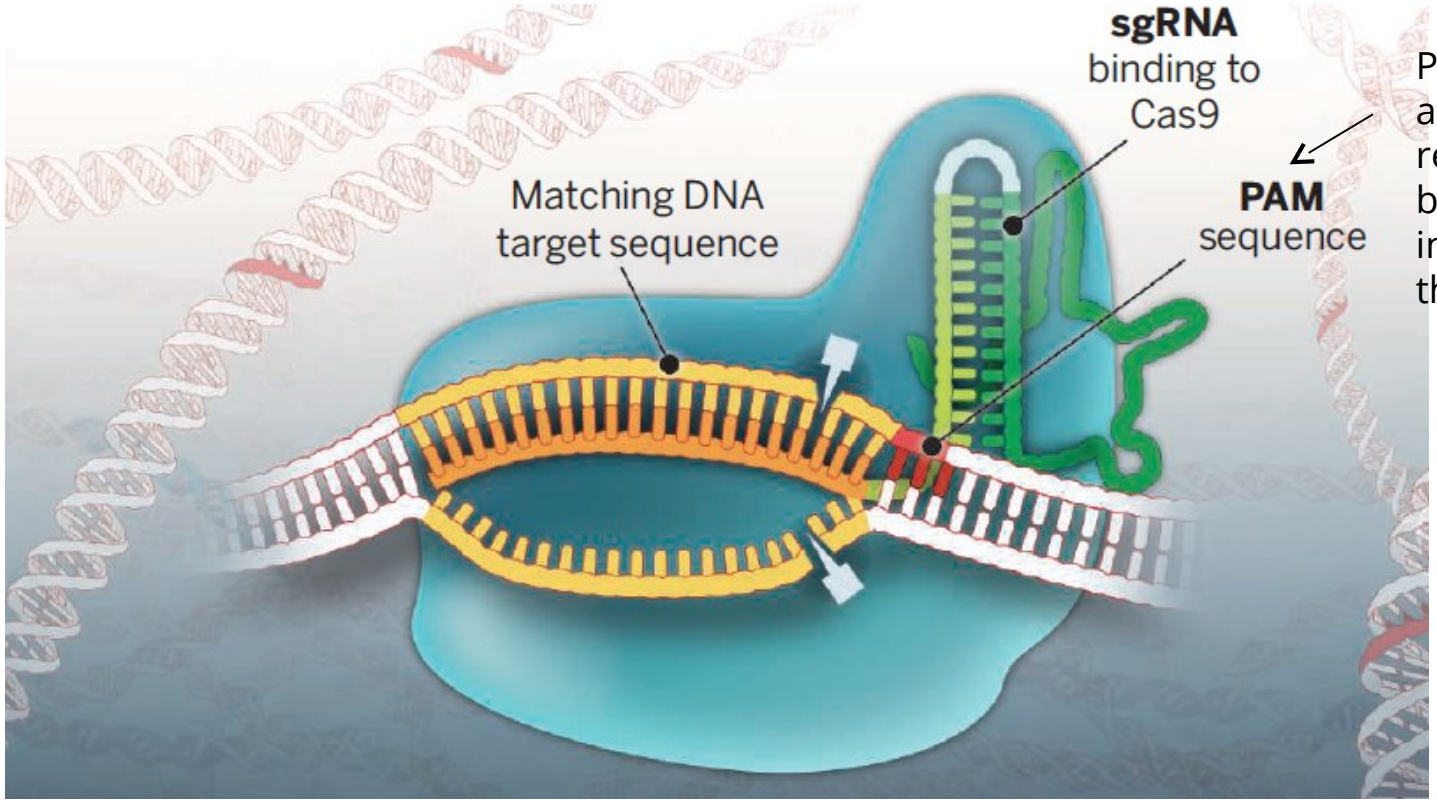
5′→3′ exo (T5 exo is heat-labile)

Annealing

Repair (Phusion DNA pol and Taq ligase are heat stable)

Gibson et al. (2009) Nature Methods **6**, p. 343

Type II CRISPR-Cas9: an RNA-guided nuclease



PAM = protospacer adjacent motif: required for Cas9 to bind DNA so strand invasion can occur by the guide RNA

The RNA-target interactions are very stable, and can also provide a tethering platform for proteins or RNAs, provided the nuclease activity is shut down

2020 Nobel Prize in Chemistry

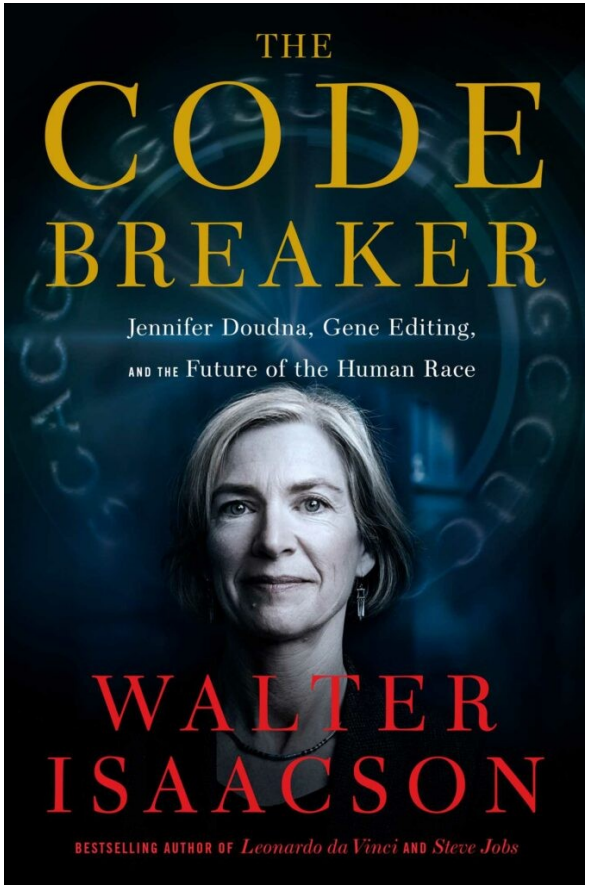
Emmanuelle Charpentier, Max Planck Institute, Berlin
Jennifer Doudna, University of California-Berkeley

https://www.nature.com/articles/d41586-020-02765-9

The Nobel Prize in Chemistry 2020 was awarded jointly to Emmanuelle Charpentier and Jennifer A. Doudna "for the development of a method for genome editing."

https://www.nobelprize.org/prizes/chemistry/2020/summary/

Also see: “Code Breaker”, book by Walter Isaacson (2021)

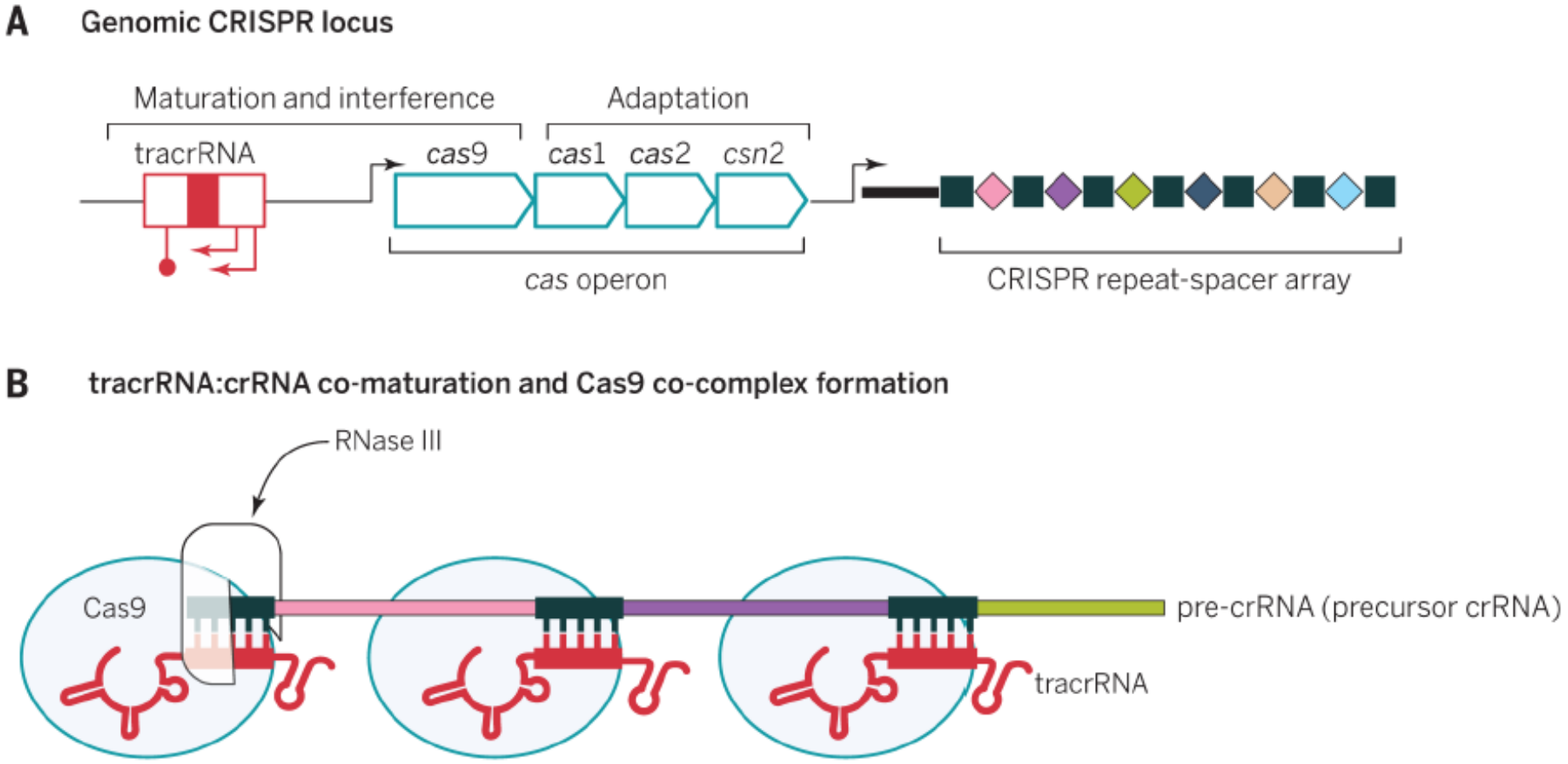


https://datebook.sfchronicle.com/books/review-in-code-breaker-how-jennifer-doudna-became-a-pioneer-of-genes

https://www.wired.com/story/the-code-breaker-is-the-crispr-chronicle-you-need-to-read/

CRISPR-Cas9 comes from prokaryotes

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

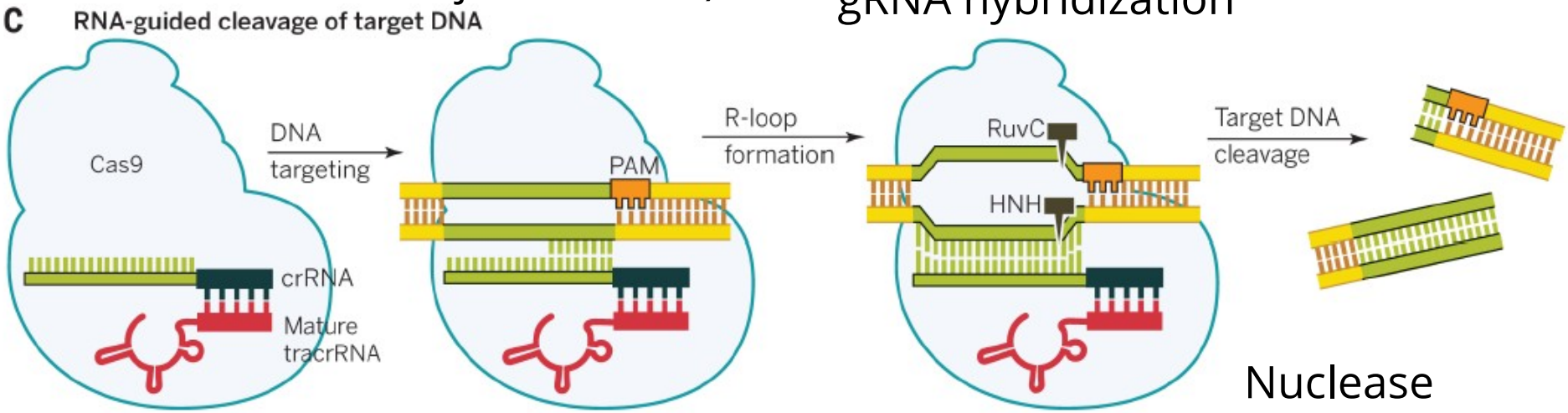


Tracr RNA interacts with Cas9, and this complex interacts with precursor crRNA to make the mature enzyme

Cas9-RNA machinery in action

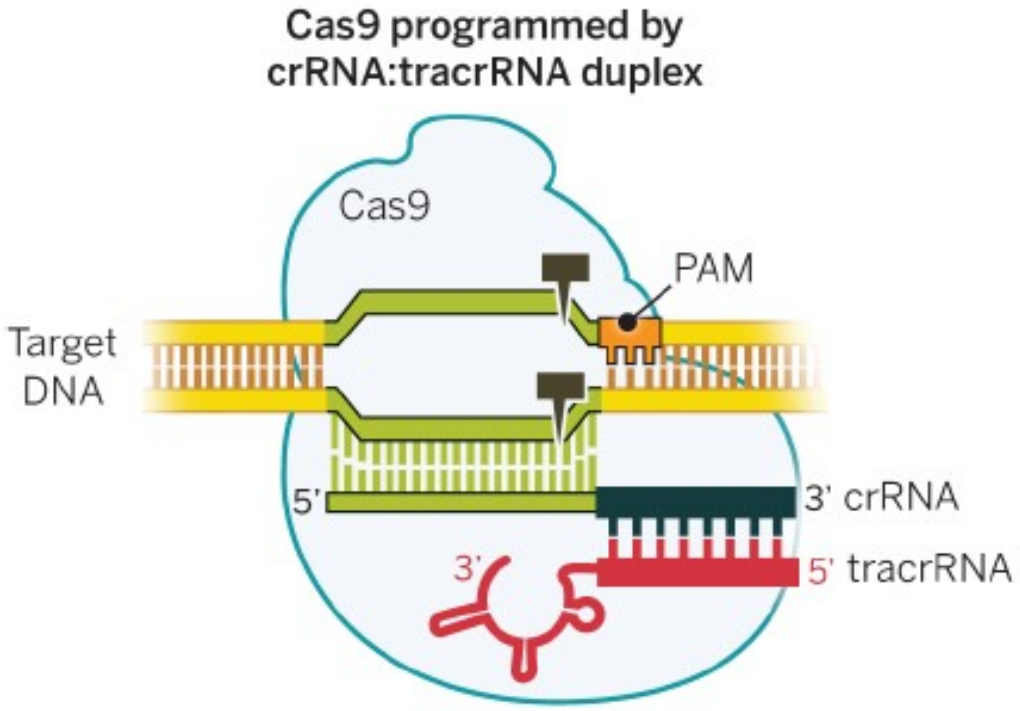
Cas9 finds PAM (protospacer adjacent motif)

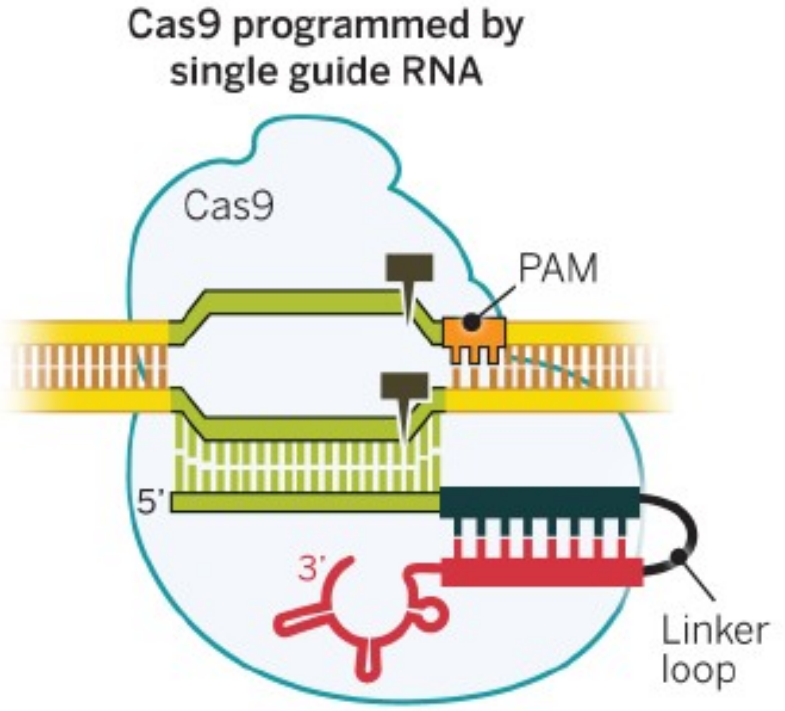
R-loop forms for gRNA hybridization



Nuclease activity cuts DNA

An important innovation in utilizing this machine for engineering: fusion of crRNA and tracrRNA



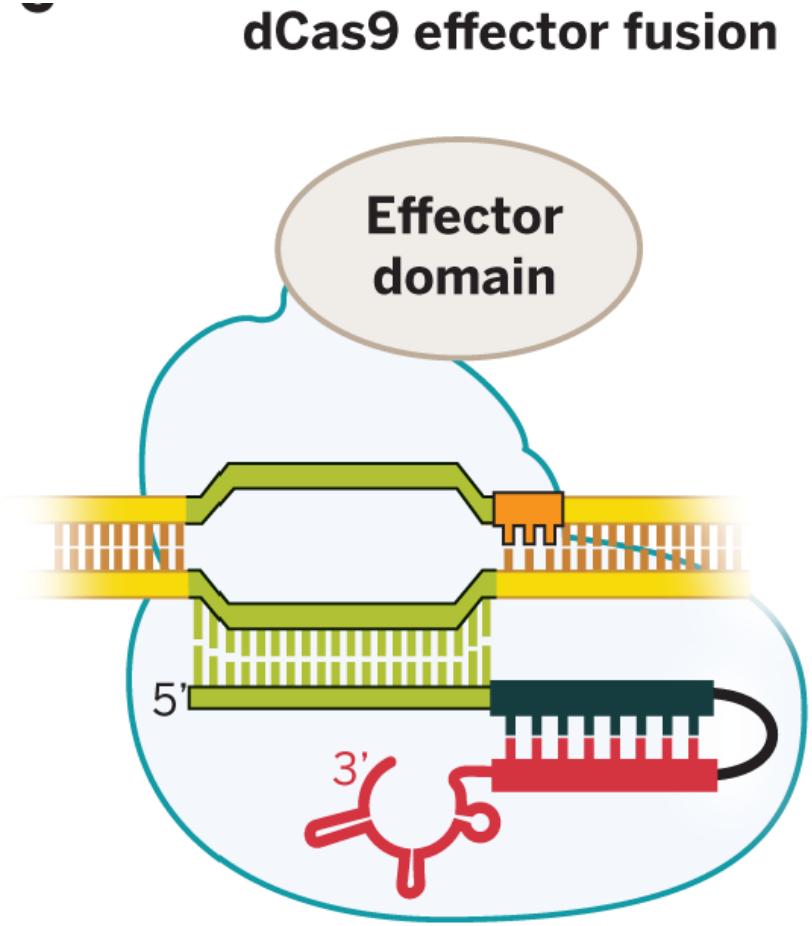


Normally, crRNA and tracrRNA are separate

They can be joined by a linker and the machine still functions

Cas9 as a programmable DNA binding protein

Both nucleases can be inactivated, making Cas9 a DNA binding protein that can be programmed, and can take effector domains any place in the genome



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