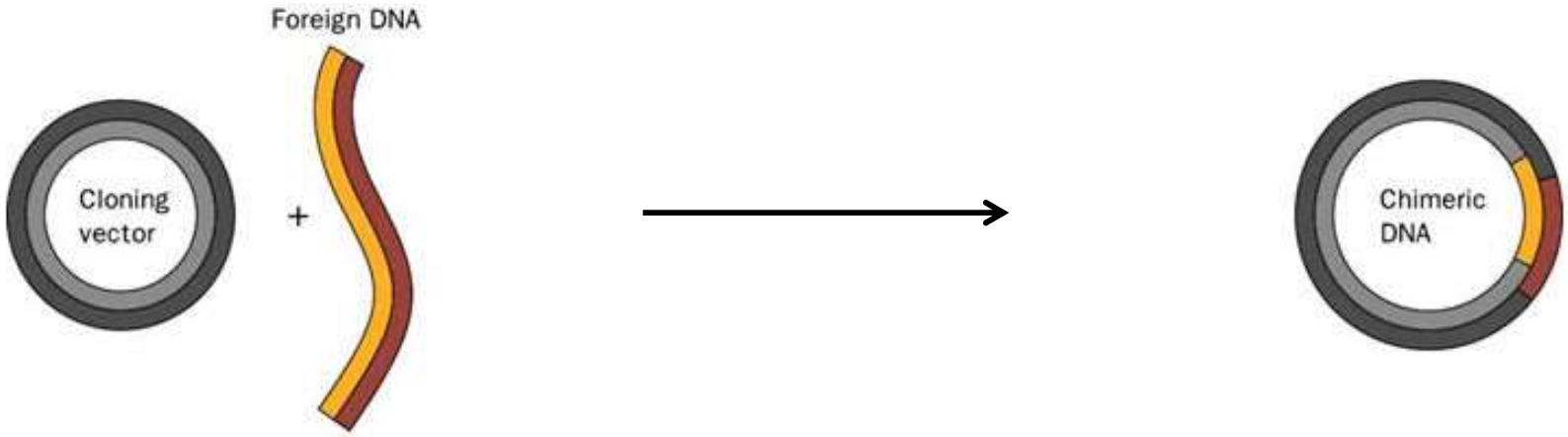
Molecular Cloning for Biological Expression Design

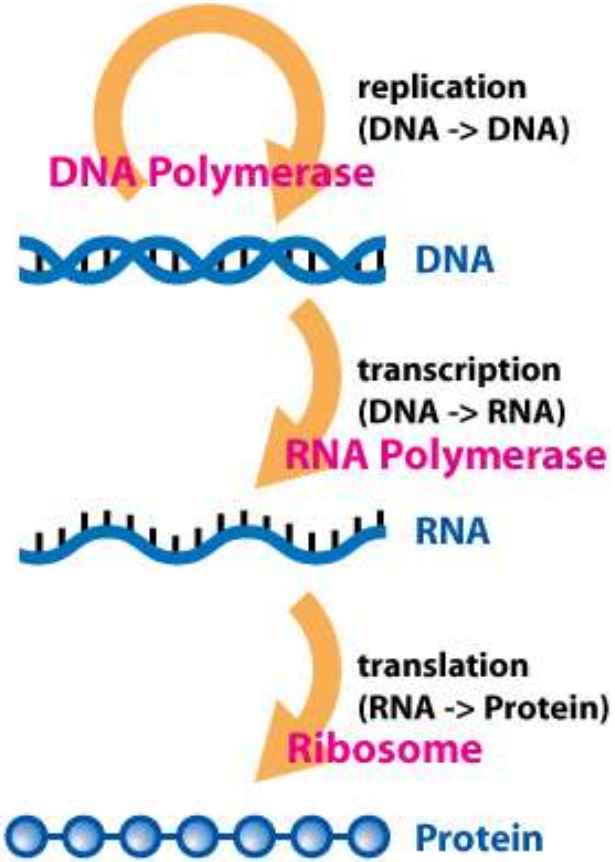
UE2.1 Biological Parts and Devices



Manish Kushwaha

4 October, 2024

Central Dogma: Relevance for Expression Design



- Replication Origin
  - Copy Number
  - Relaxed/ Stringent control

- Promoter
  - Constitutive
  - Inducible
- Terminator
  - Intrinsic
  - Rho-dependent

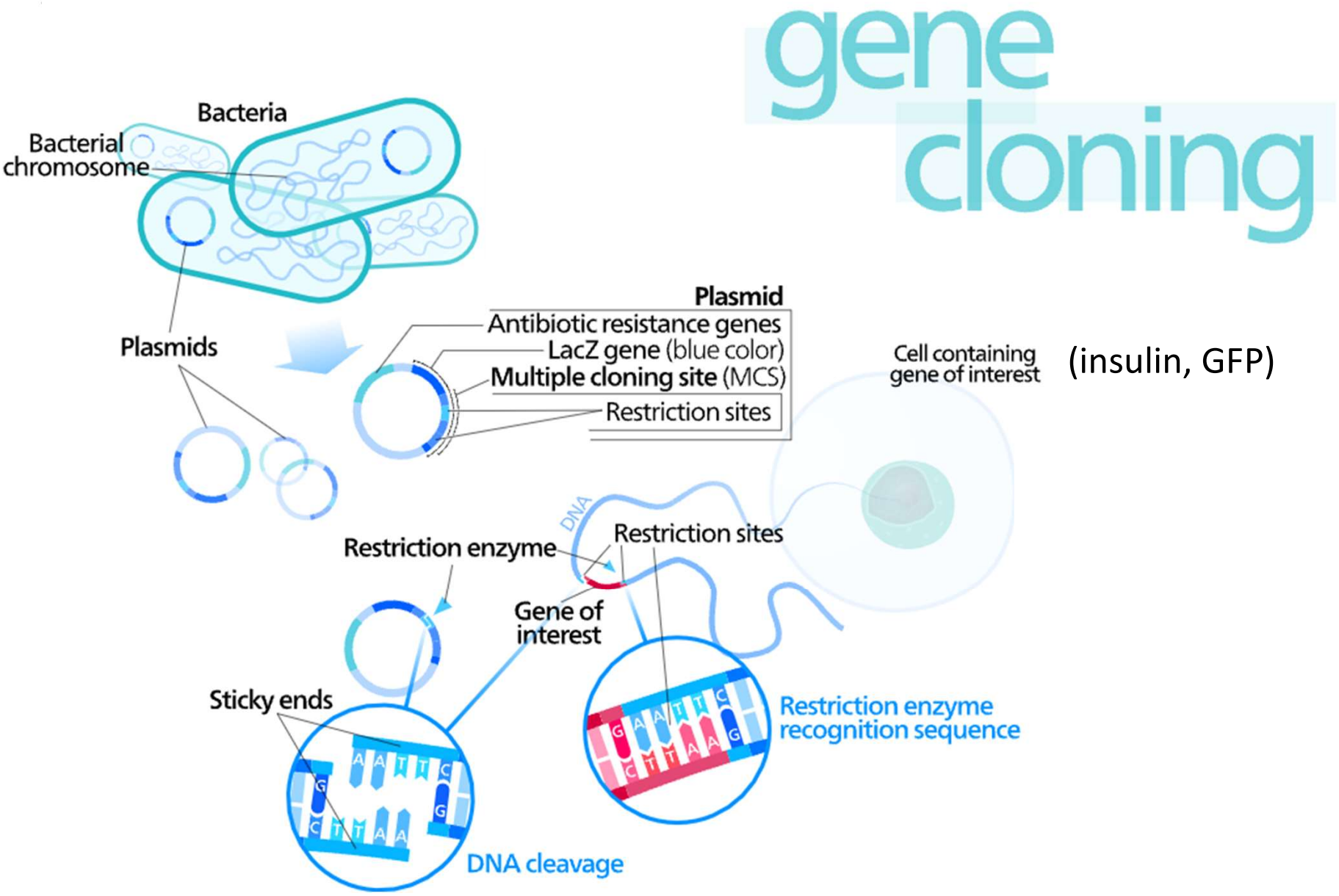
- Ribosome Binding Site (RBS)
- Kozak Sequence
- IRES

- Gene/s of interest (Coding sequence)

- Antibiotic Selection Marker

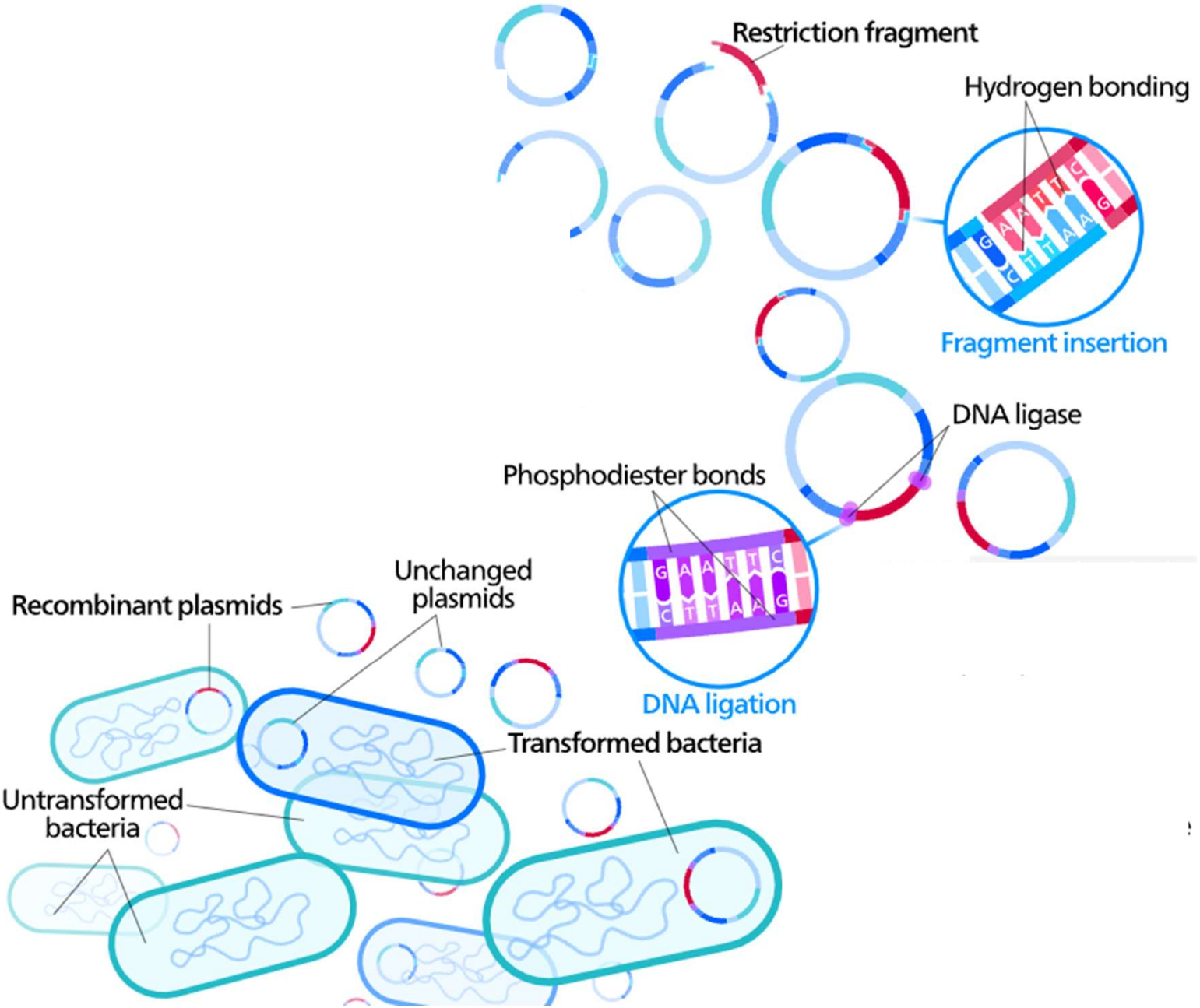
Image source:
https://en.wikipedia.org/wiki/Central_dogma_of_molecular_biology

Molecular Cloning: Overview



Source: https://en.wikipedia.org/wiki/Molecular_cloning

Molecular Cloning: Overview



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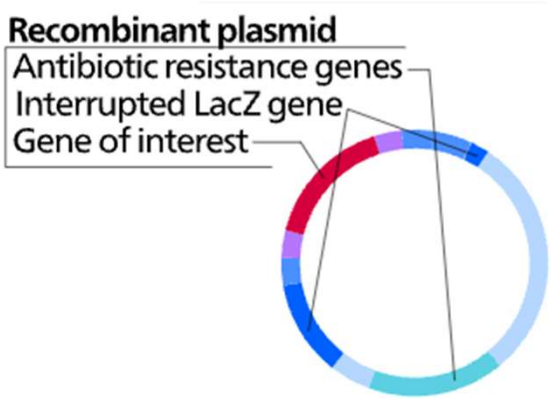
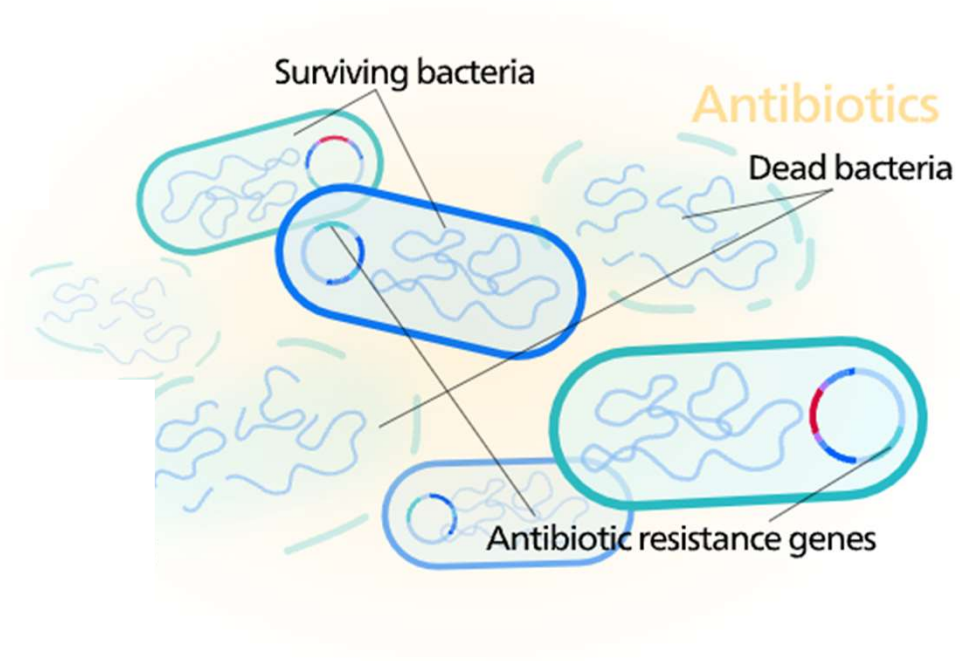
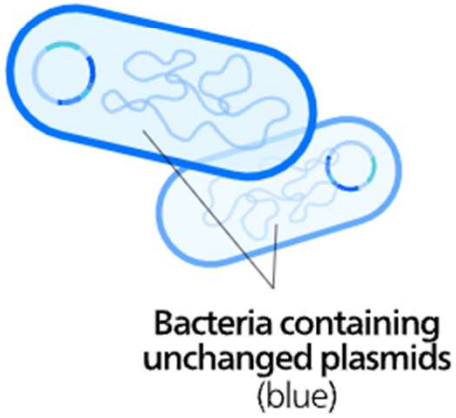
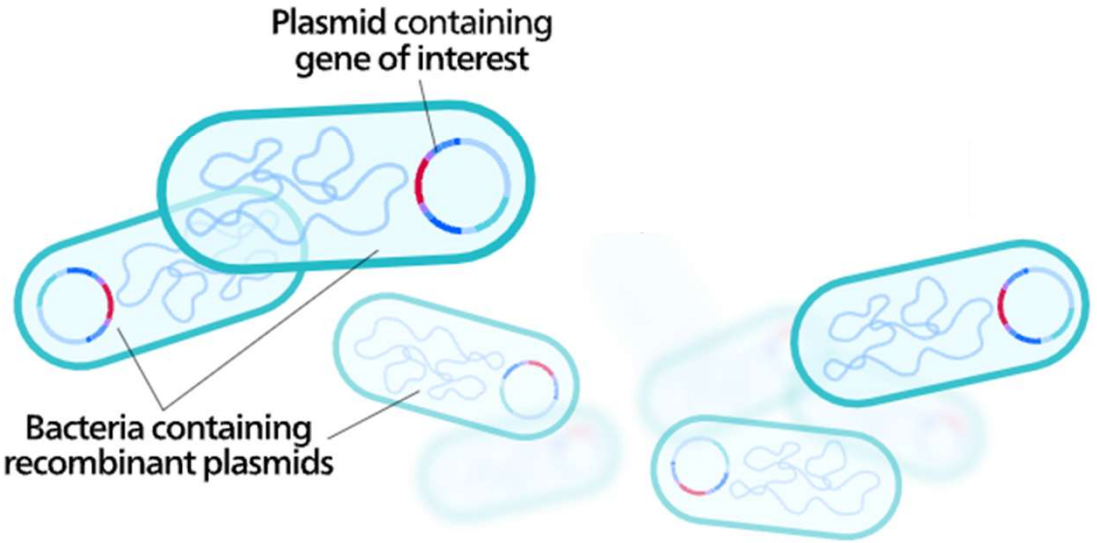
Molecular Cloning: Overview









Source: https://en.wikipedia.org/wiki/Molecular_cloning

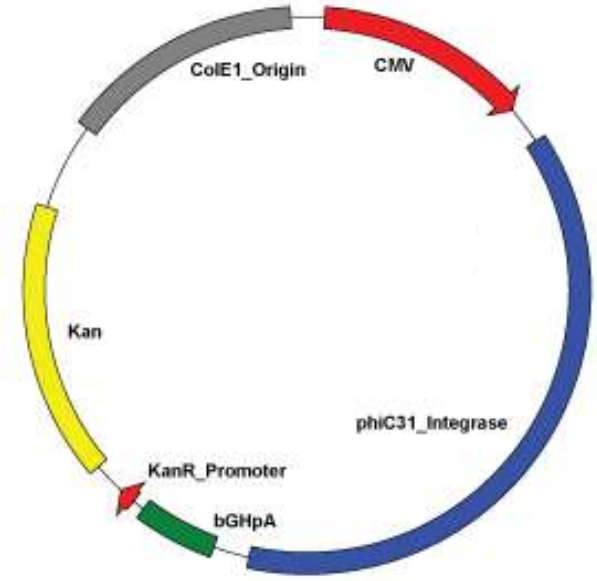
Molecular Cloning: the Plasmid Vector

Plasmid Vectors

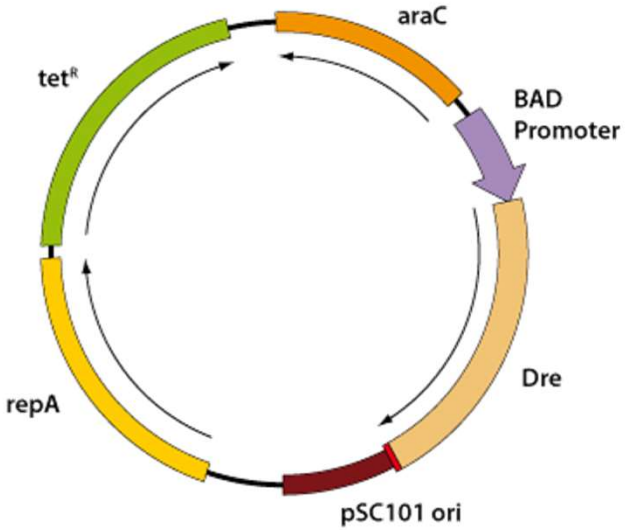
Plasmids must have two required signals:

1. An **origin of replication** to use the host's DNA replication machinery for self-maintenance.
2. An **antibiotic selection marker** to provide a selective advantage to the cells that contain the plasmid.

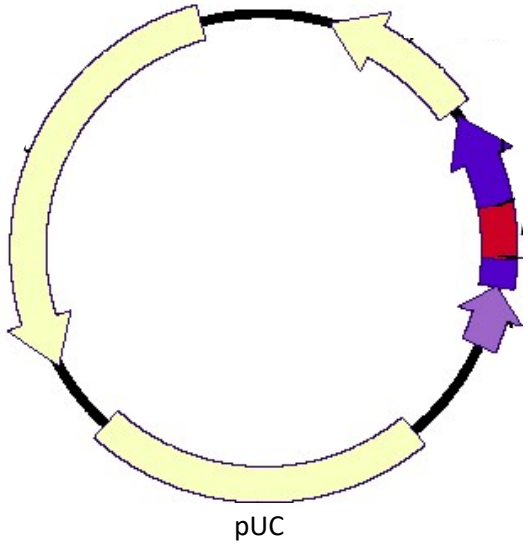
1. ColE1 origin, 40-60 copies
2. Kanamycin resistance



1. pSC101 origin, ~5 copies
2. Tetracycline resistance



1. pUC origin, ~200 copies
2. Ampilicin resistance



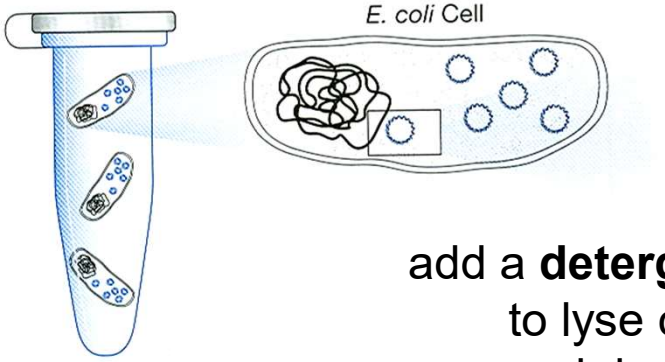
Adapted from ChE340 teaching material (Howard Salis, PennState, 2014)

Molecular Cloning: DNA purification

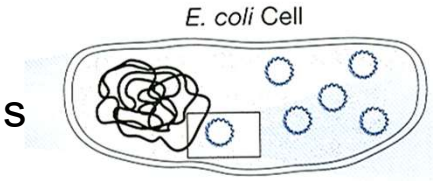
Once your microbial cells have a plasmid, ***they amplify it****.*

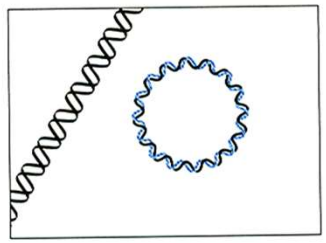
DNA Extraction

- DNA is very hydrophilic, and can be separated & purified using silica columns.
- Standard protocols are available for purifying small DNA molecules (e.g. plasmids) and large DNA molecules (whole chromosomes).

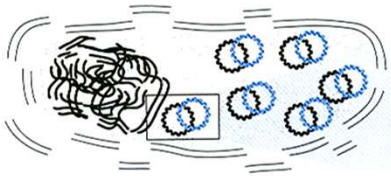


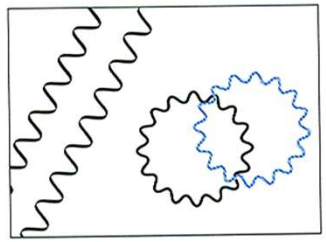
centrifuge
to pellet your cells



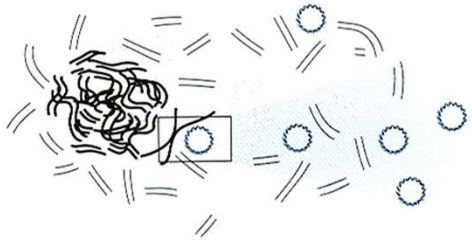


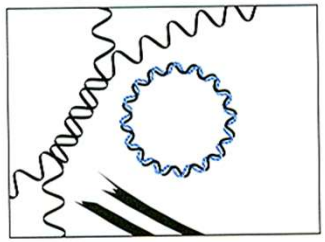
add a **detergent + strong base**
to lyse open your cells
and denature the DNA





add a **weak acid** to neutralize
The plasmid DNA re-anneals quickly,
but not the chromosomal DNA
(**too big for quick reannealing!**)





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Molecular Cloning: DNA purification

From your amplified microbial cells, you can purify the plasmid.

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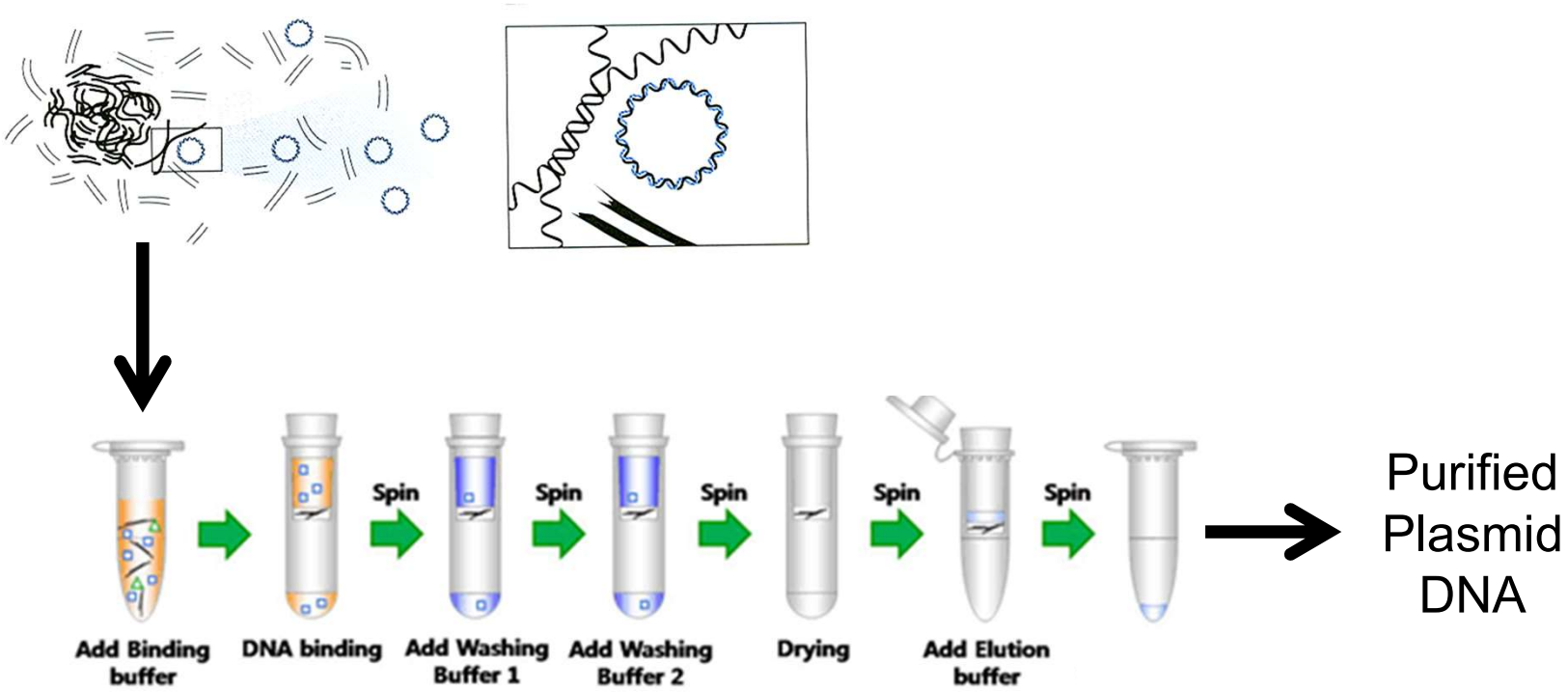
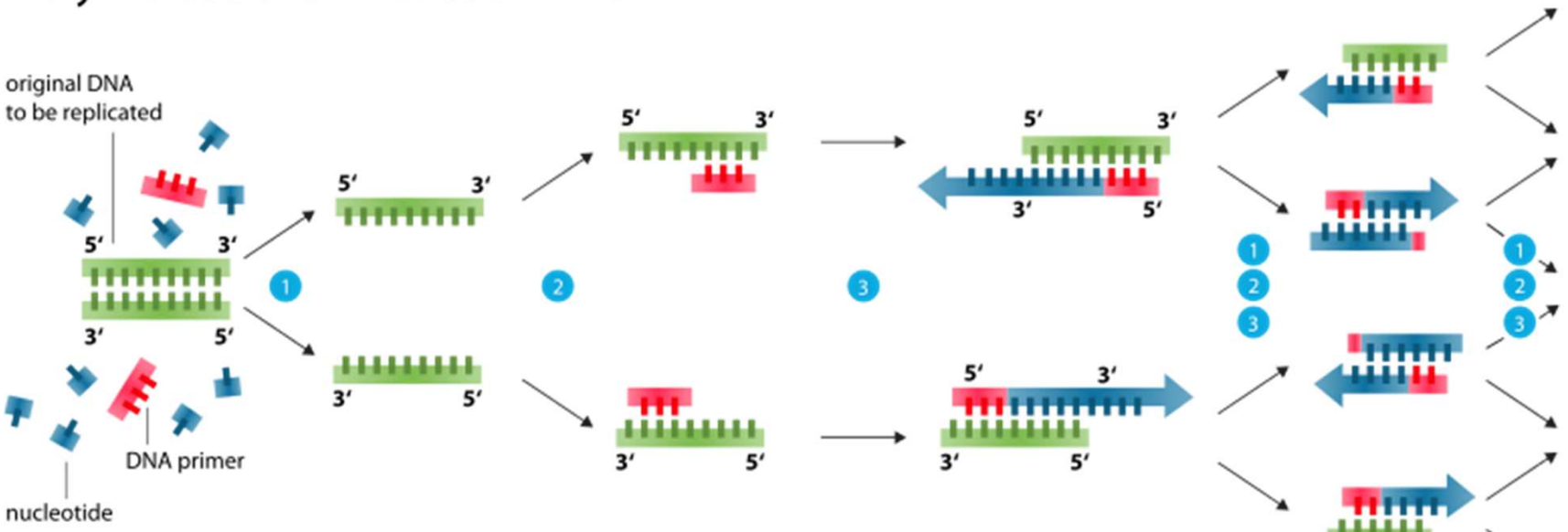


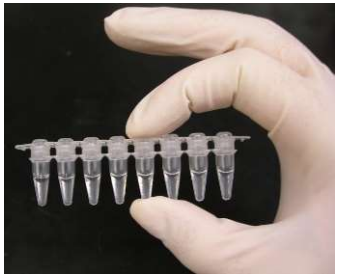
Adapted from ChE340 teaching material (Howard Salis, PennState, 2014)

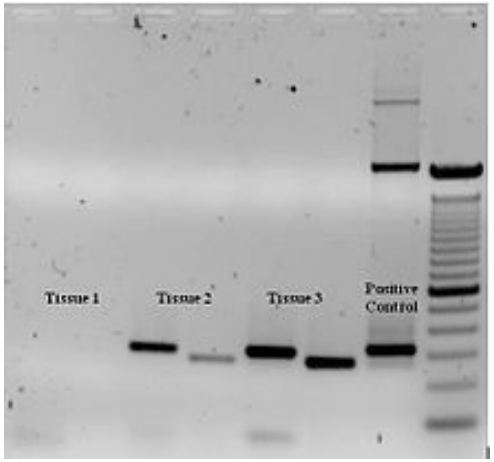
How else can we amplify DNA?

Polymerase chain reaction - PCR

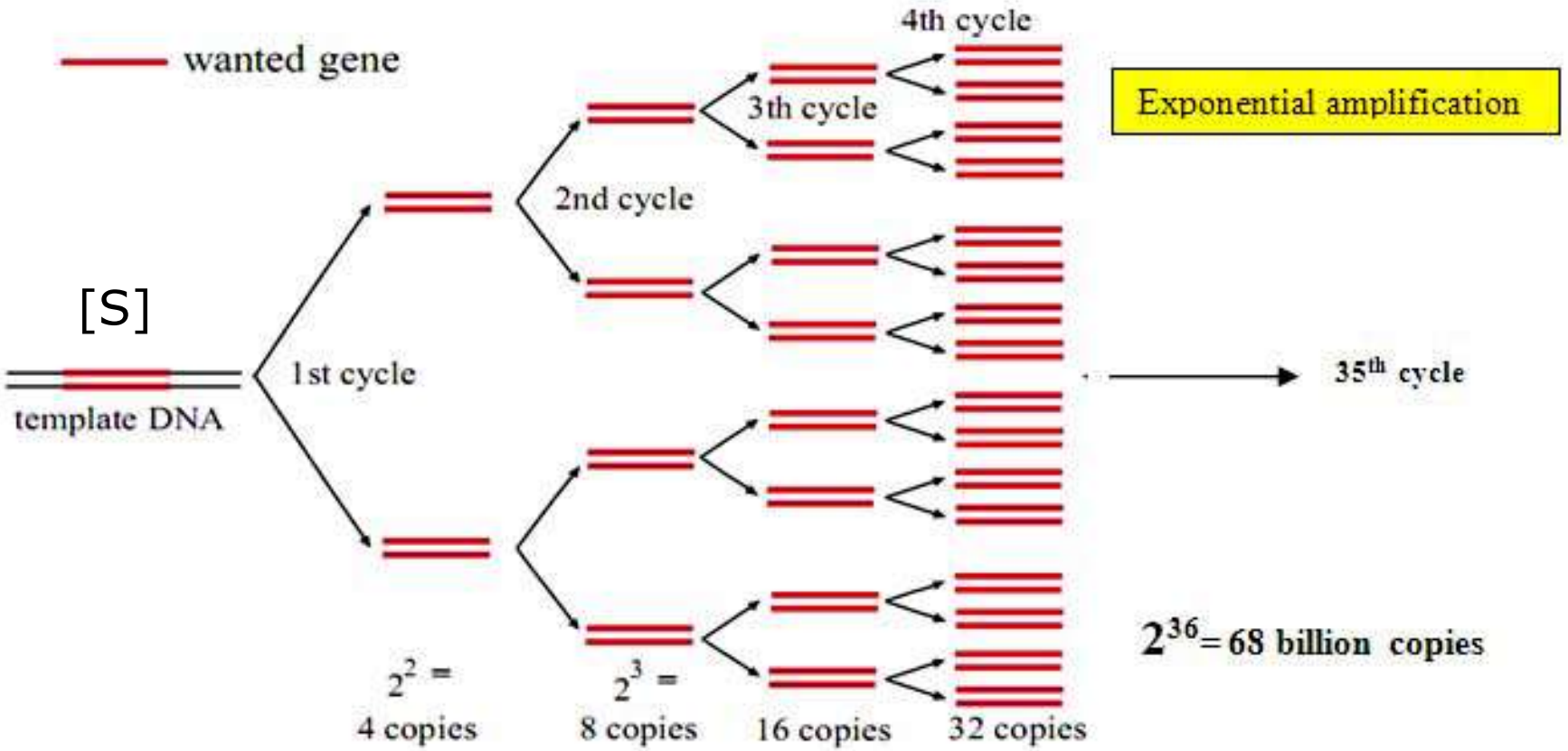


1. **Denaturation** at 94-96°C
2. **Annealing** at ~68°C
3. **Elongation** at ca. 72 °C





Amplifying DNA using PCR



Exponential Amplification

Number of copies of ssDNA obtained after 'n' cycles = [S] * 2^n

Primer design

- Ideal primer length: ~20 nt
- Ideal primer Tm: 60 C
- 3’ GC clamp: 3 out of the last 5 nt should be G/C

Basic Melting Temperature (Tm) Calculations

Two standard approximation calculations are used.

- For sequences less than 14 nucleotides the formula is:

  Tm= (wA+xT) * 2 + (yG+zC) * 4

  where w,x,y,z are the number of the bases A,T,G,C in the sequence, respectively.

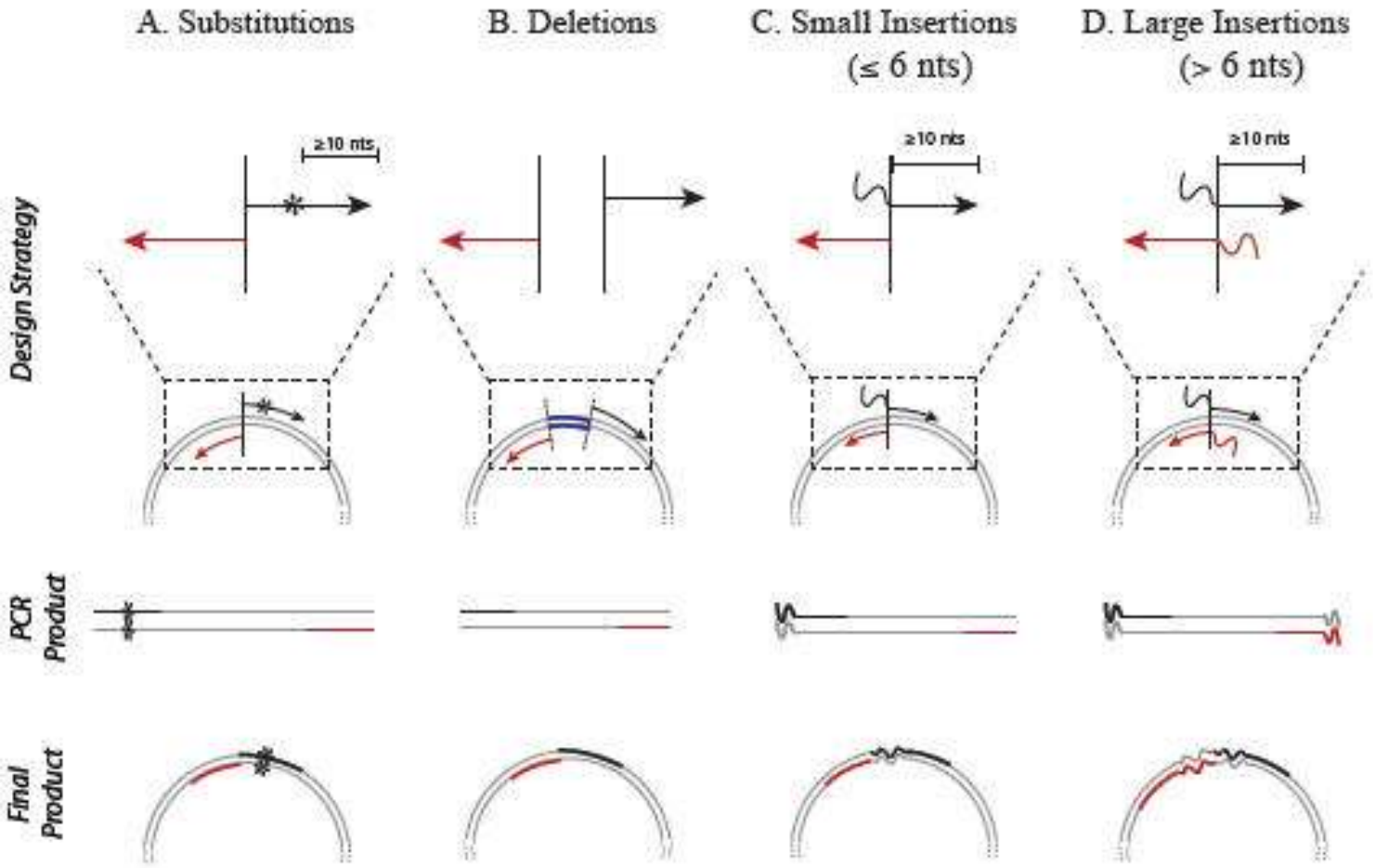
- For sequences longer than 13 nucleotides, the equation used is

  Tm= 64.9 +41*(yG+zC-16.4)/(wA+xT+yG+zC)

http://insilico.ehu.es/tm.php?formula=basic

Primer3
https://bioinfo.ut.ee/primer3-0.4.0/

PCR to modify DNA (ends)



Molecular Cloning Toolkit

Once you have DNA in a tube, you can cut, paste, & modify it.

Enzymes to Chemically Modify DNA

Restriction endonuclease:
- Binds to a defined DNA sequence, and cuts the DNA.
- Creates "sticky" or "blunt" ends to be used for ligation.

DNA Ligase:
- Binds to two DNAs and creates a covalent bond

Two types of ligation reactions: sticky end & blunt end
- DNA ends must have 3'-OH and 5'-PO_4

T4 Polynucleotide kinase (PNK): Adds a phosphate to the 5' ends of DNA fragments

DNA Phosphatases:
- Removes the 5'-PO_4 from DNA fragment ends

Antartic Phosphatase (5' only), Shrimp Alkaline Phosphatase (5' & 3')

Exonucleases:
- Chews back sticky ends (single-stranded DNA)

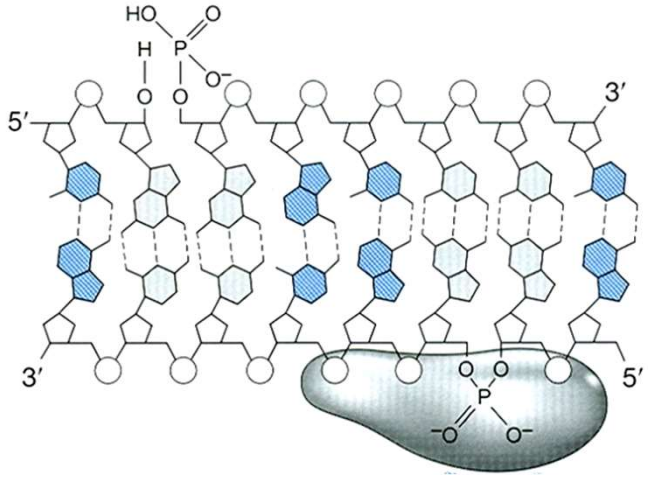
Mung Bean Nuclease:
- Chews back both 5' and 3' sticky ends.

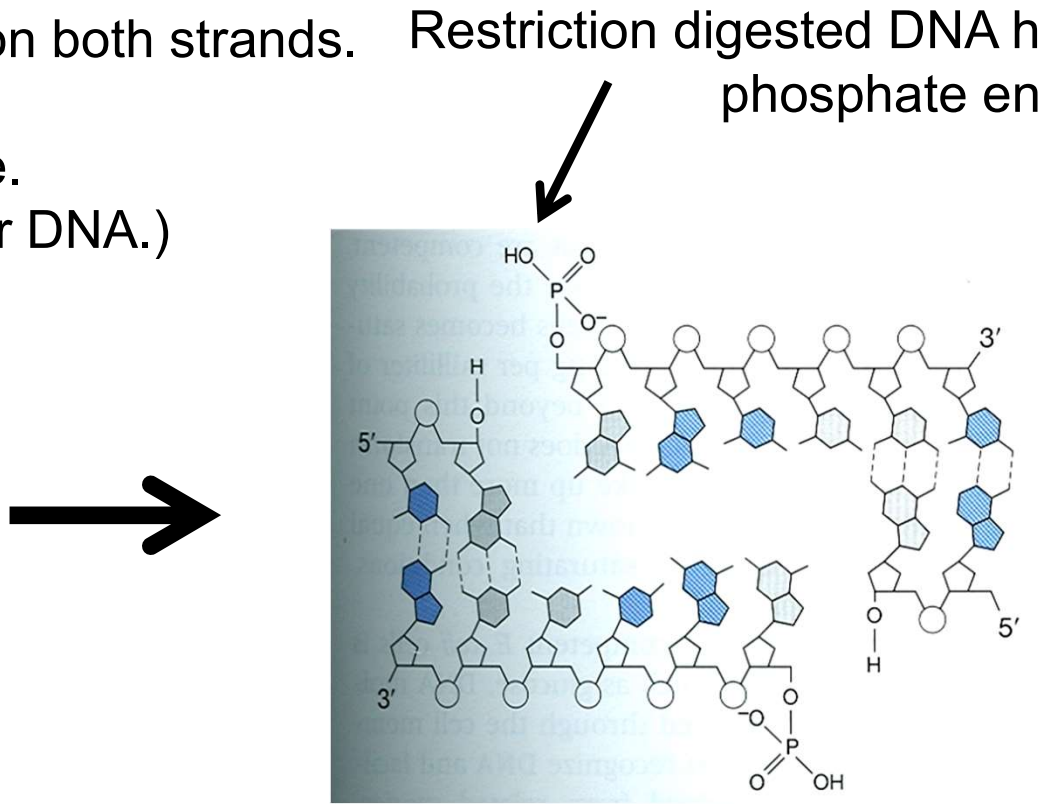
T7 Exonuclease:
- Chews back 5' sticky ends

Adapted from ChE340 teaching material (Howard Salis, PennState, 2014)

Restriction Digestion

- Restriction Endonucleases cut DNA on both strands.
- Creates two DNA fragments from one.
  (Or one DNA fragment from a circular DNA.)





- The recognition sequence for a restriction enzyme is 4 to 8 base pairs long (typically a palindrome)

EcoRI: G^AATTC
AscI: GG^CGCGCC

- Many restriction enzymes need some **flanking/ landing space** (they may not bind to their DNA sequence if it is too close to the end)

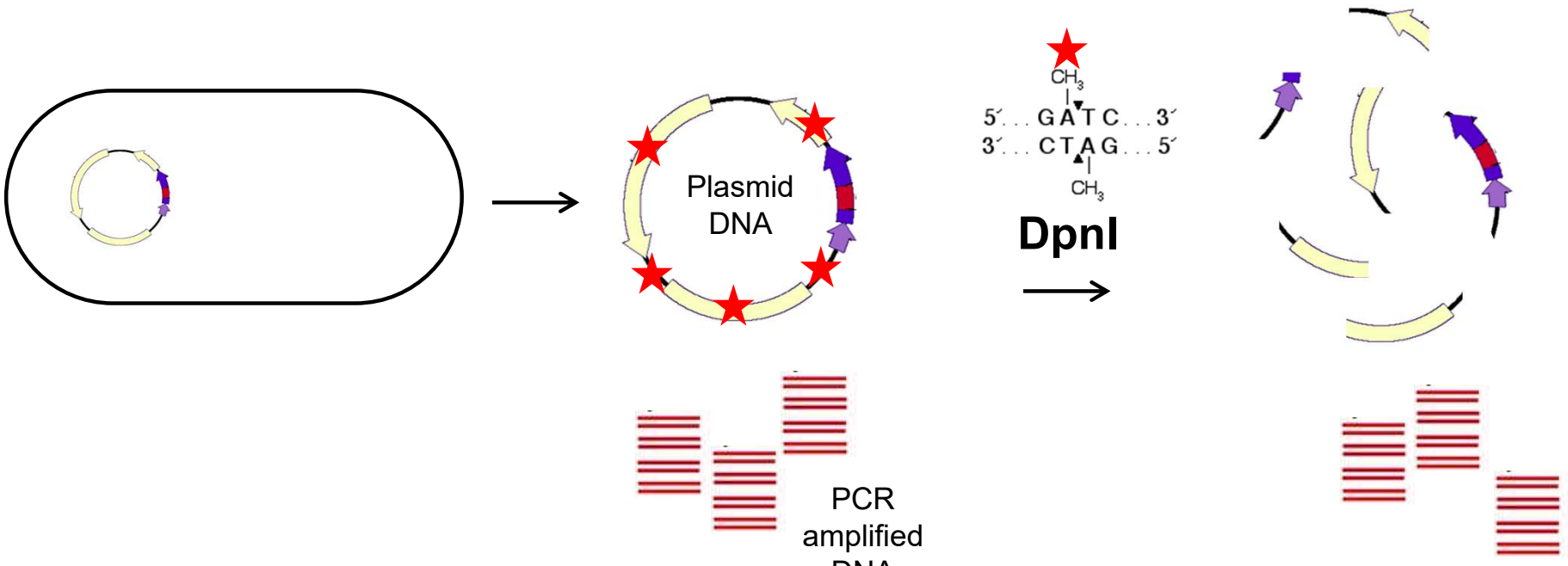
Adapted from ChE340 teaching material (Howard Salis, PennState, 2014)

Restriction Digestion

Dam methylase (*dam* gene): GATC (methylates N6 in A)
Mec methylase (*dcm* gene): CCAGG and CCTGG (methylates C5 position in the 2nd C)

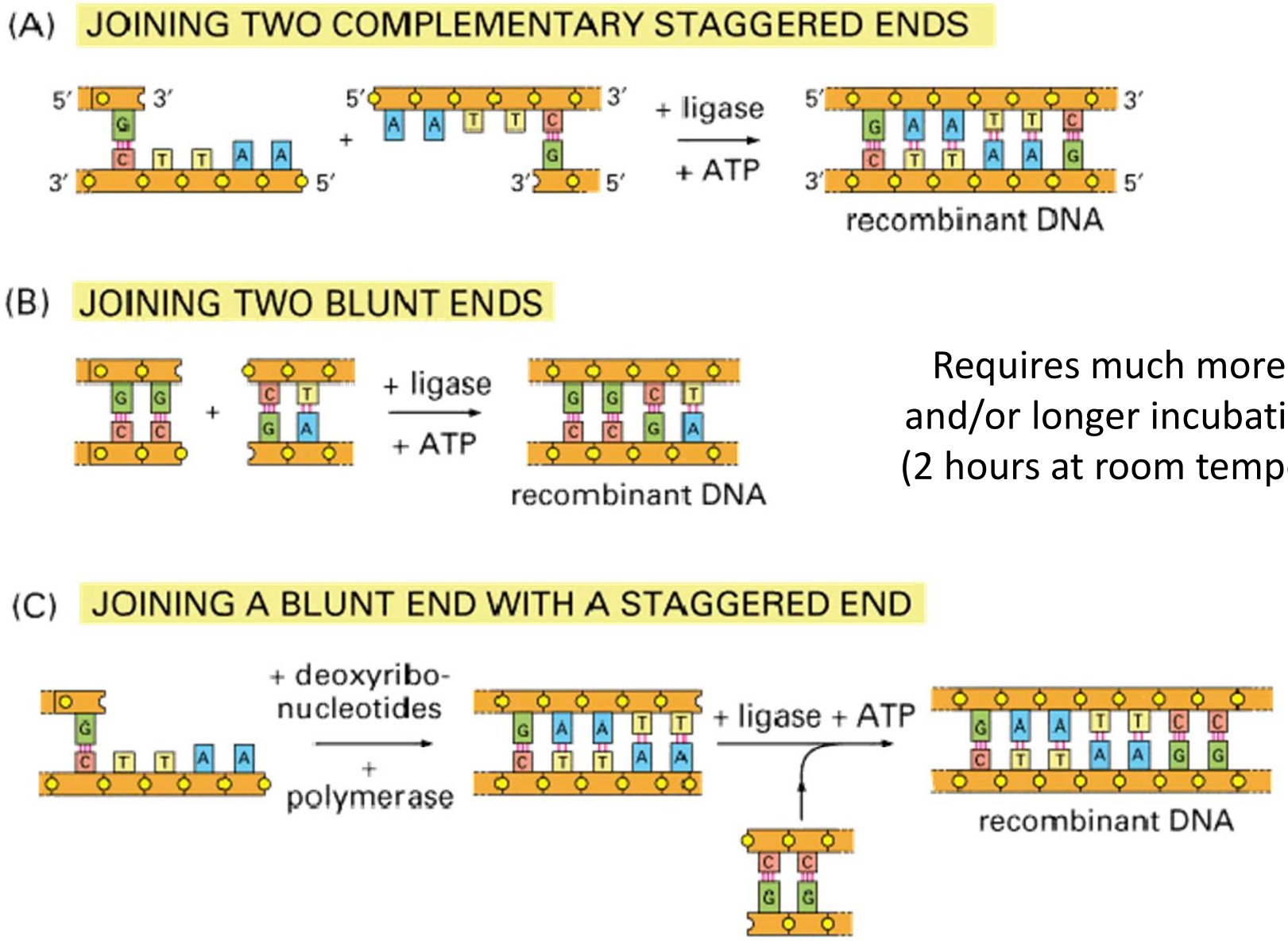
- Methylation of restriction sites often makes them resistant to restriction digestion.

- However, some methylated sites become sensitive to specific restriction enzymes.
- **This can be used to remove template DNA (methylated) after PCR**.



DNA Ligase connects sticky ends and blunt ends

3'-OH and 5'-PO_4

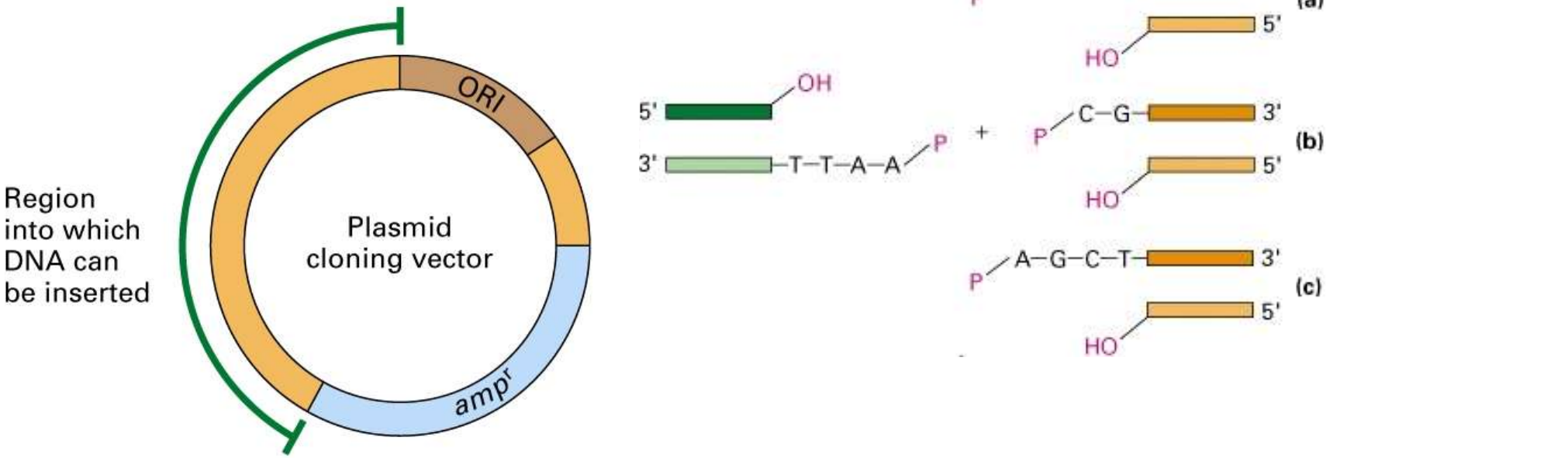


Requires much more ligase and/or longer incubation time (2 hours at room temperature)

Figure 10-18 Essential Cell Biology, 2/e. (© 2004 Garland Science)

Adapted from ChE340 teaching material (Howard Salis, PennState, 2014)

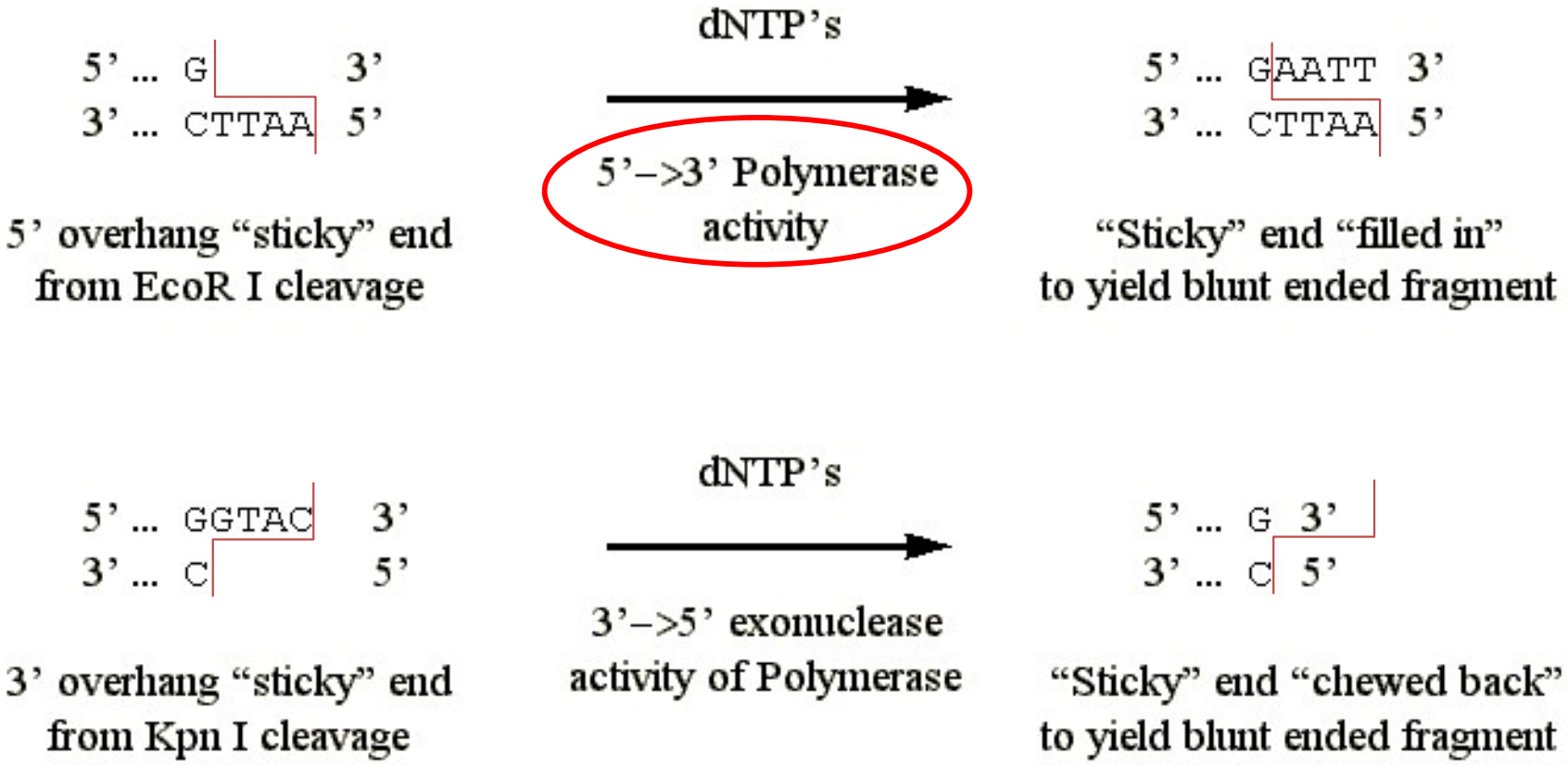
Restriction fragments with complementary “sticky ends” are ligated easily



Adapted from ChE340 teaching material (Howard Salis, PennState, 2014)

Other DNA modifying enzymes (Blunting)

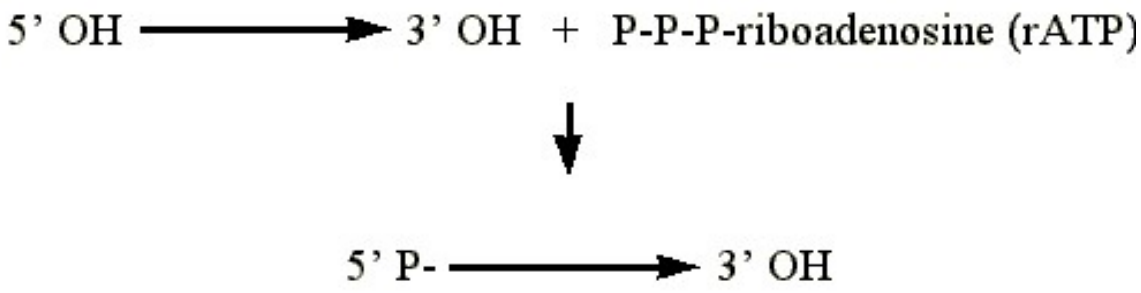
- T4 DNA polymerase
  - Polymerase activity (fill, 5' → 3')
  - Nuclease activity (chew, 3' exonuclease)



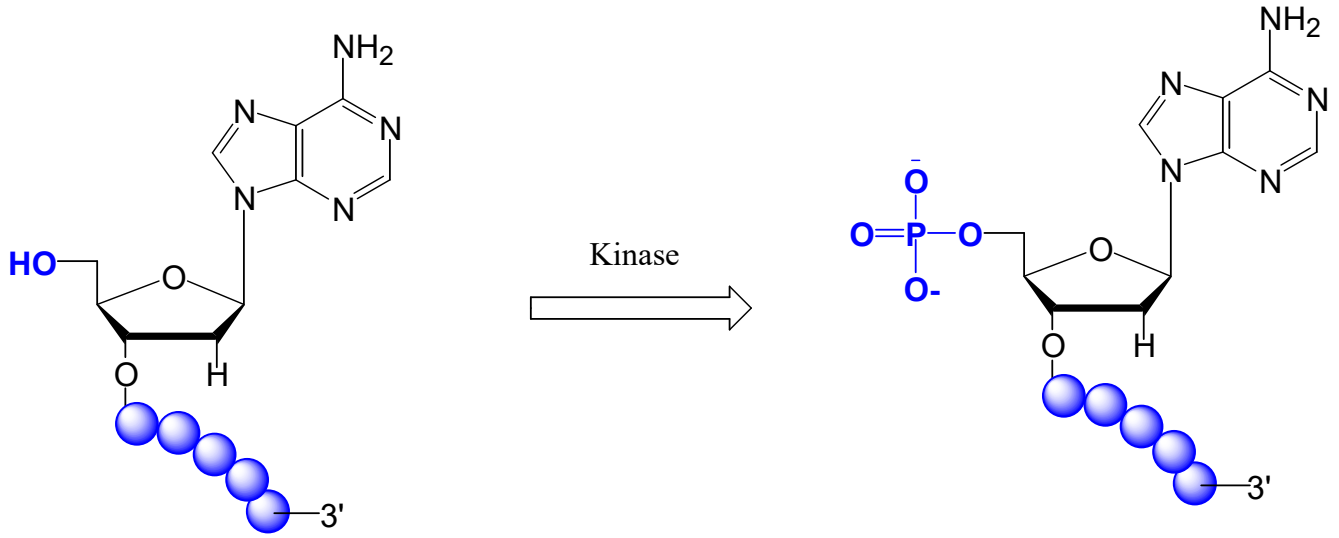
Adapted from ChE340 teaching material (Howard Salis, PennState, 2014)

Polynucleotide Kinase (PNK)

Adds 5'phosphate



γ-Phosphate group is transfered from rATP

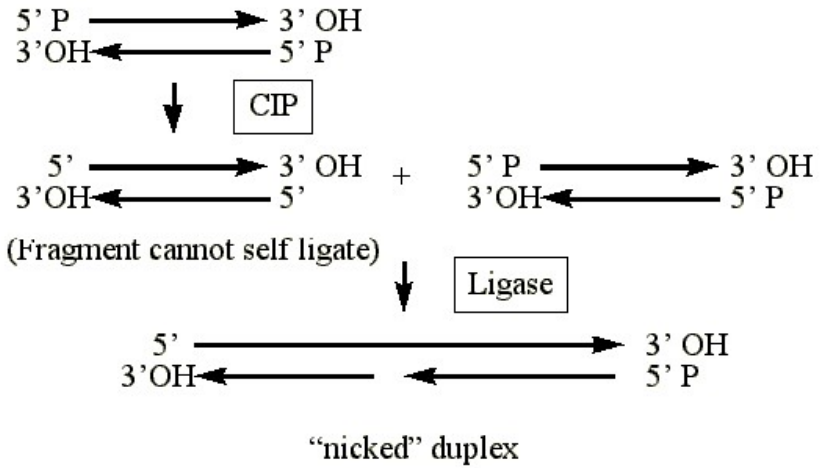


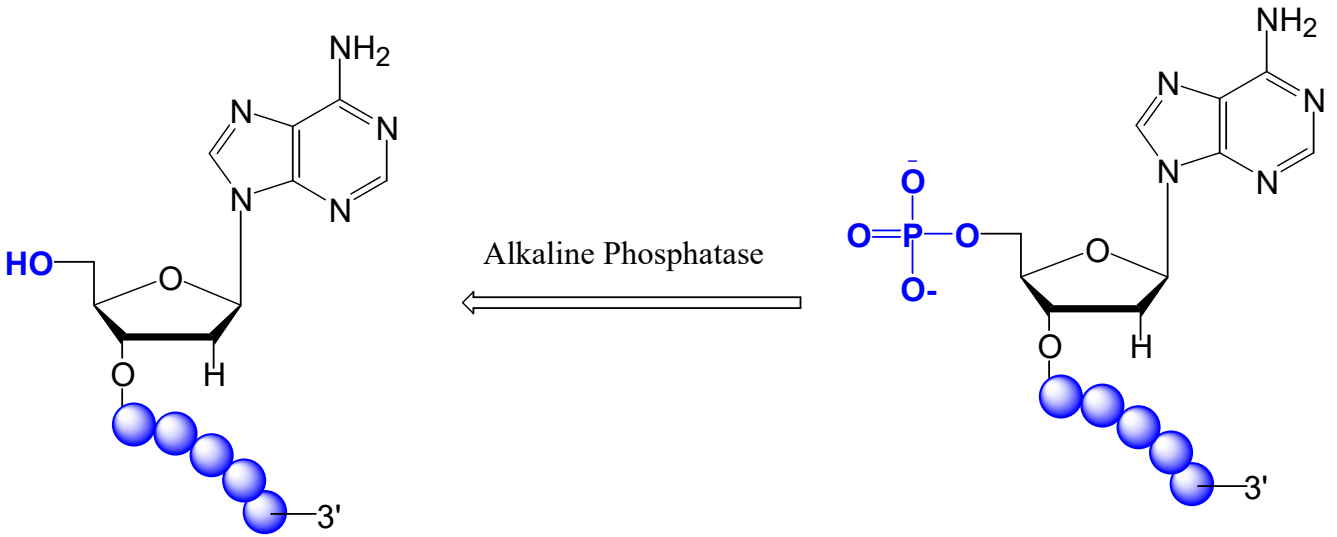
Synthetic oligonucleotides are unphosphorylated at the 5' end.
A very useful enzyme to radiolabel DNA fragments.

Adapted from ChE340 teaching material (Howard Salis, PennState, 2014)

Phosphatase

Removes 5’ phosphate

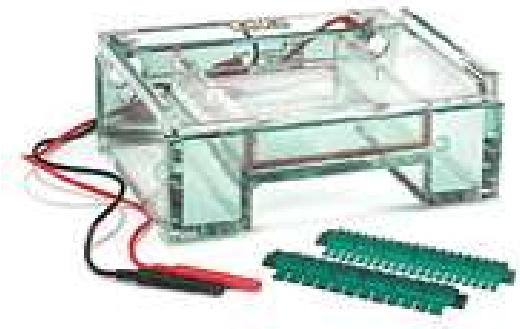


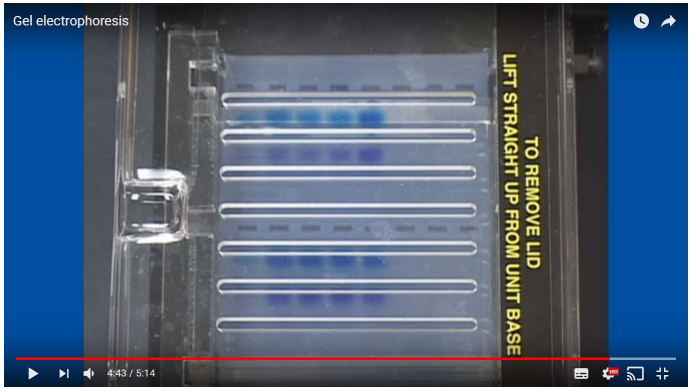


A useful enzyme to reduce the background of cloning.

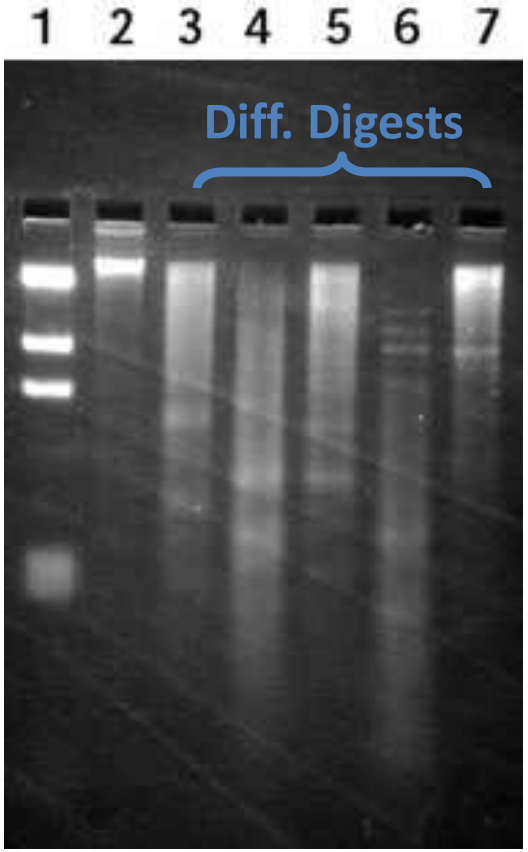
Adapted from ChE340 teaching material (Howard Salis, PennState, 2014)

Visualization and Separation of DNA





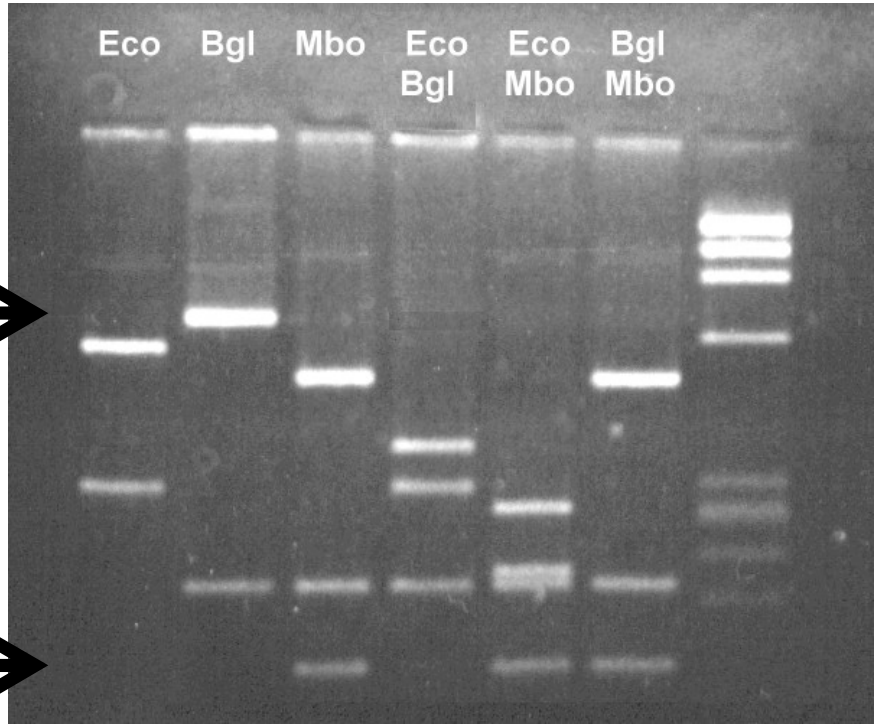
Gel Electrophoresis in Action.
http://www.youtube.com/watch?v=QEG8dz7cbnY



Genomic DNA

Longer DNA fragments

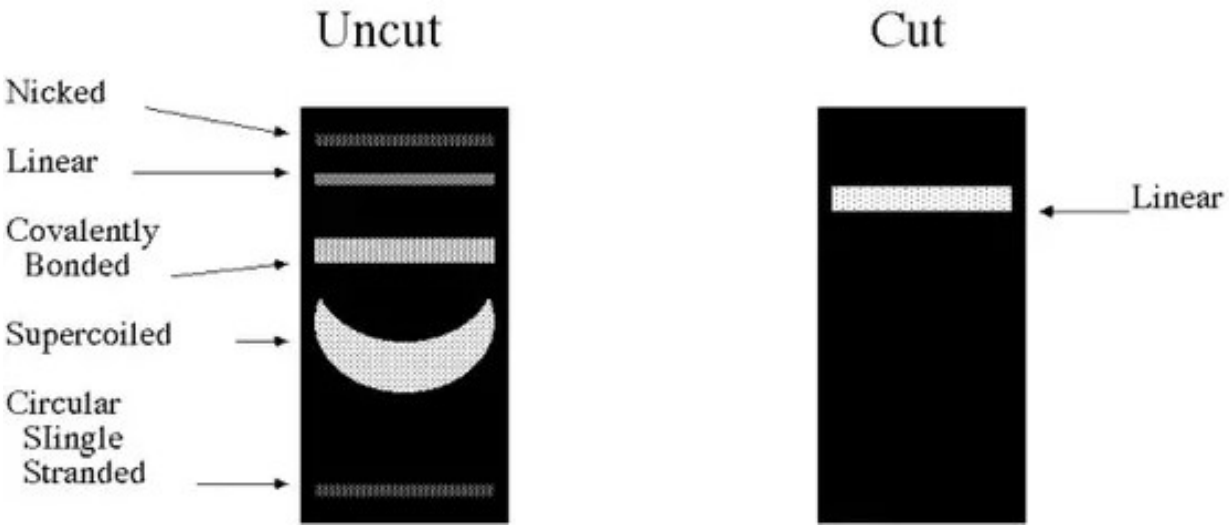
Shorter DNA fragments

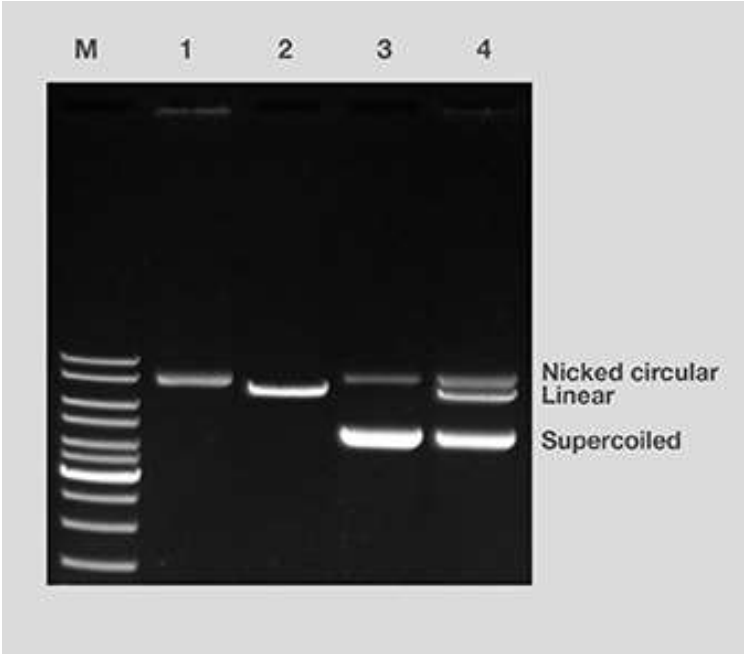


Plasmid DNA

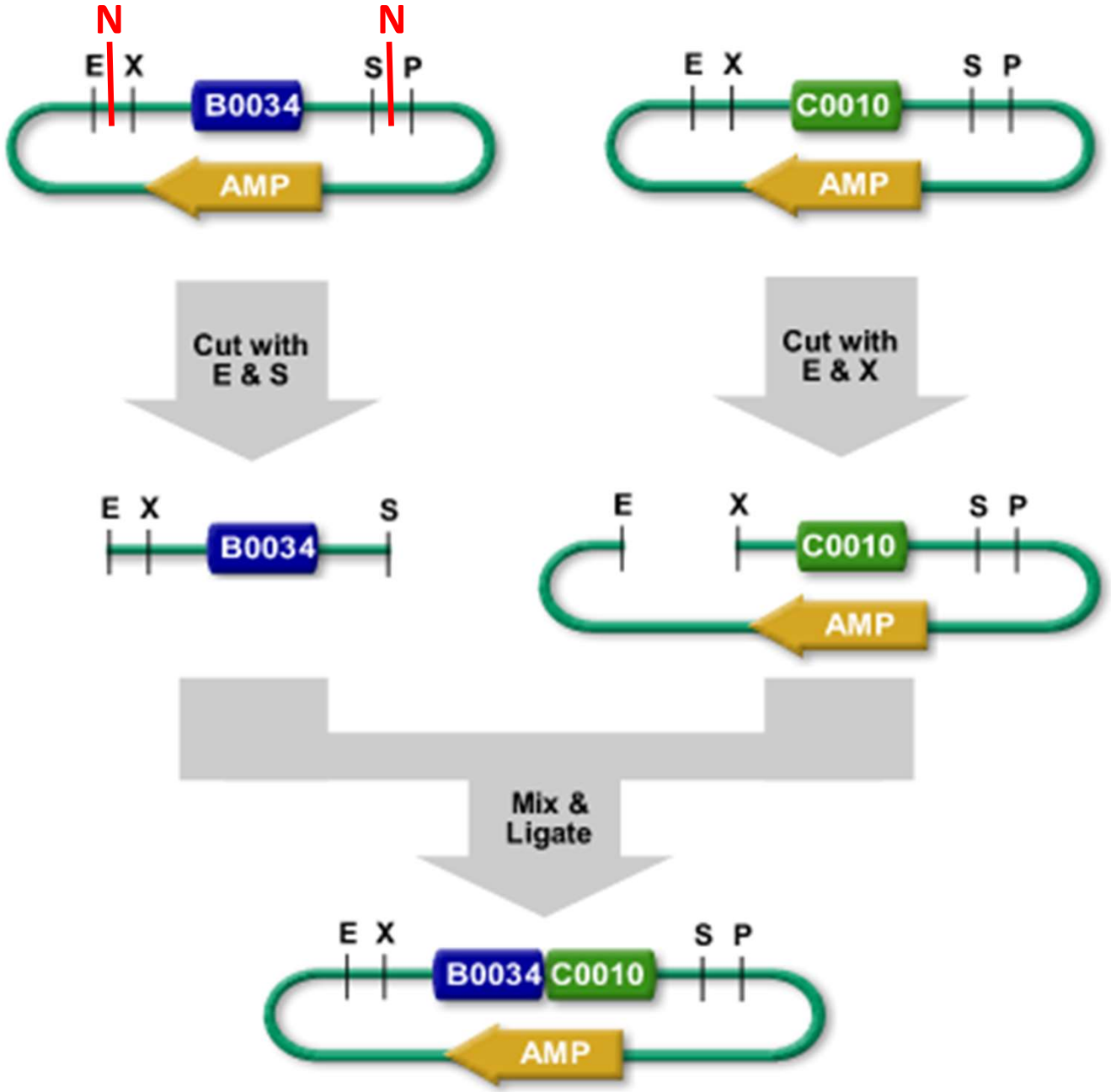
Visualization and Separation of DNA

Plasmid DNA





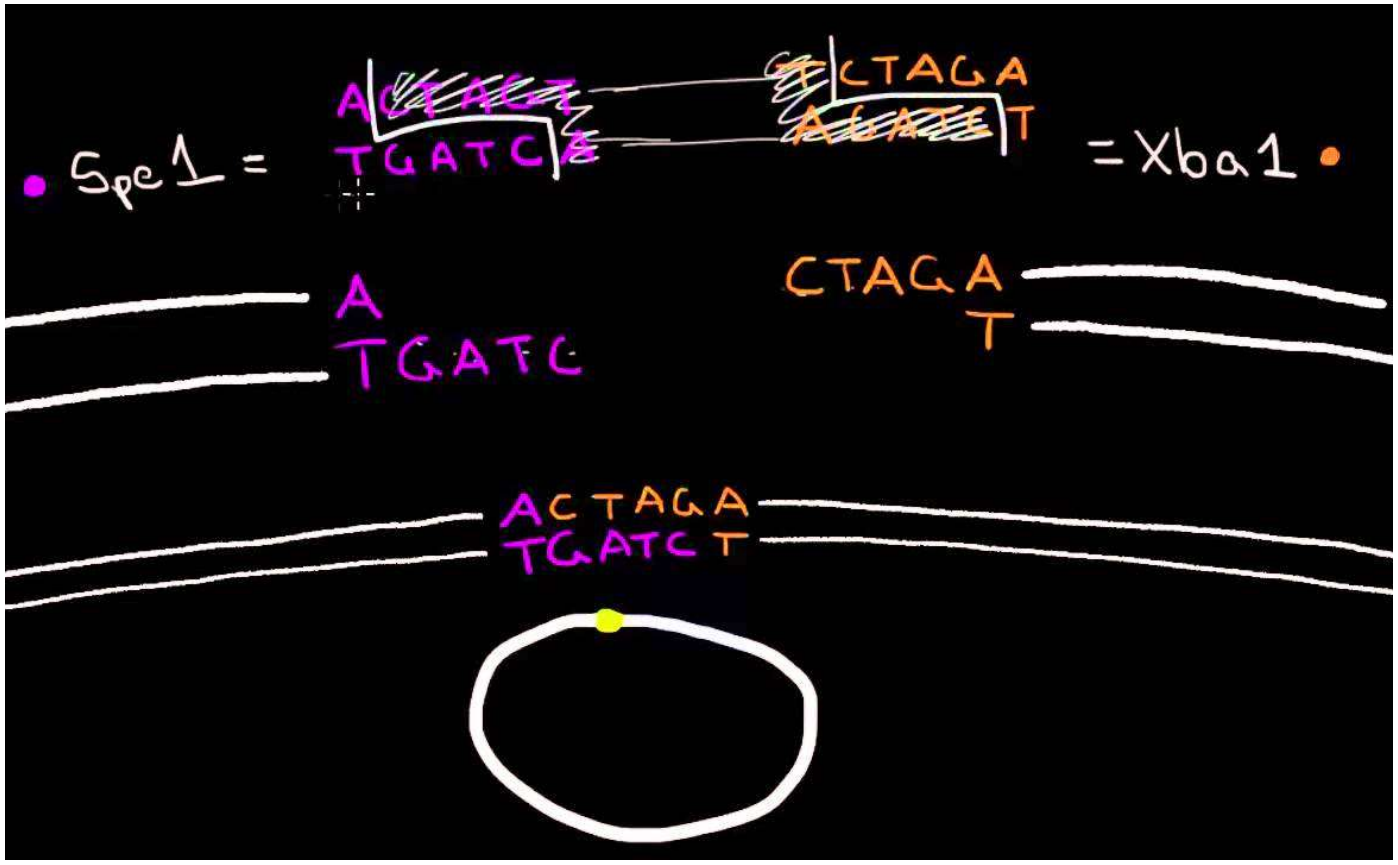
BioBrick Cloning: step-wise addition of parts



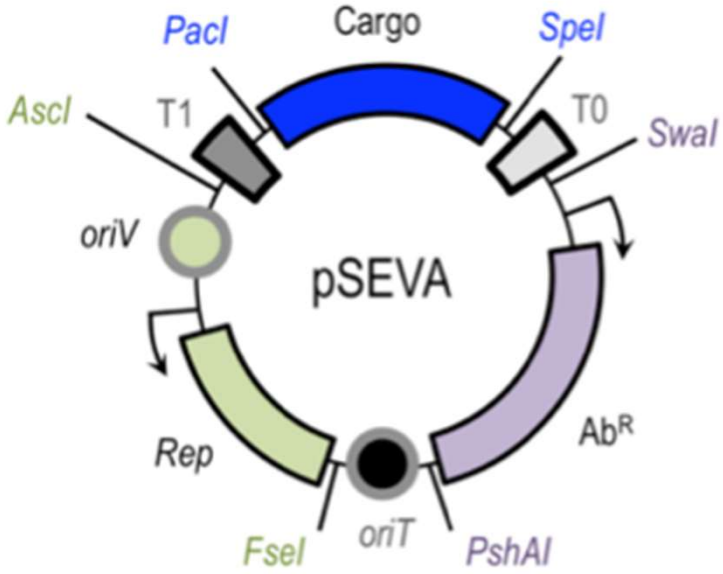
BioBrick vector standards

E = EcoRI
X = XbaI
S = SpeI
P = PstI
N = NotI

BioBrick Cloning
SpeI and XbaI have compatible ends



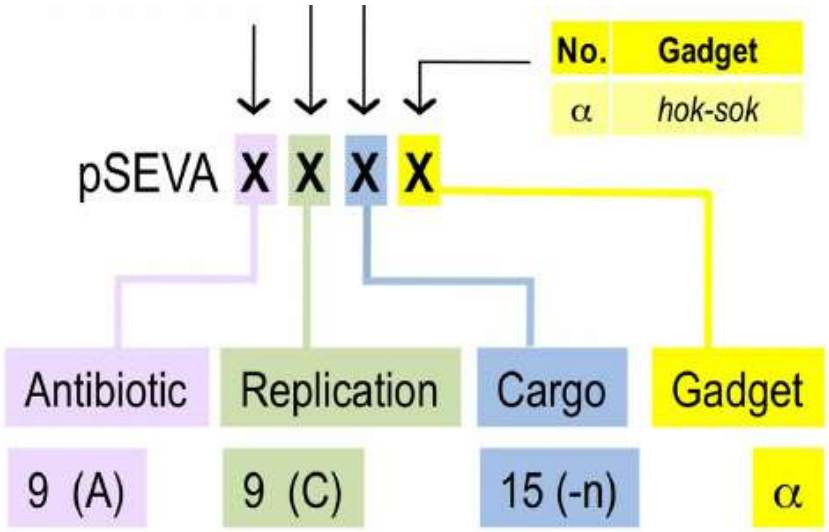
The Standard European Vector Architecture (SEVA 2.0)



No.	Ori V
1	R6K
2	RK2
3	pBBR1
4	pRO1600/ColE1
5	RSF1010
6	p15A
7	pSC101
8	pUC
9	pBBR322/ROP

No.	Cargo
1	MCS-default
2	*lacZα*-pUC19
3	*lacZα*-pUC18
4	*lacI^q-Ptrc*
5	*lacZ*
6	*luxCDABE*
7	*GFP*
8	*xylS-Pm*
9	*alkS-Palk*
10	*araC-Pbad*
11	*chnR-PchnB*
12	*cprK1-PDB3*
13	PEM7

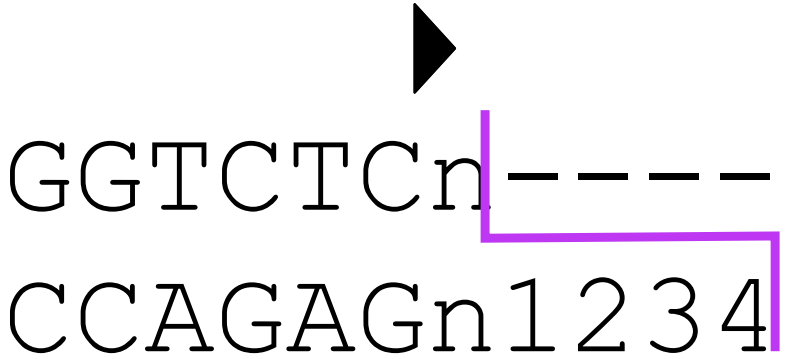
No.	Antb
1	Ap
2	Km
3	Cm
4	Sm/Sp
5	Tc
6	Gm



Martínez-García *et al.*, 2014. NAR.

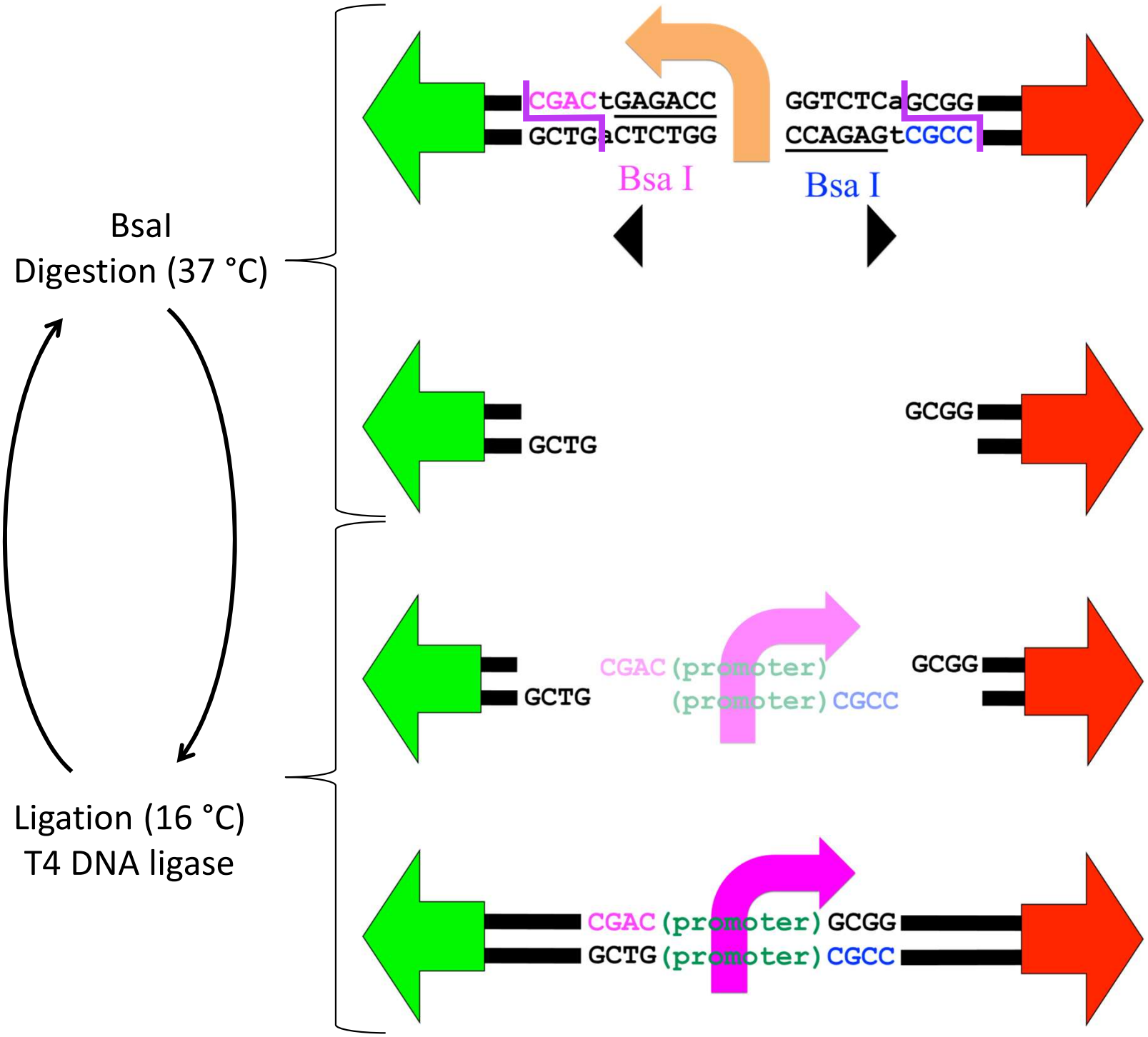
Golden Gate cloning enzyme

Bsa I

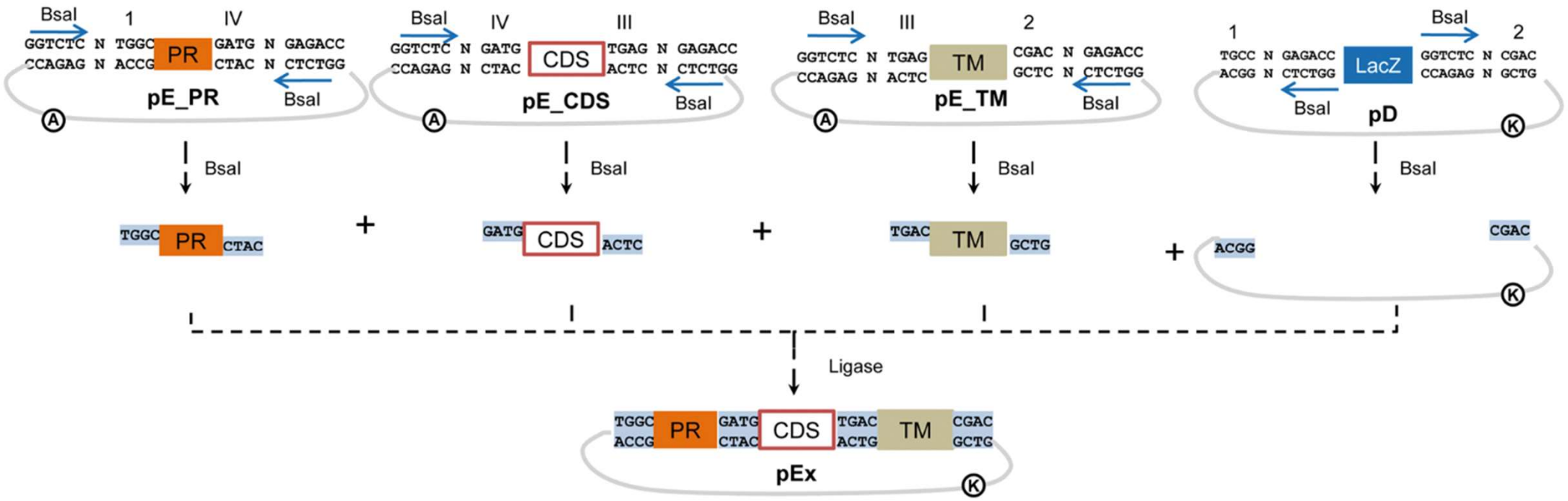


- Not Palindromic
- Type II S

Golden Gate cloning

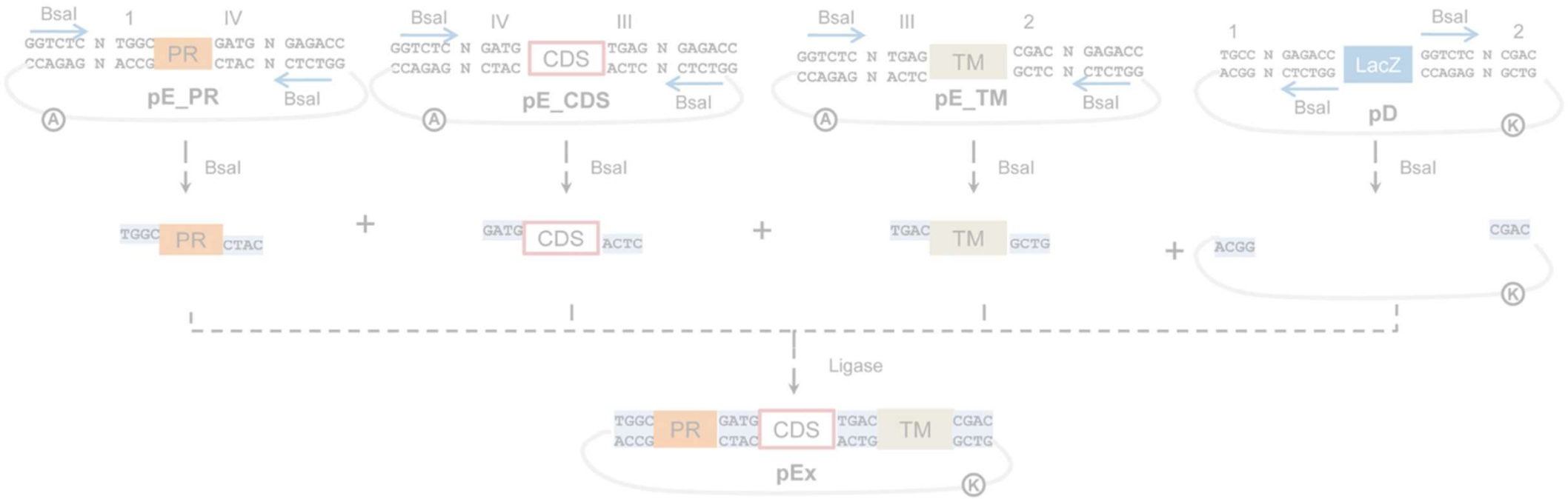


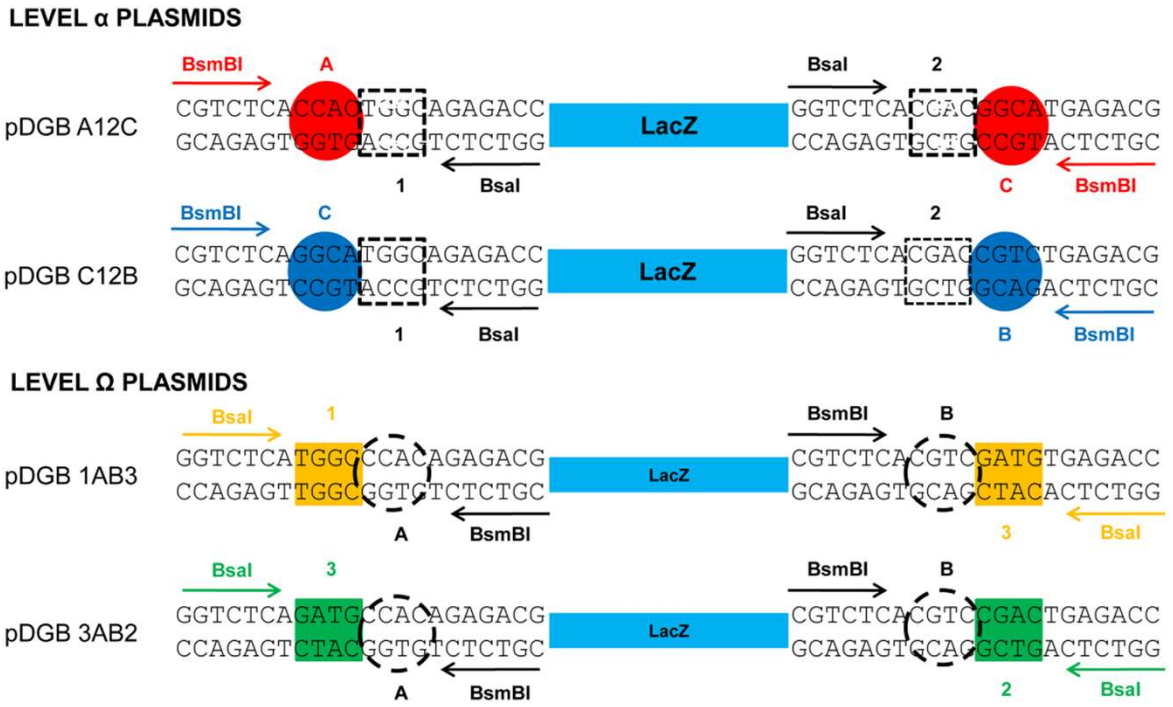
Golden Braid cloning



Sarrion-Perdigones *et al.*, 2011. PLoS One.

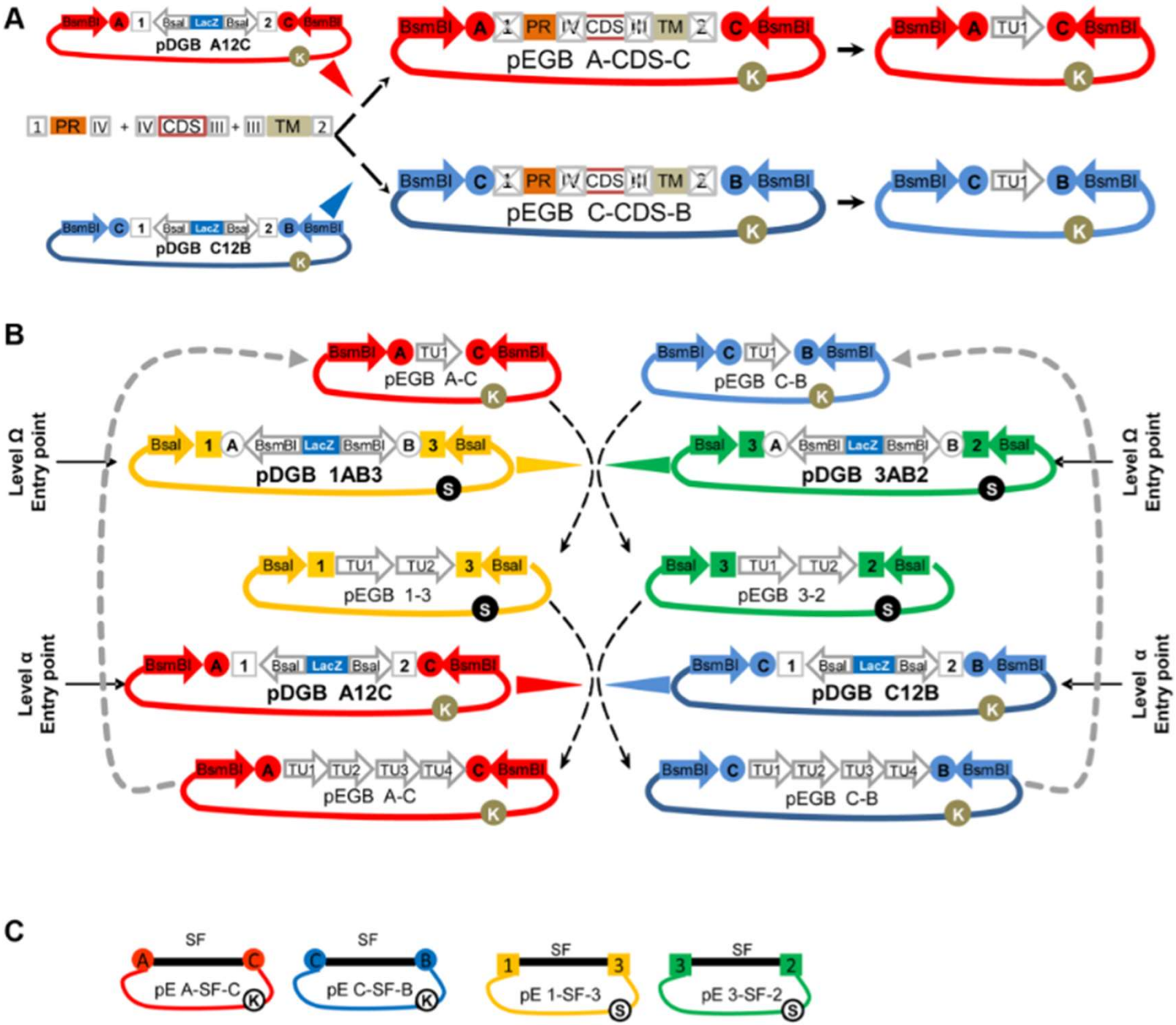
Golden Braid cloning





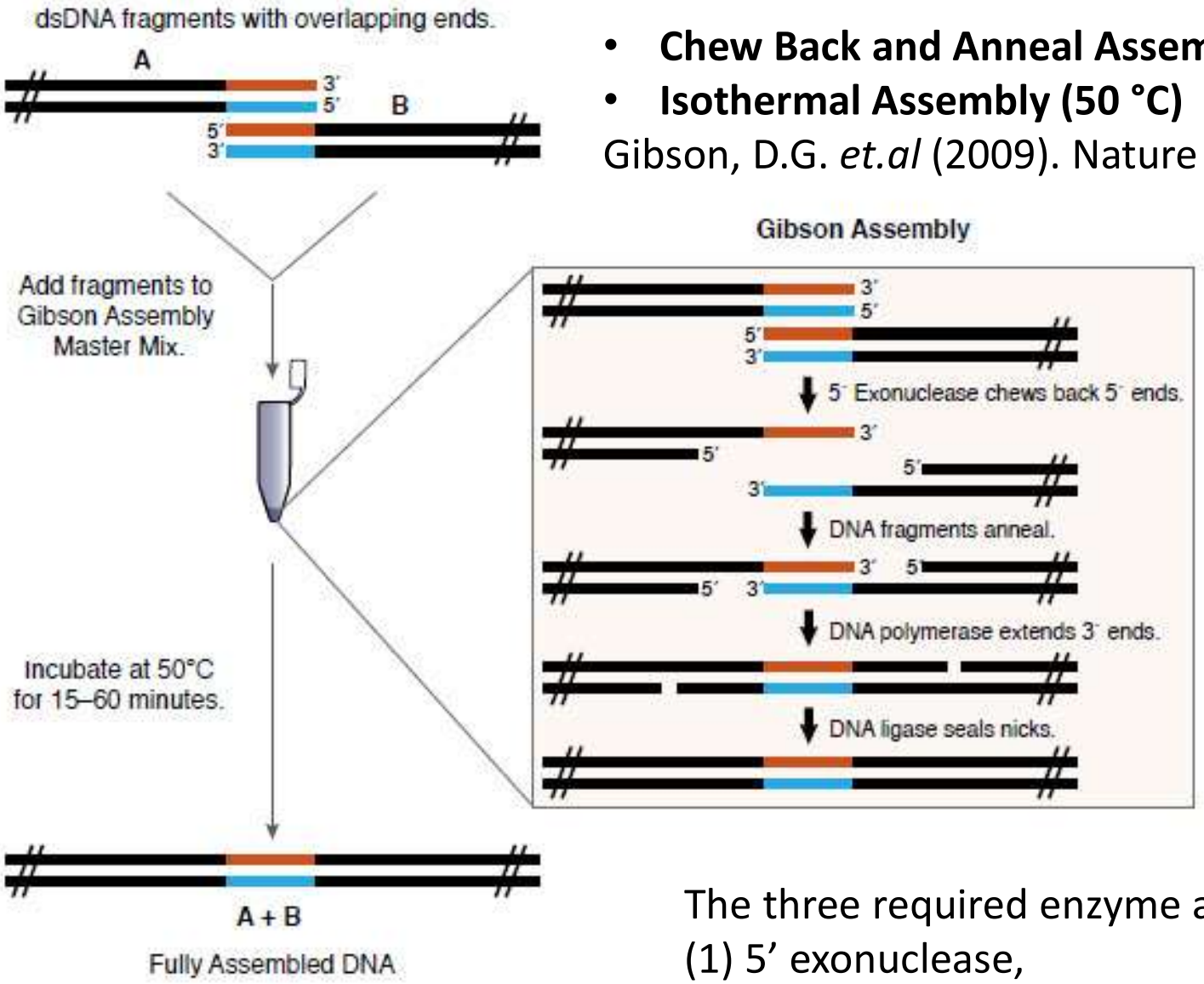
Sarrion-Perdigones *et al.*, 2011. PLoS One.

Golden Braid cloning



Sarrion-Perdigones *et al.*, 2011. PLoS One.

Gibson cloning

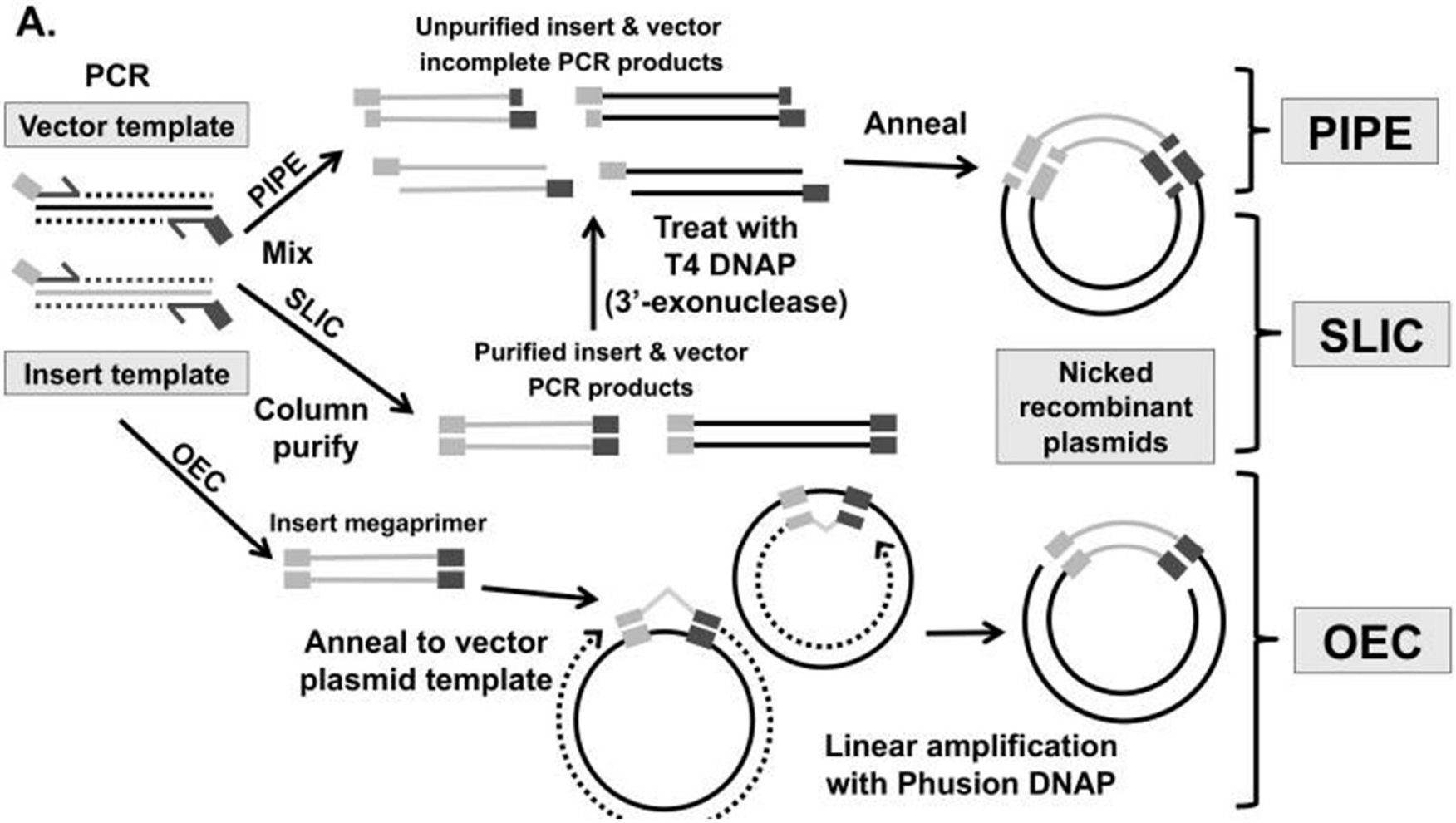


- **Chew Back and Anneal Assembly (CBA)**
- **Isothermal Assembly (50 °C)**

Gibson, D.G. *et.al* (2009). Nature Methods. 343-345.

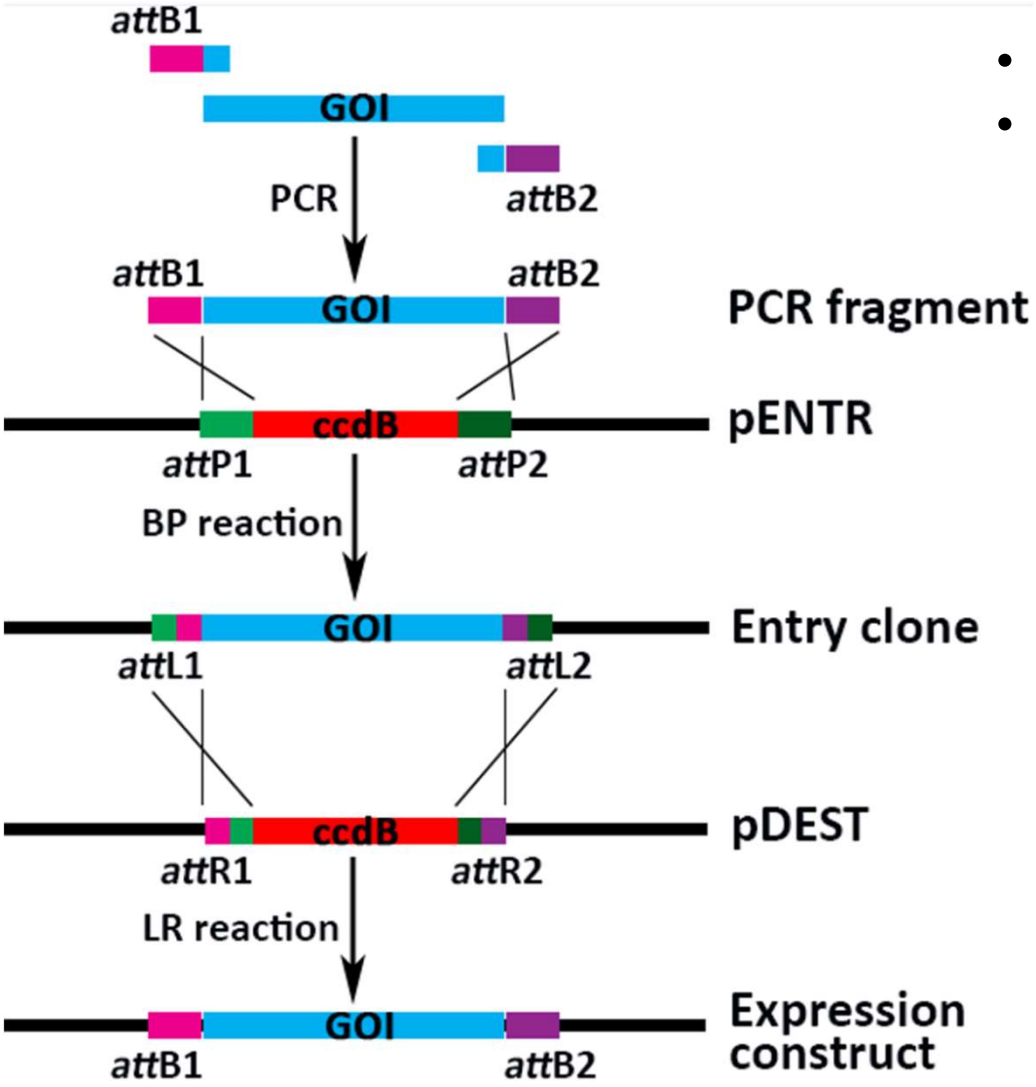
The three required enzyme activities are:
(1) 5’ exonuclease,
(2) DNA polymerase (Phusion), and
(3) DNA ligase (Taq ligase)

Other “Ligation Independent” Cloning methods



Review: Stevenson et al., 2013. PLoS One.

Recombination based cloning



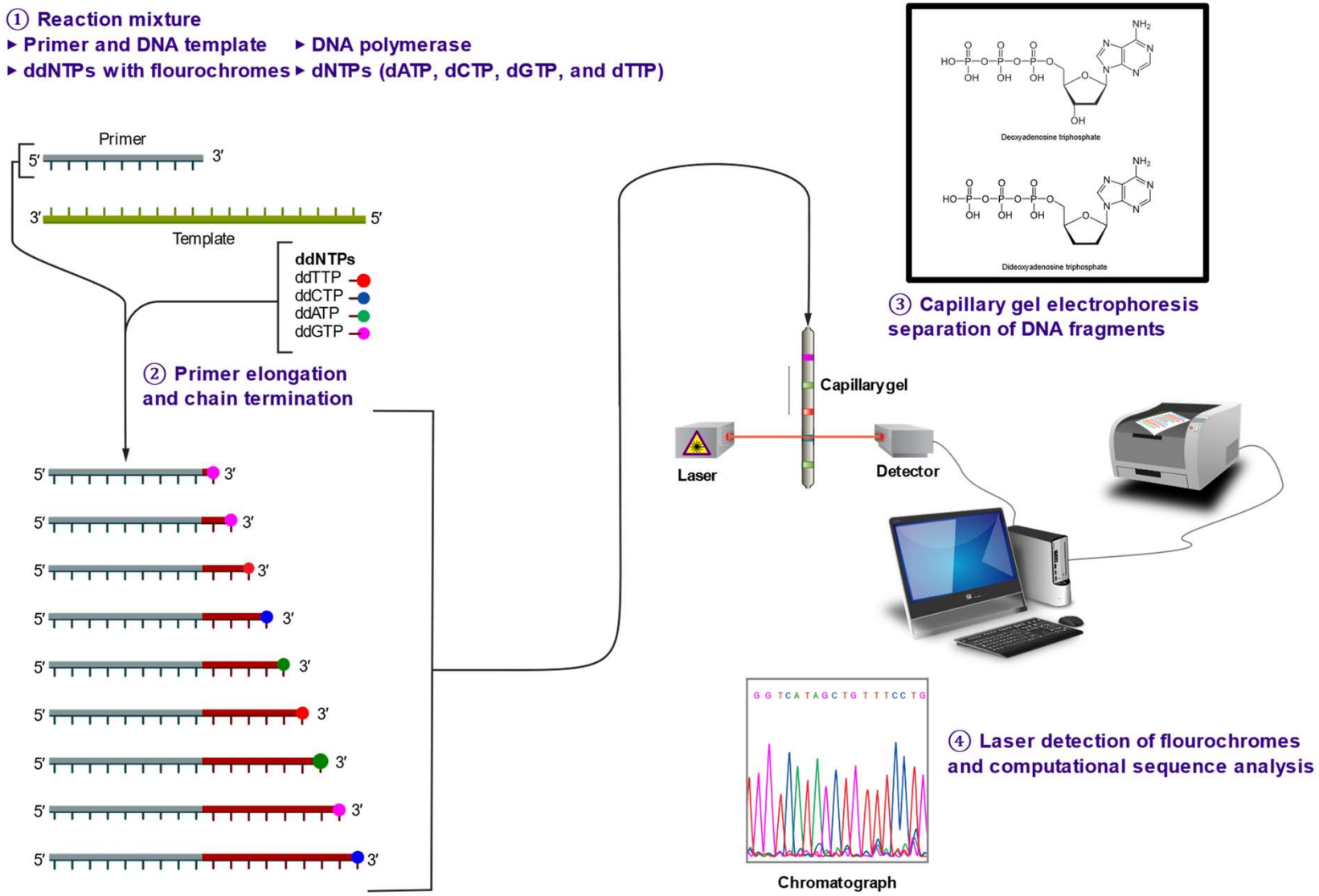
- Gateway cloning
- Phage lambda (λ) recombination system

https://www.embl.org/groups/protein-expression-purification/services/strategy-and-construct-design/recombination-based-cloning-gateway/

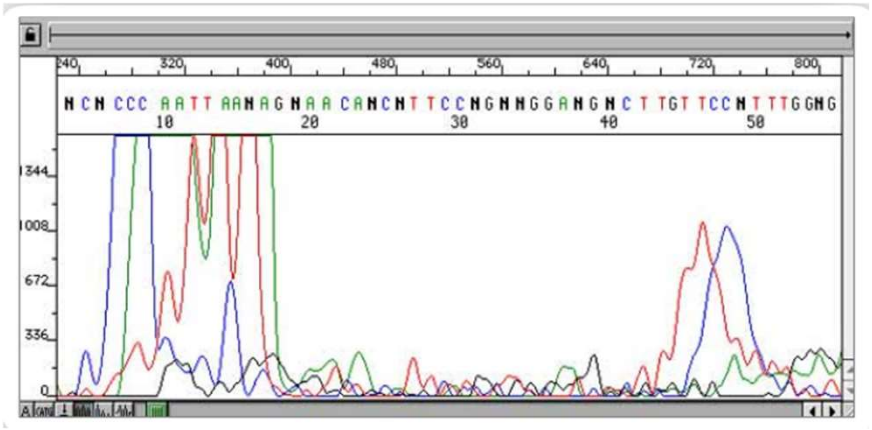
DNA Sequencing

- **Sanger sequencing**: the most common sequencing method after cloning: (di-deoxy method)

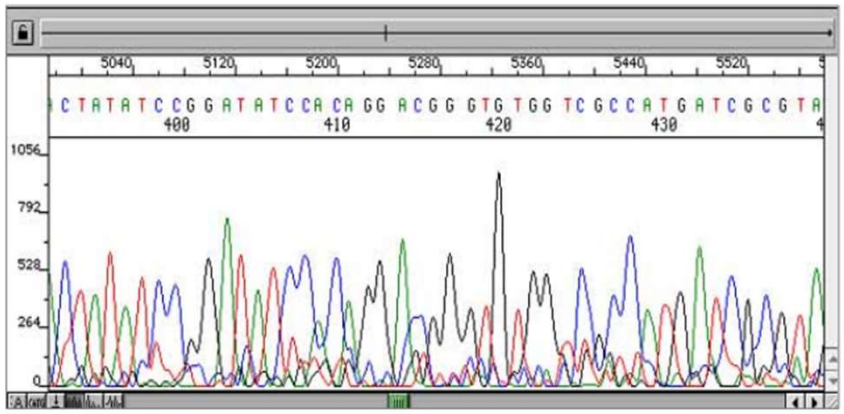
https://en.wikipedia.org/wiki/Sanger_sequencing



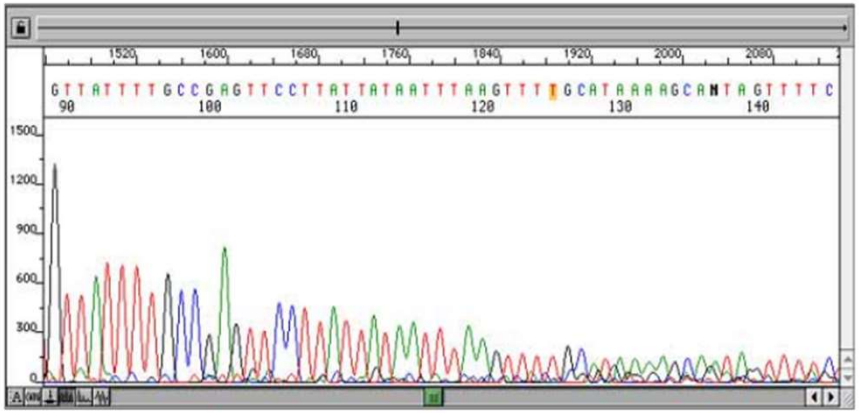
Sanger Sequencing Troubleshooting



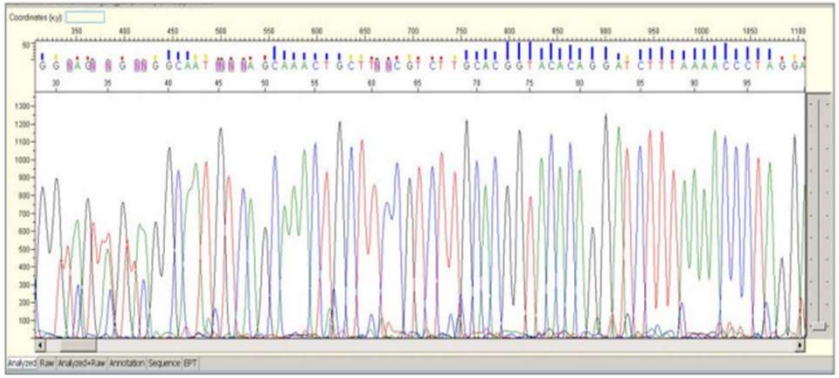
Poor quality template or primer, missing binding site



Multiple priming, mixed templates



Low template/ primer concentrations, poor primer design/ binding

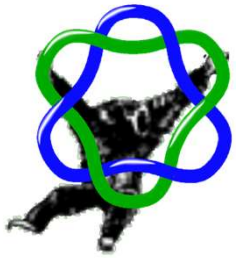


Too much template

https://www.eurofinsgenomics.eu/media/962732/sequencing-result-guide_sen_2014.pdf
https://www.operon.com/services/dna-sequencing/troubleshooting.pdf

Preparing for the Practical Exercise

(1) Download and install: A Plasmid Editor (ApE)



ApE plasmid editor

Download: https://jorgensen.biology.utah.edu/wayned/ape/

Tutorials: https://www.youtube.com/channel/UC_-pObWrnUZRhsO8YblX6gQ

(2) Make an account on: Benchling

Benchling

Starting Tutorial: https://benchling.com/tutorials/49/nav-redesign-overview

Molecular Biology: https://www.youtube.com/watch?v=rhamB8IiWxA

(3) Make an account on: DeNovoDNA / RBS calculator

- https://www.denovodna.com/software/login

- Use academic email ID (.edu, universite-paris-saclay.fr, inra.fr)

Questions welcome.

manish.kushwaha@inrae.fr