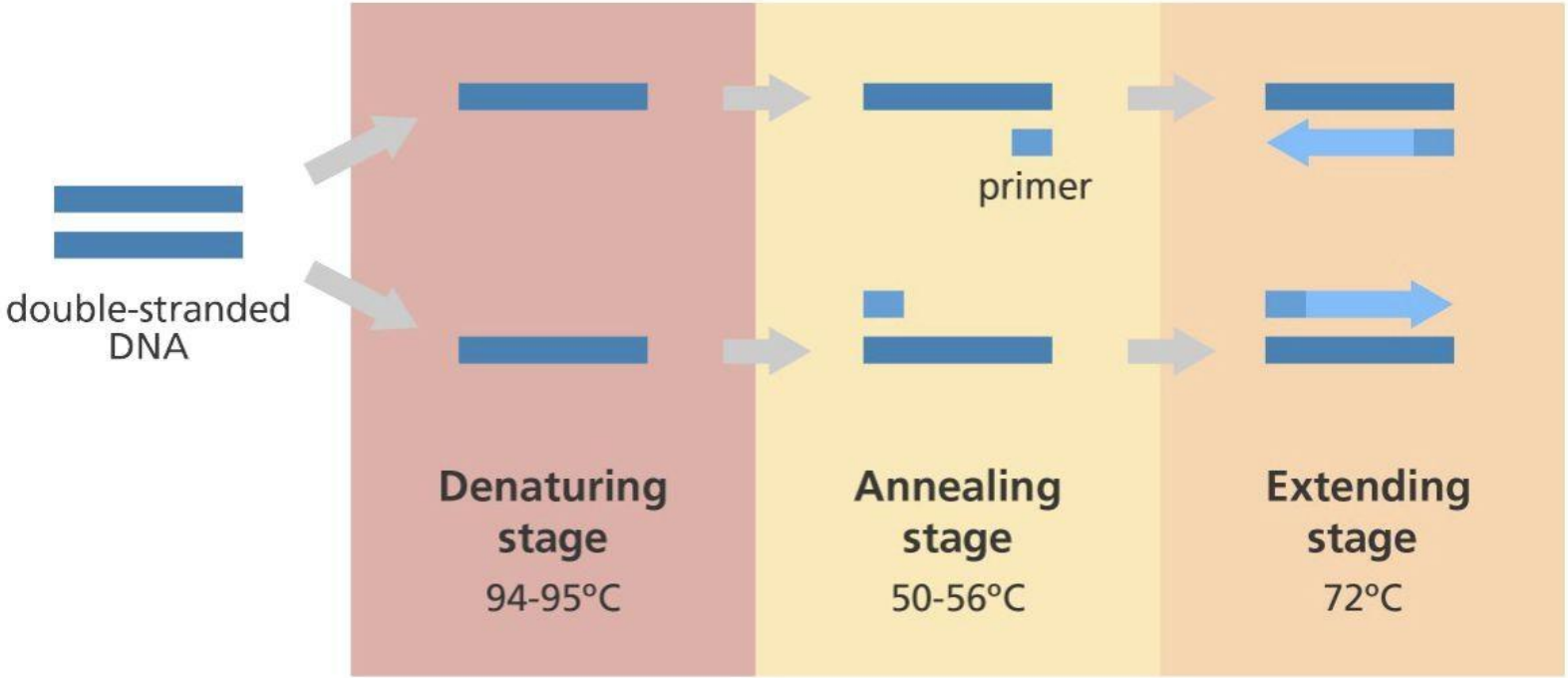
Molecular cloning techniques

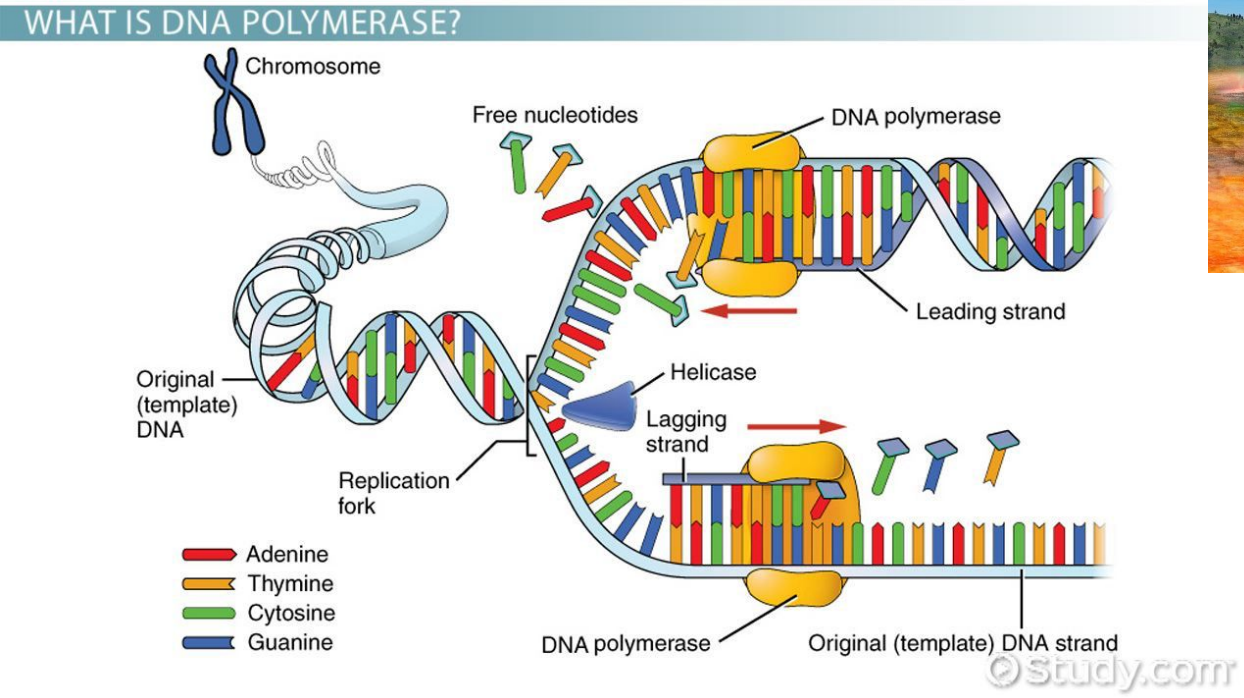
Benjamin Arias-Almeida, , MSc.

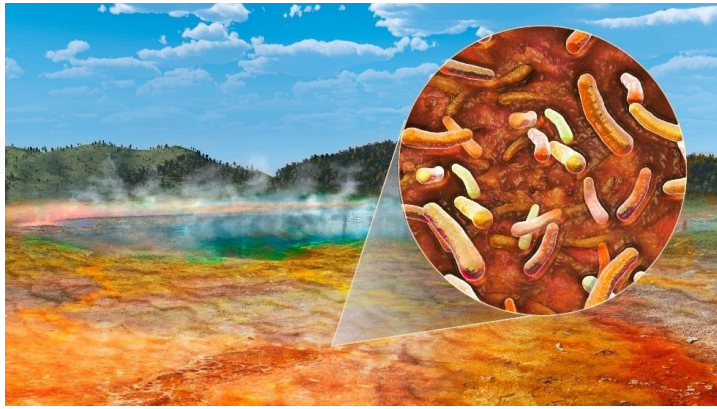
DNA cloning: PCR

Polymerase chain reaction (abbreviated PCR) is a laboratory technique for rapidly producing (amplifying) millions to billions of copies of a specific segment of DNA without the use of living cells

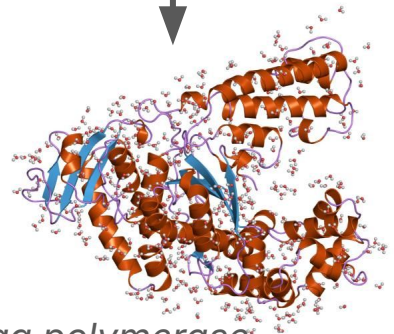


DNA cloning: PCR





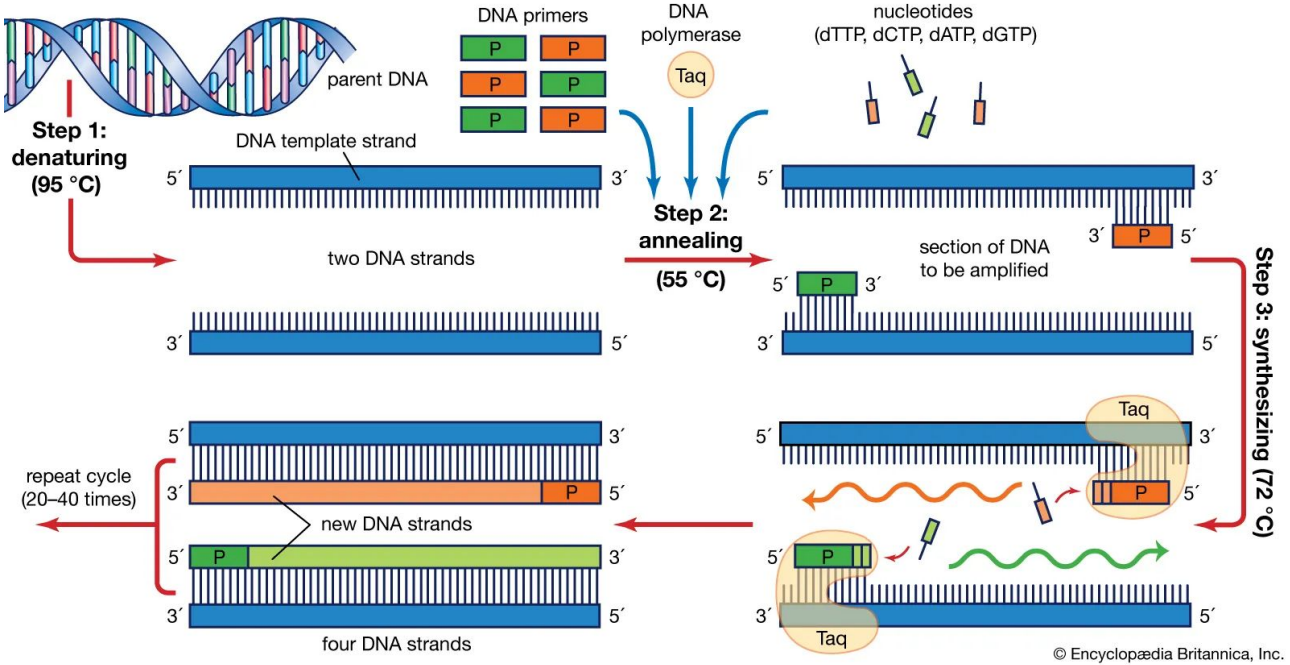
Thermus aquaticus



Taq polymerase

DNA cloning: PCR

Polymerase chain reaction (abbreviated PCR) is a laboratory technique for rapidly producing (amplifying) millions to billions of copies of a specific segment of DNA without the use of living cells



Cake recipe = Enzyme recipe

Component	20 µl Reaction	50 µl Reaction	Final Concentration
Nuclease-free water	to 20 µl	to 50 µl	
5X Phusion HF or GC Buffer	4 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 250 ng
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Cake recipe = Enzyme recipe

Component	20 µl Reaction	50 µl Reaction
Nuclease-free water	to 20 µl	to 50 µl
5X Phusion HF or GC Buffer	4 µl	10 µl
10 mM dNTPs	0.4 µl	1 µl
10 µM Forward Primer	1 µl	2.5 µl
10 µM Reverse Primer	1 µl	2.5 µl
Template DNA	variable	variable
DMSO (optional)	(0.6 µl)	(1.5 µl)
Phusion DNA Polymerase	0.2 µl	0.5 µl

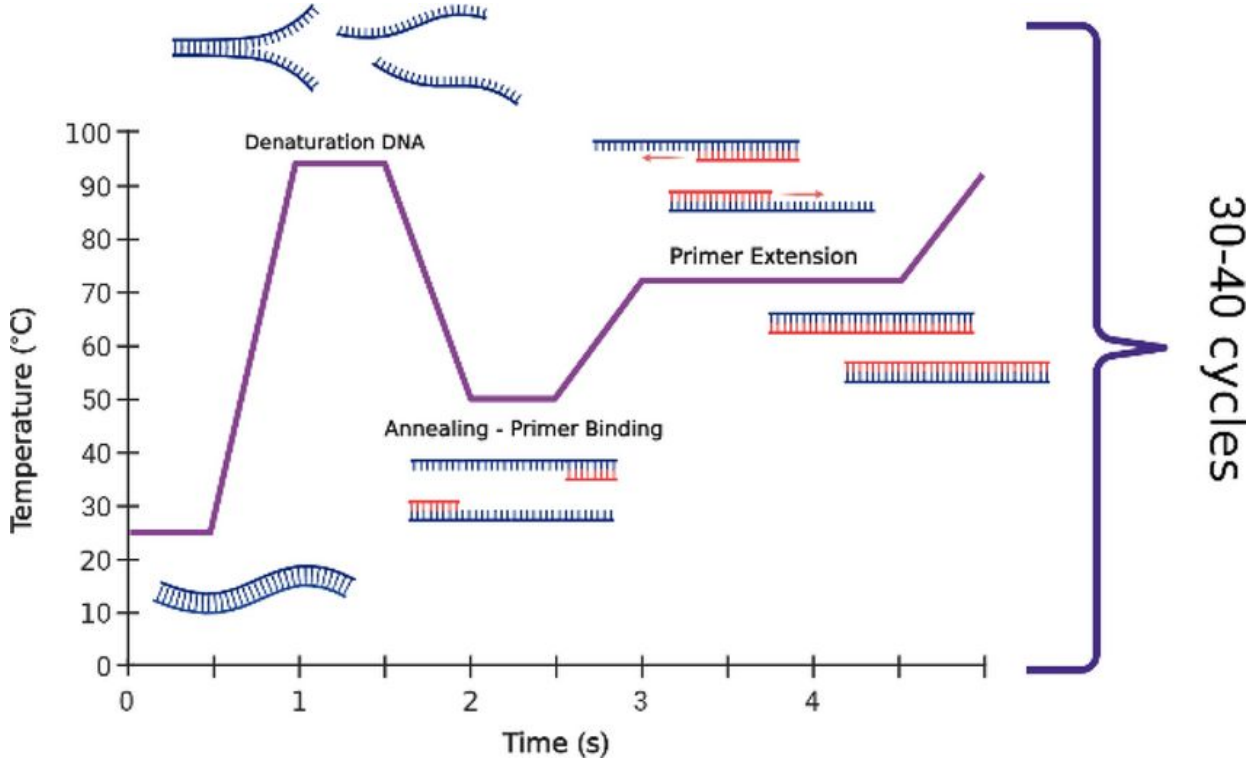
Notes: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

DNA cloning: PCR

A **thermocycler** (also called a PCR machine) is a laboratory instrument that precisely and repeatedly changes sample temperatures in programmed cycles, enabling DNA denaturation, primer annealing, and extension steps required to amplify specific DNA segments during PCR.

DNA cloning: PCR

Each cycle is composed of:



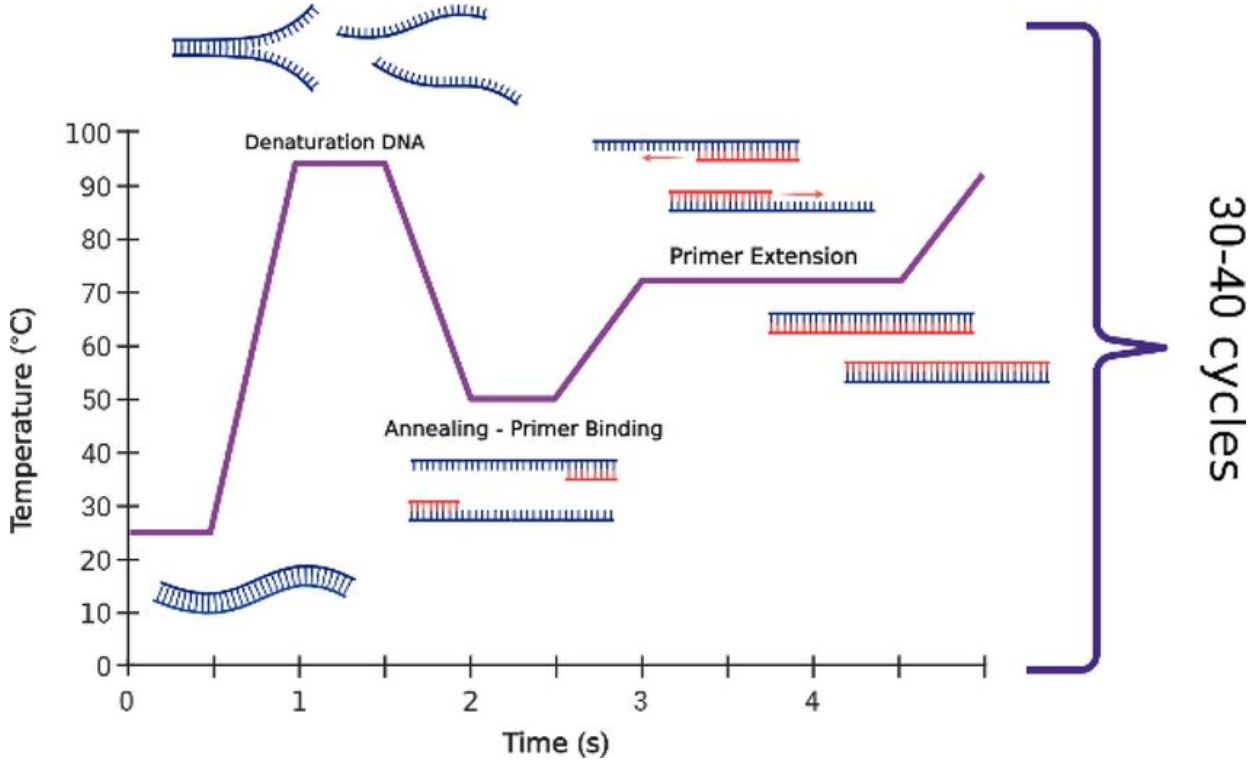
DNA cloning: PCR

Each cycle is composed of:

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C 45-72°C 72°C	5-10 seconds 10-30 seconds 15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	4-10°C	

DNA cloning: PCR

Each cycle is composed of:



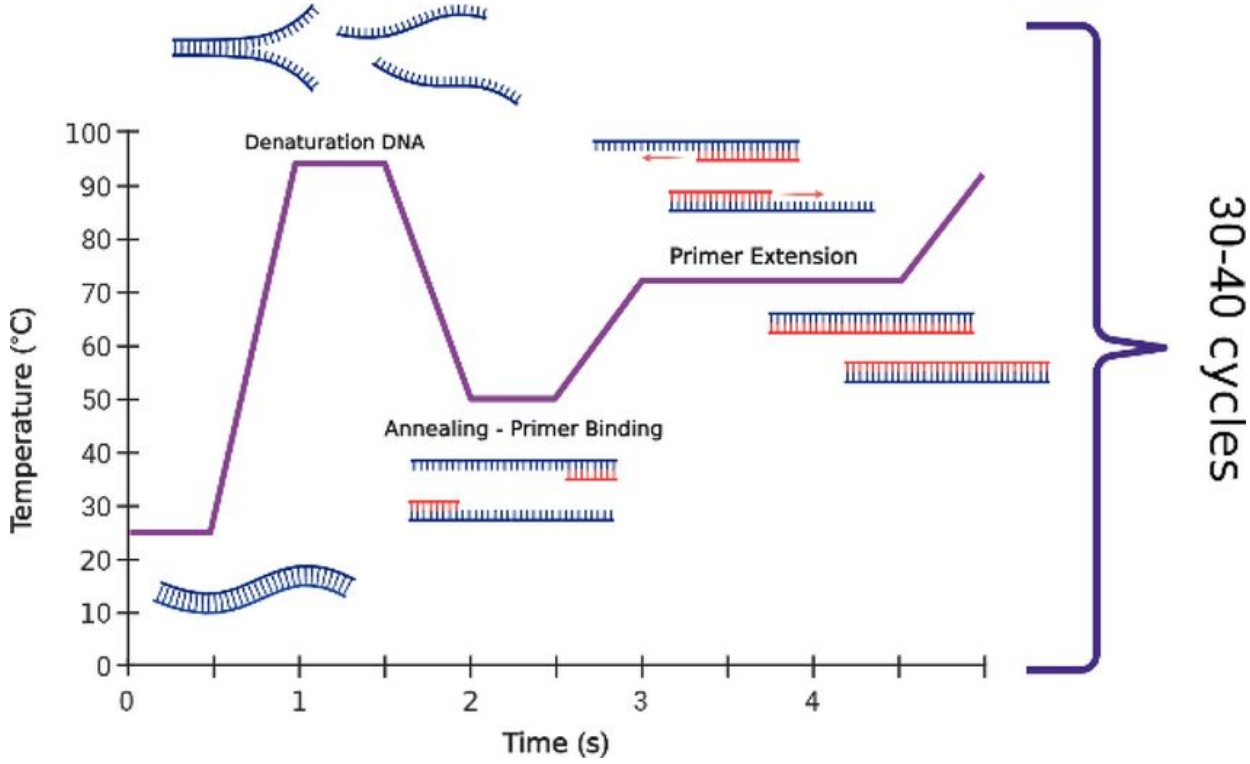
DNA cloning: PCR

Each cycle is composed of:

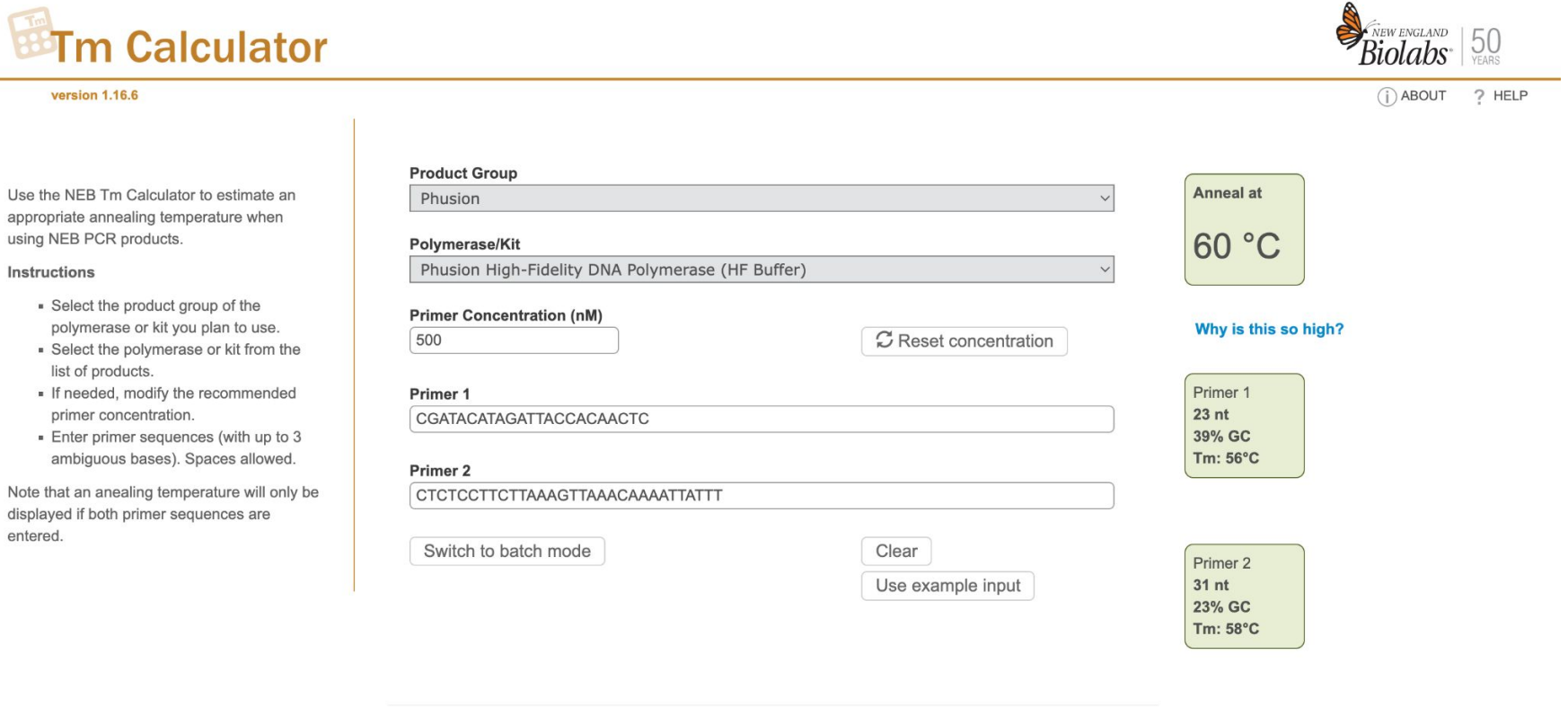
STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C	5-10 seconds
	45-72°C	10-30 seconds
	72°C	15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	4-10°C	

DNA cloning: PCR

Each cycle is composed of:



DNA cloning: PCR



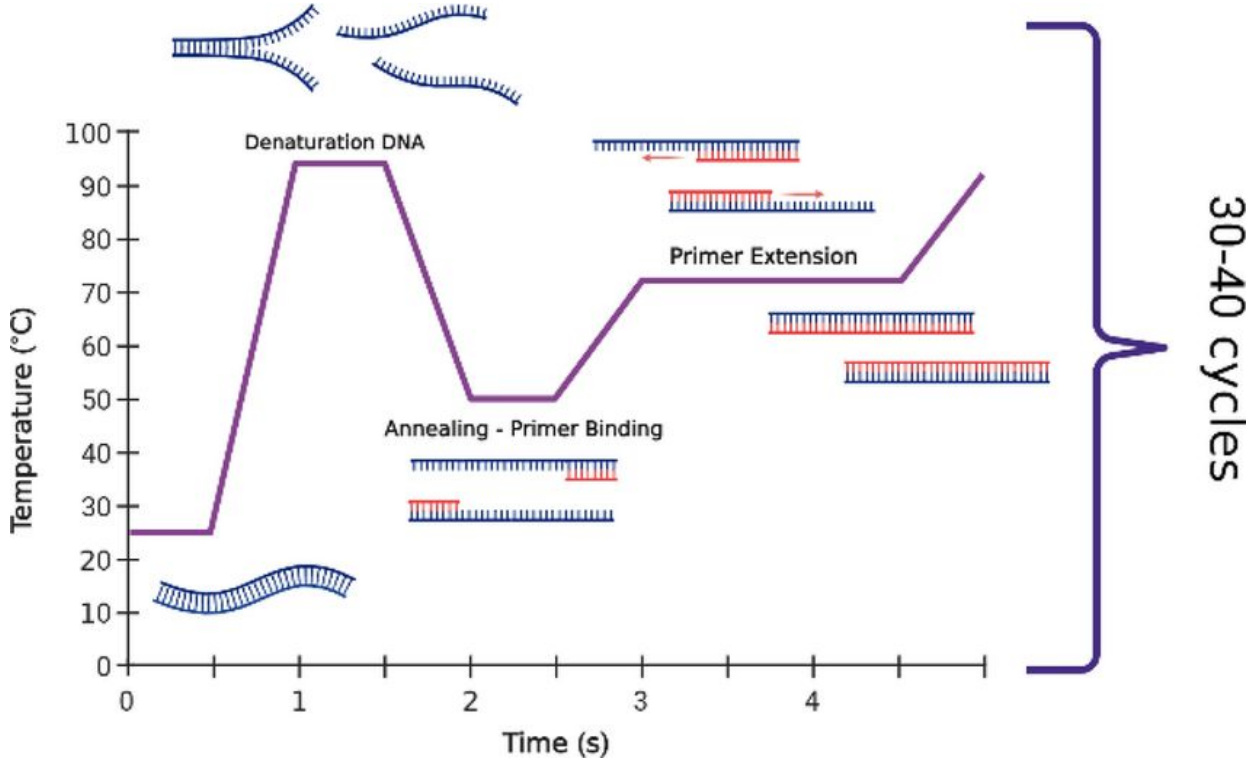
DNA cloning: PCR

Each cycle is composed of:

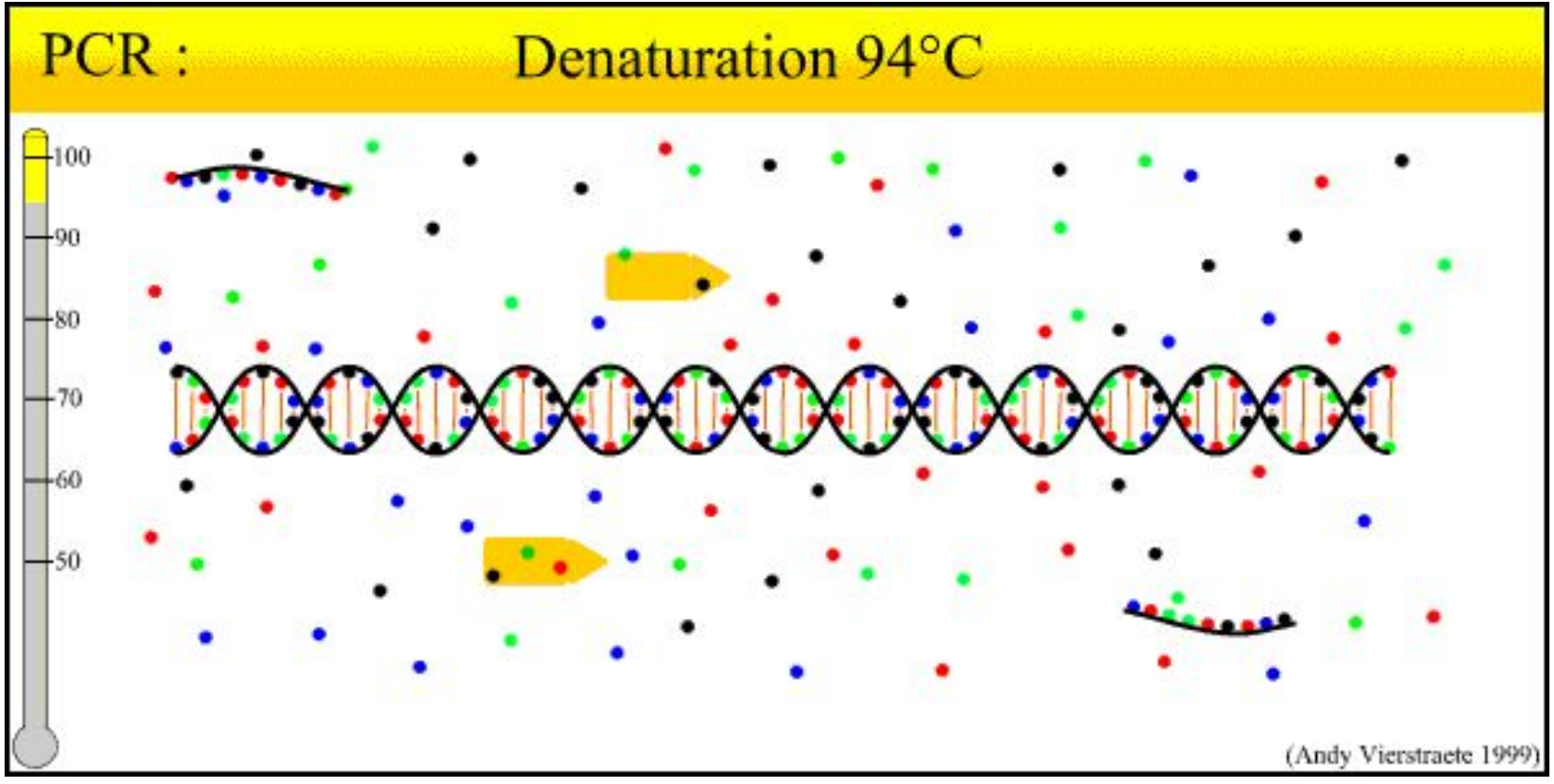
STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C 45-72°C 72°C	5-10 seconds 10-30 seconds 15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	4-10°C	

DNA cloning: PCR

Each cycle is composed of:

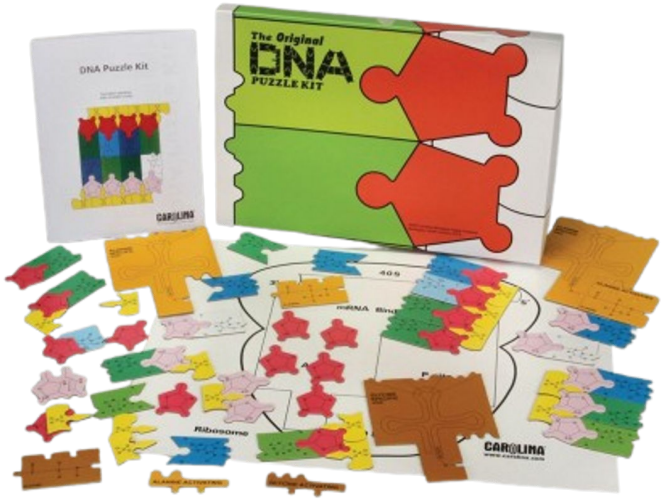


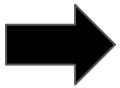
DNA cloning: PCR

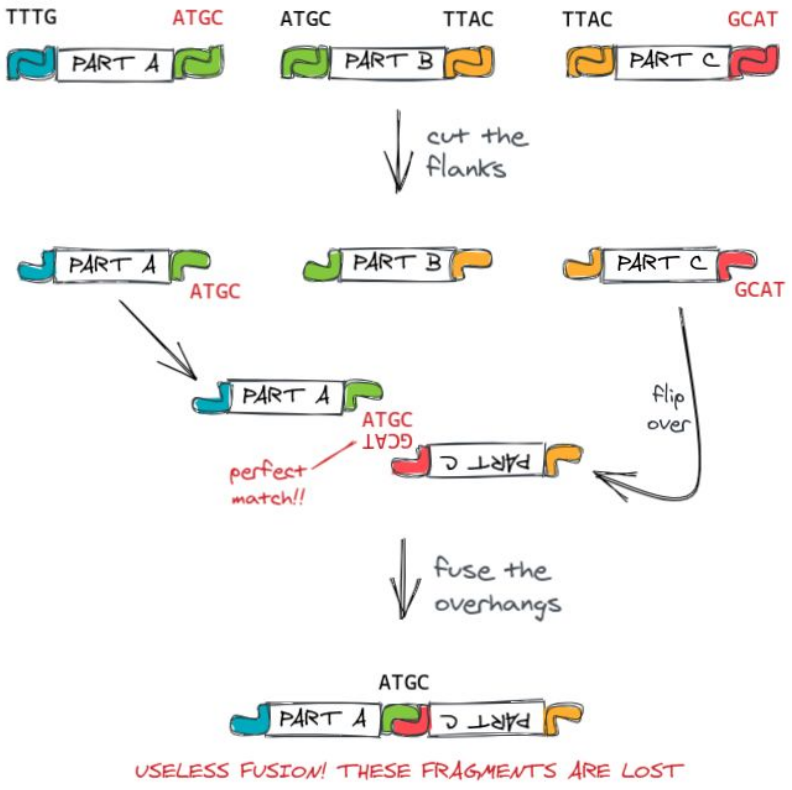


Different methods for DNA cloning/assembly

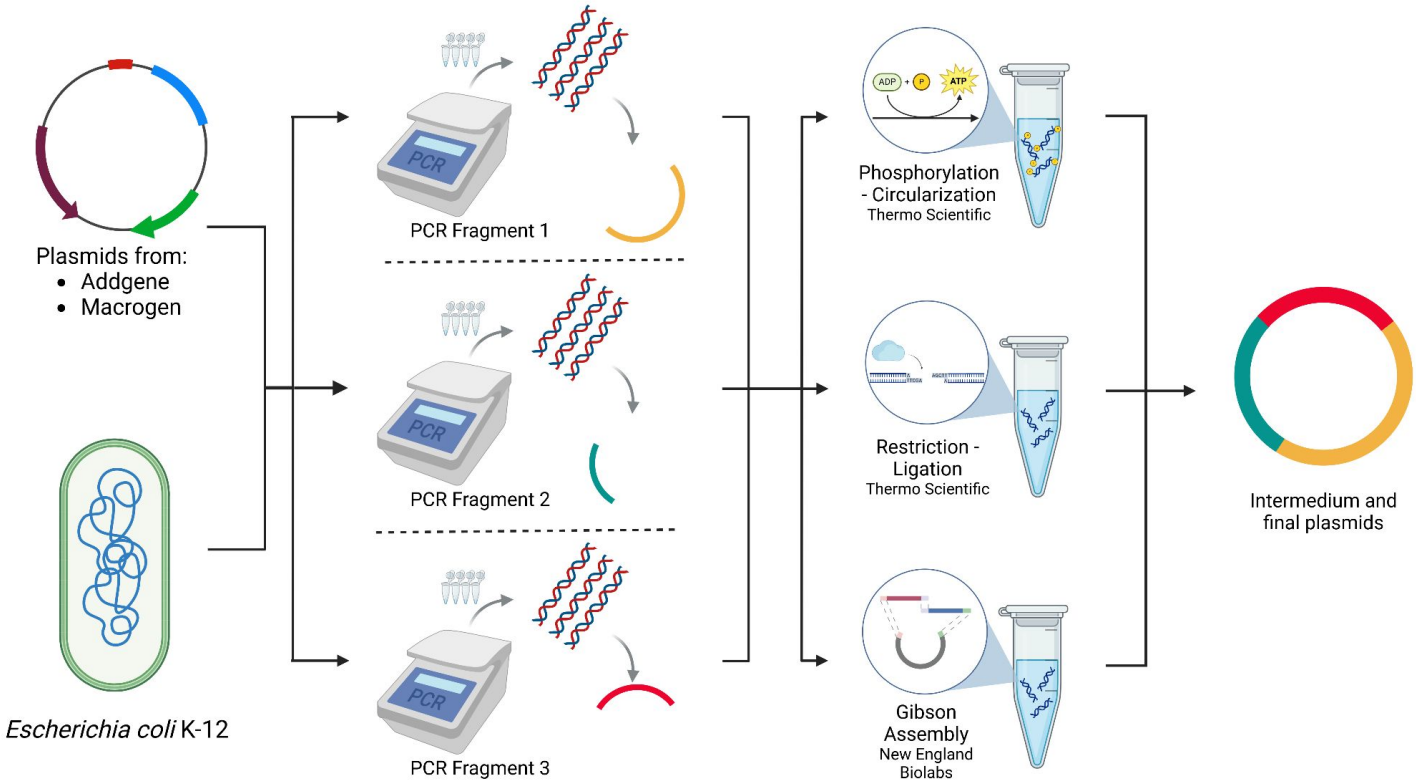
Lab methods for joining different parts of DNA together





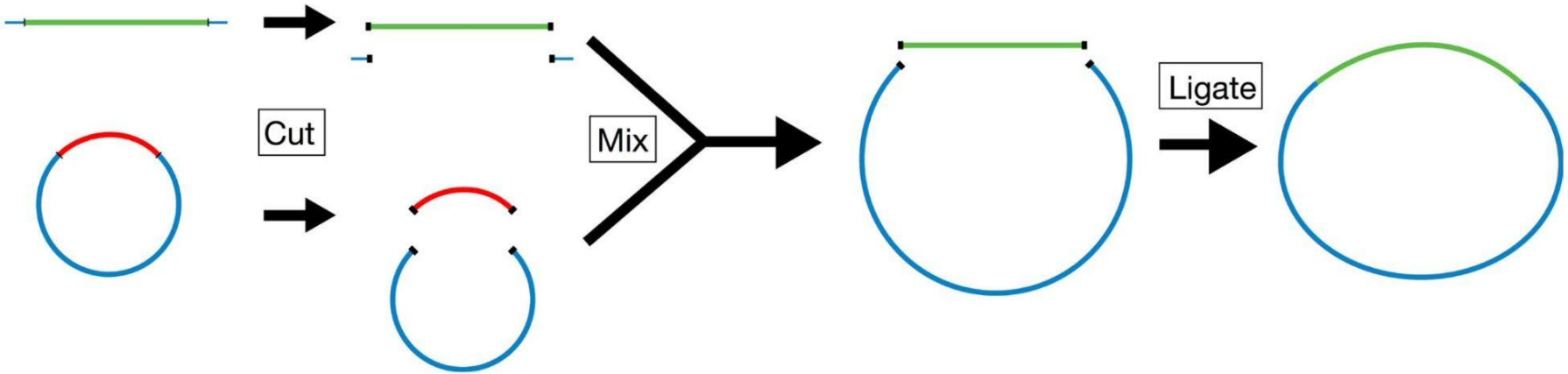


Different methods for DNA cloning/assembly



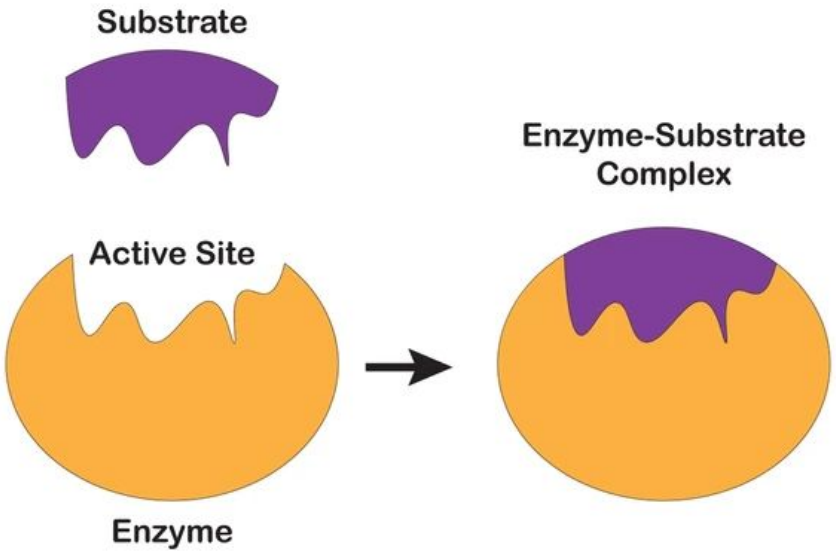
Restriction enzyme based assembly

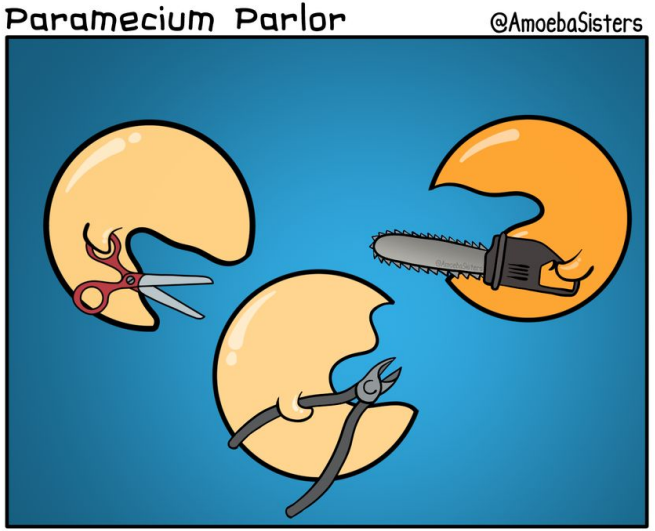
Restriction enzymes are a type of enzymes that recognize a specific DNA sequence and make a specific pattern of cut. This pattern can be used to join two DNA molecules by this extremes.



What we use?

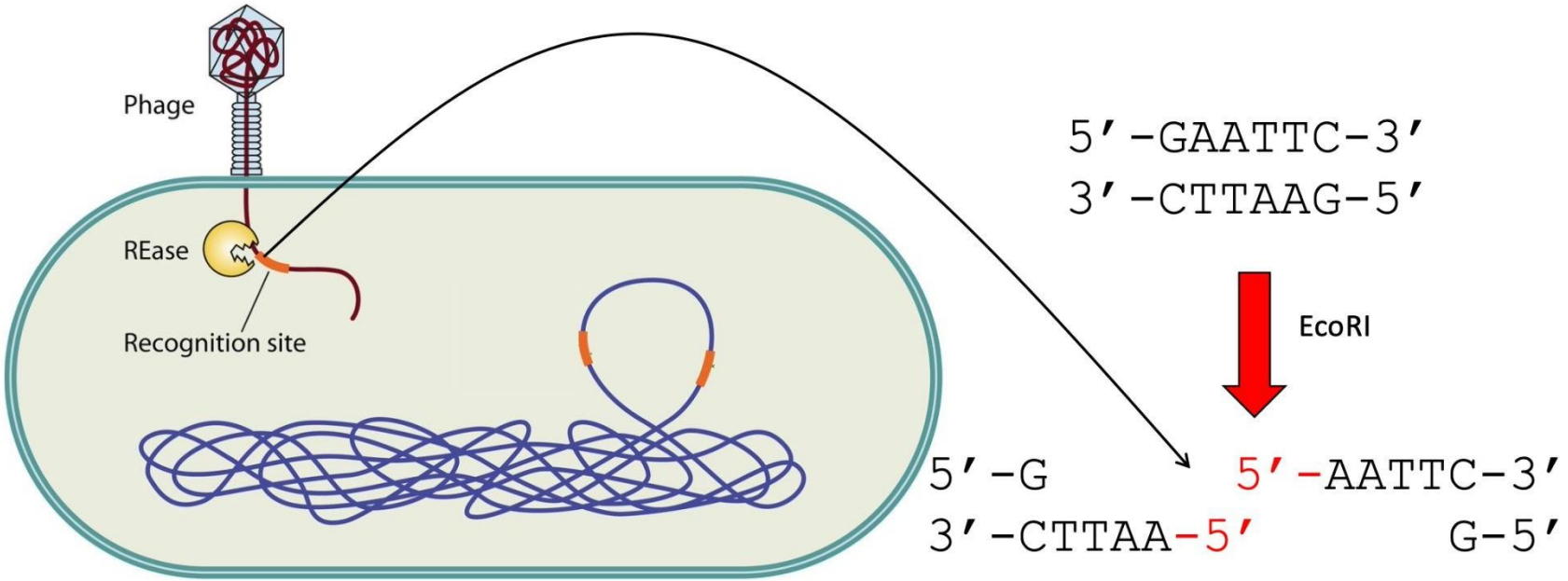
Tiny molecular machines called enzymes !!!





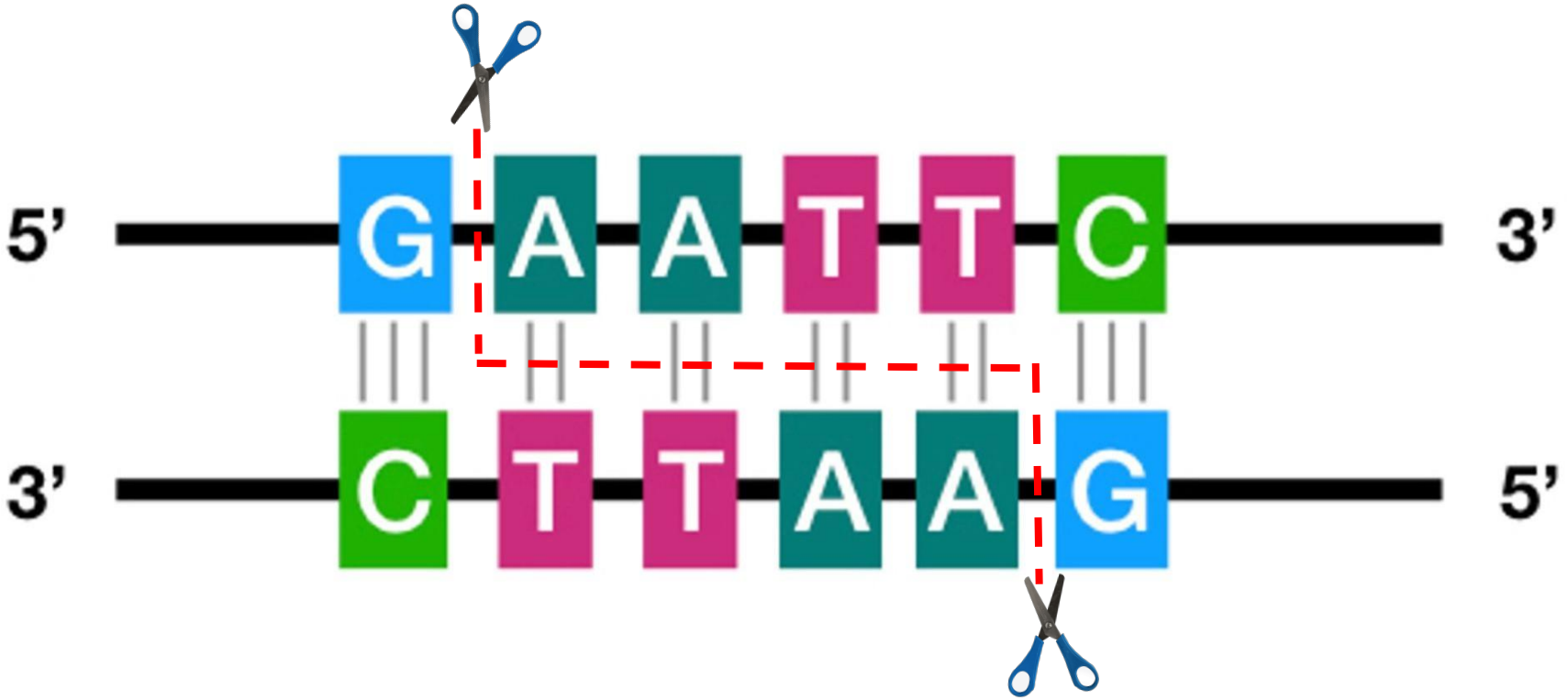
Restriction Enzymes: A variety of itty bitty DNA scissors

What are restriction enzymes?

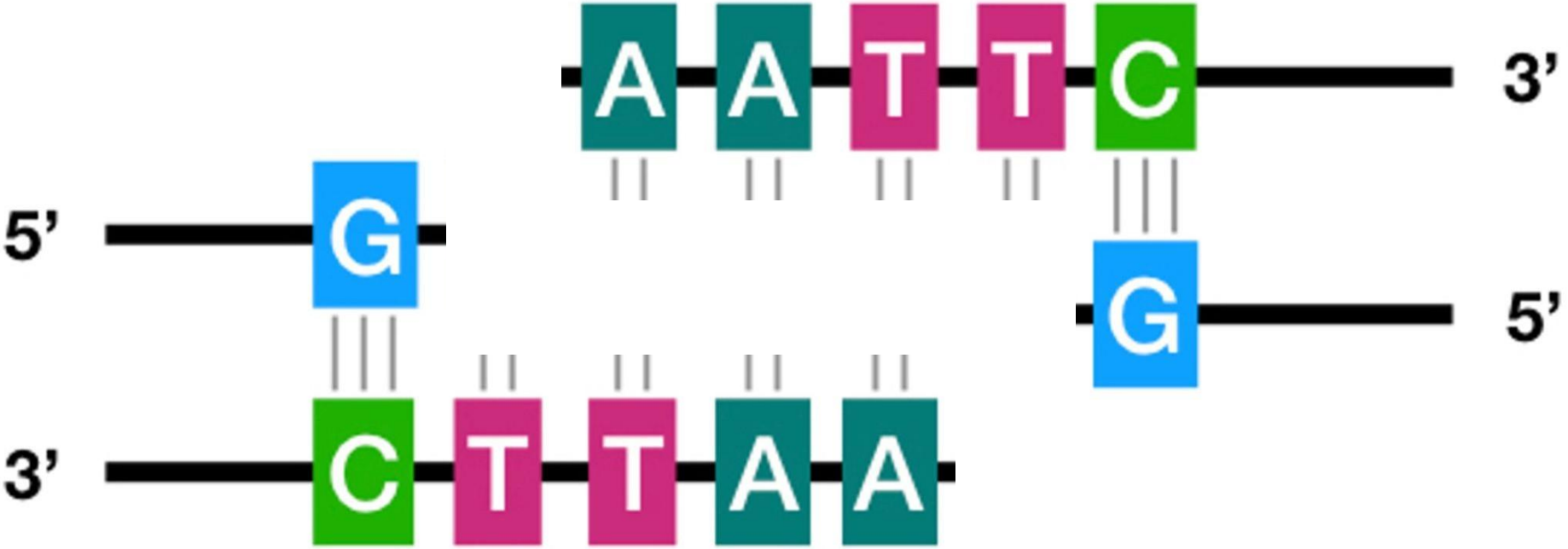


Recognition site: palindromic sequence

Recognition site: palindromic sequence



Recognition site: palindromic sequence



Type II Restriction Enzymes

Enzyme	Source	Recognition Sequence	Cut
EcoR1	*Escherichia coli*	5'GAATTC 3'CTTAAG	5'---G AATTC---3' 3'---CTTAA G---5'
BamH1	*Bacillus amyloliquefaciens*	5'GGATCC 3'CCTAGG	5'---G GATCC---3' 3'---CCTAG G---5'
Taq1	*Thermus aquaticus*	5'TCGA 3'AGCT	5'---T CGA---3' 3'---AGC T---5'
Alu1*	*Arthrobacter luteus*	5'AGCT 3'TCGA	5'---AG CT---3' 3'---TC GA---5'
* = blunt ends			

Cake recipe = Enzyme recipe

Protocol for Fast Digestion of Different DNA

1. Combine the following reaction components at room temperature in the order indicated:

	Plasmid DNA	PCR product	Genomic DNA
Water, nuclease-free (#R0581)	15 µL	17 µL	30 µL
10X FastDigest or 10X FastDigest Green Buffer	2 µL	2 µL	5 µL
DNA	2 µL (up to 1 µg)	10 µL (~0.2 µg)	10 µL (5 µg)
FastDigest enzyme	1 µL	1 µL	5 µL
Total volume:	20 µL	30 µL	50 µL

2. Mix gently and spin down.
3. Incubate at 37°C in a heat block or water thermostat for 15 min (plasmid and genomic DNA) or for 20 min (PCR product). Optional: Inactivate the enzyme by heating for 5 min at 80°C.
4. If the FastDigest Green Buffer was used in the reaction, load an aliquot of the reaction mixture directly on a gel.

Cake recipe = Enzyme recipe

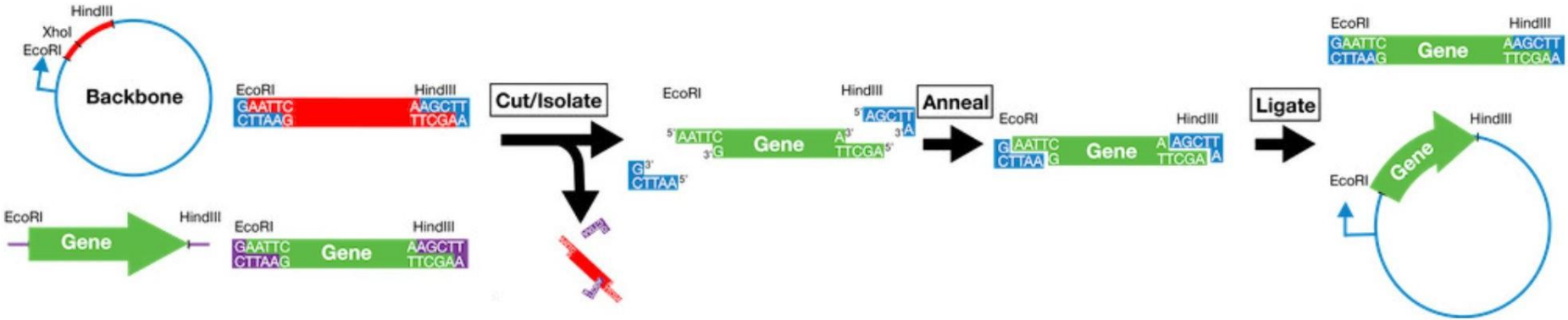
Protocol for Fast Digestion of Different DNA

1. Combine the following reaction components at room temperature in the order indicated:

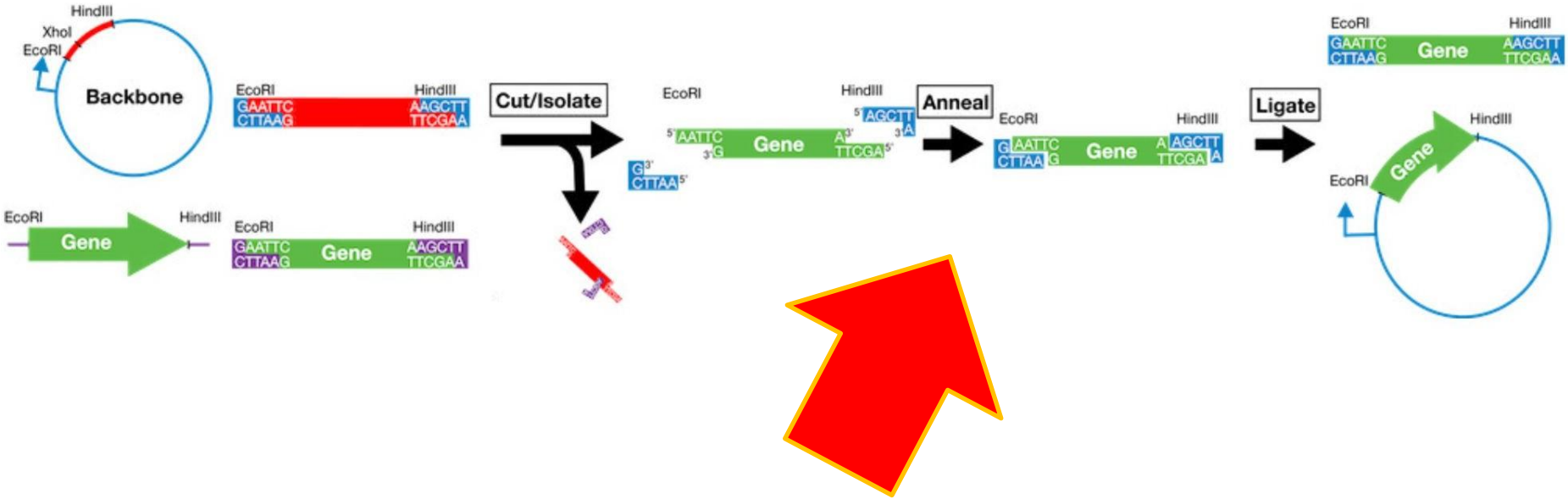
	Plasmid DNA	PCR product	Genomic DNA
Water, nuclease-free (#R0581)	15 µL	17 µL	30 µL
10X FastDigest or 10X FastDigest Green Buffer	2 µL	2 µL	5 µL
DNA	2 µL (up to 1 µg)	10 µL (~0.2 µg)	10 µL (5 µg)
FastDigest enzyme	1 µL	1 µL	5 µL
Total volume:	20 µL	30 µL	50 µL

2. Mix gently and spin down.
3. Incubate at 37°C in a heat block or water thermostat for 15 min (plasmid and genomic DNA) or for 20 min (PCR product). Optional: Inactivate the enzyme by heating for 5 min at 80°C.
4. If the FastDigest Green Buffer was used in the reaction, load an aliquot of the reaction mixture directly on a gel.

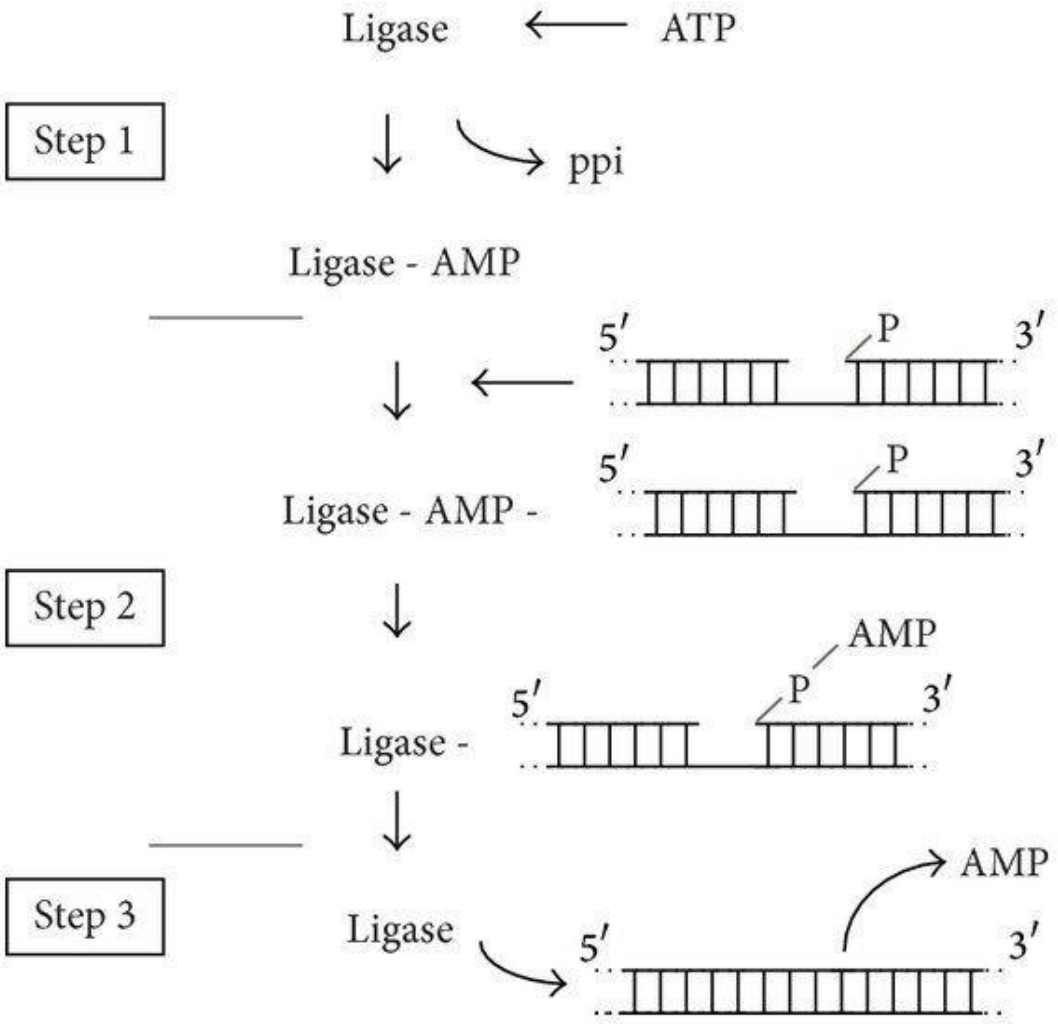
Restriction enzyme based assembly



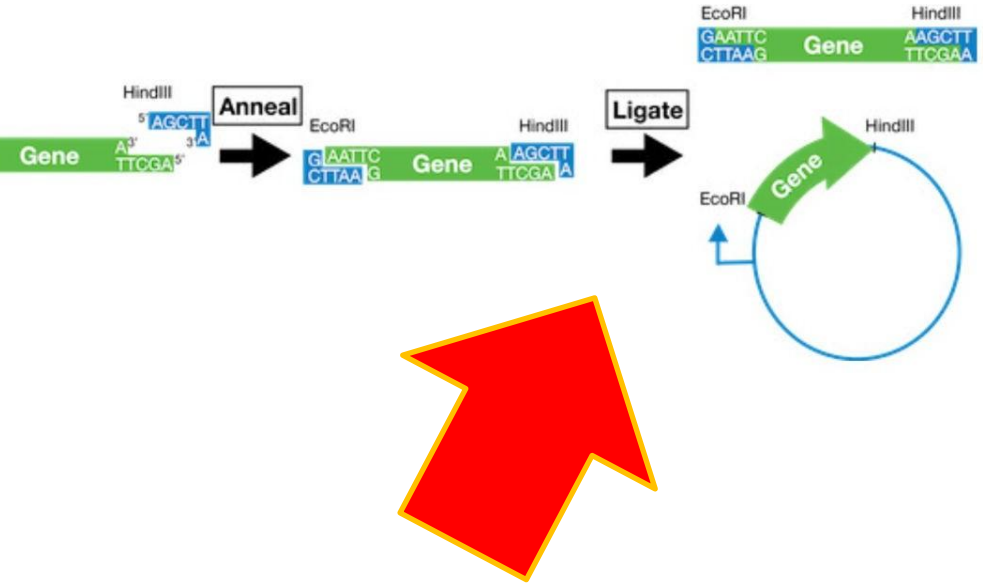
Restriction enzyme based assembly



Ligation reaction



Ligation reaction



Sticky-end Ligation

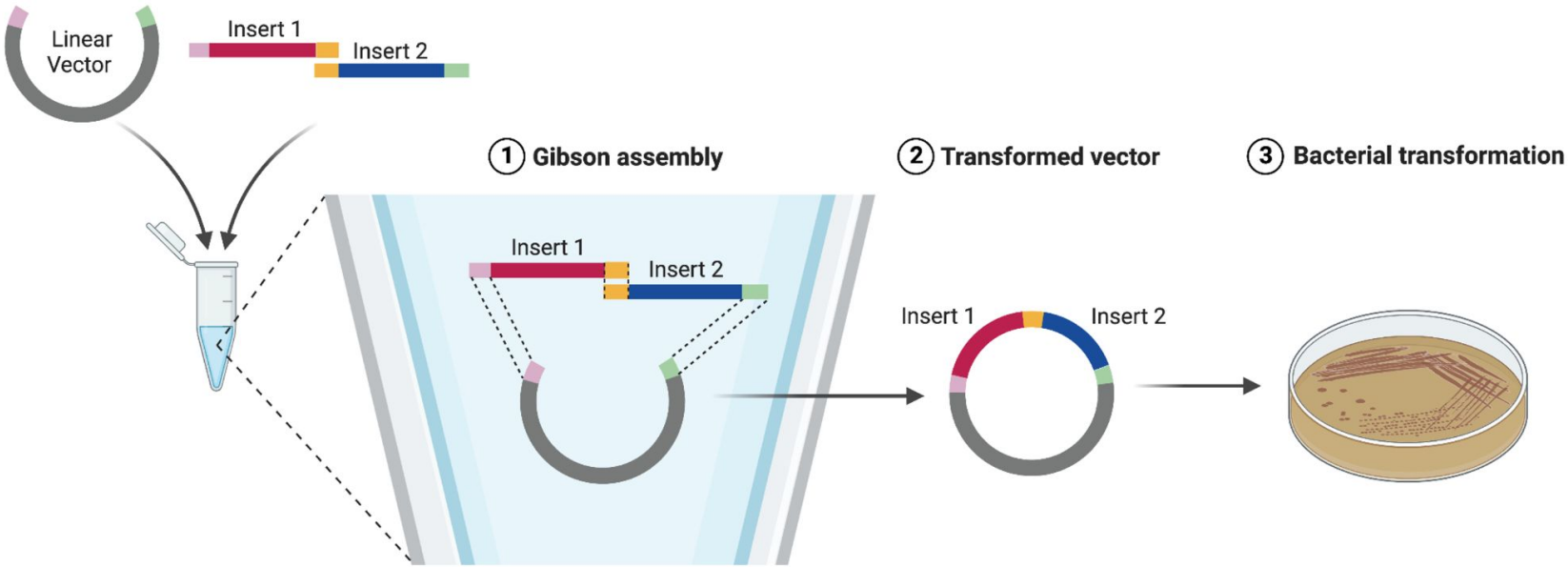
1. Prepare the following reaction mixture:

Linear vector DNA	20-100 ng
Insert DNA	1:1 to 5:1 molar ratio over vector
10x T4 DNA Ligase buffer	2 µL
Thermo Scientific T4 DNA Ligase (Cat #EL0016)	1 U
Water, nuclease-free	to 20 µL
Total volume	**20 µL**

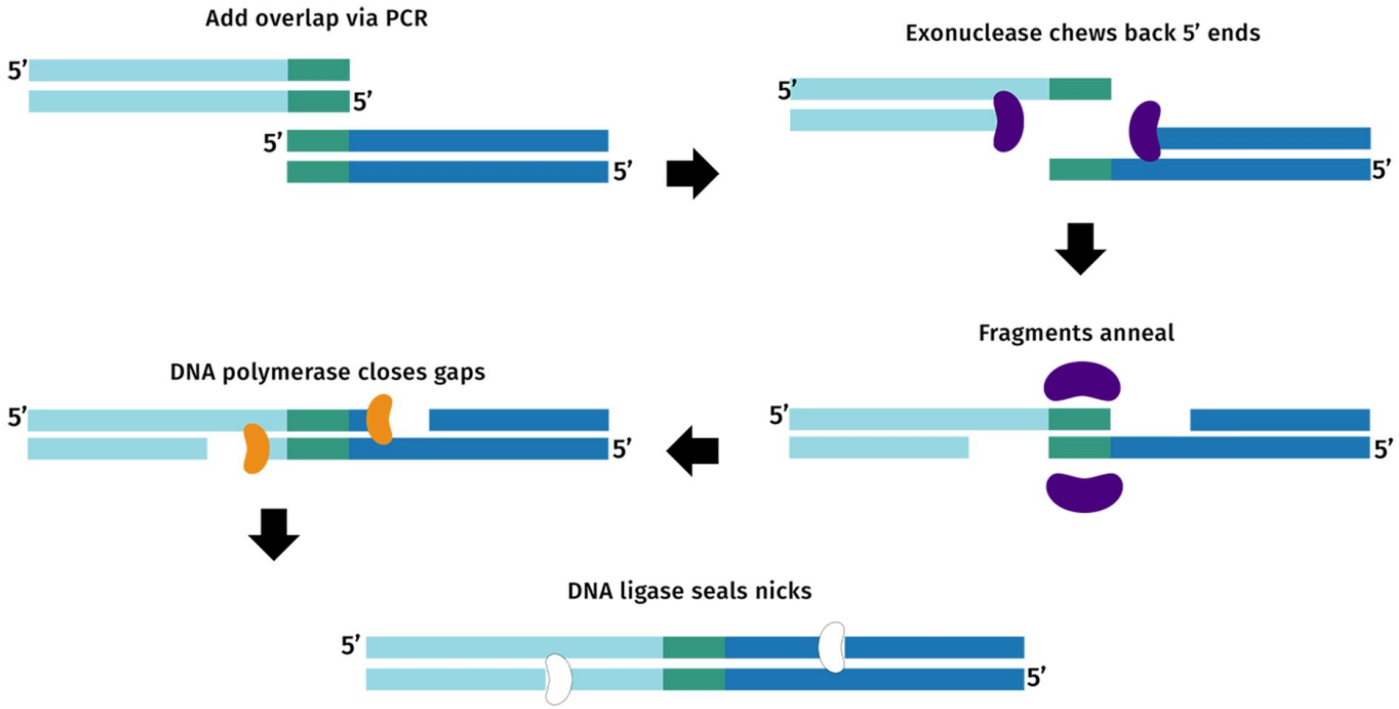
2. Incubate 10 minutes at 22 °C.
3. Use up to 5 µL of the mixture for transformation of 50 µL of chemically competent cells or use 1-2 µL per 50 µL electrocompetent cells.

Gibson Assembly

Gibson Assembly is a DNA assembly method used for joint 2 or more DNA fragments by using homologous DNA sequences in the extremes of the DNA molecule.

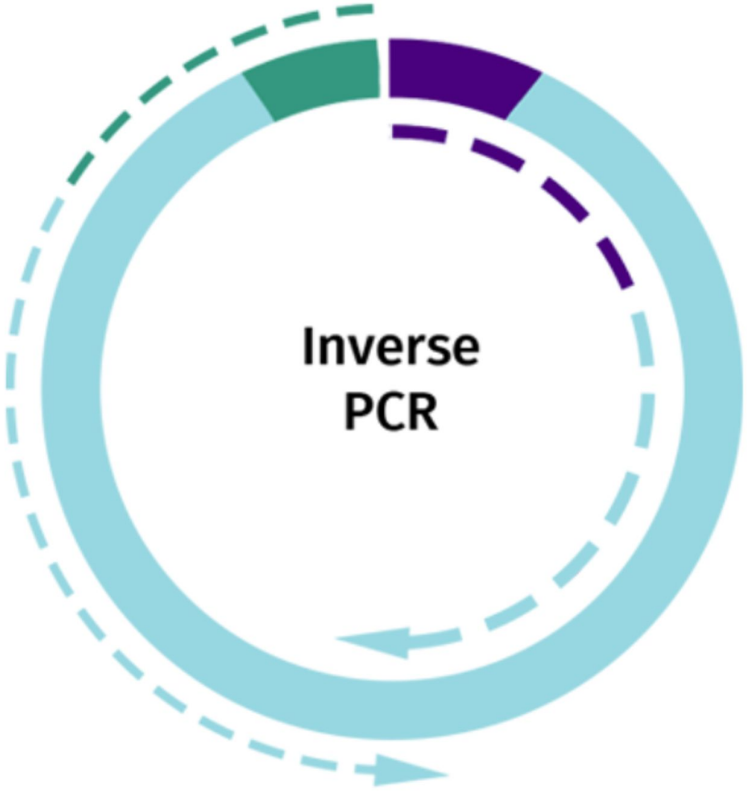


Gibson Assembly



Gibson assembly cloning technique

Gibson Assembly

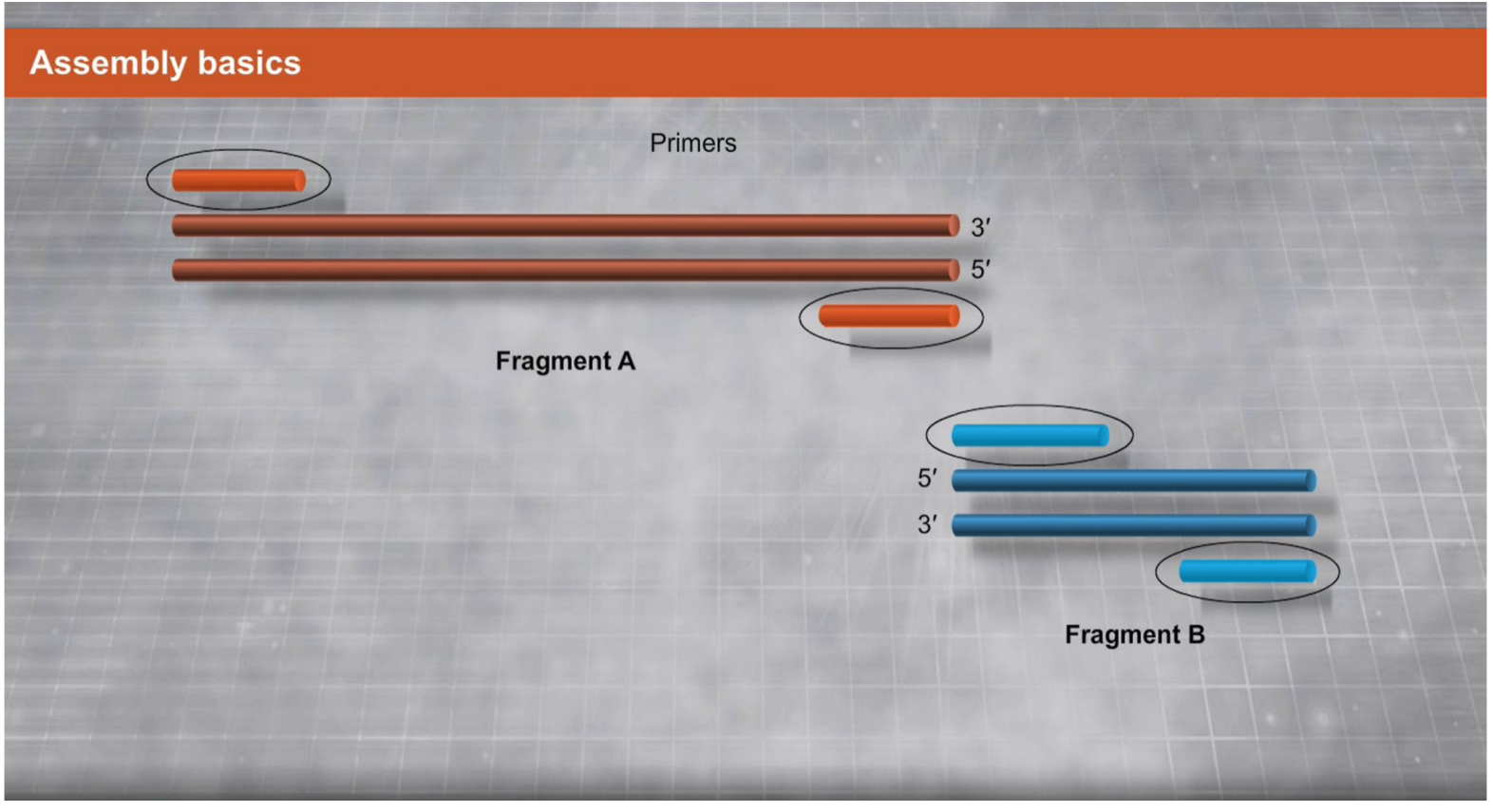


Inverse PCR for Gibson assembly

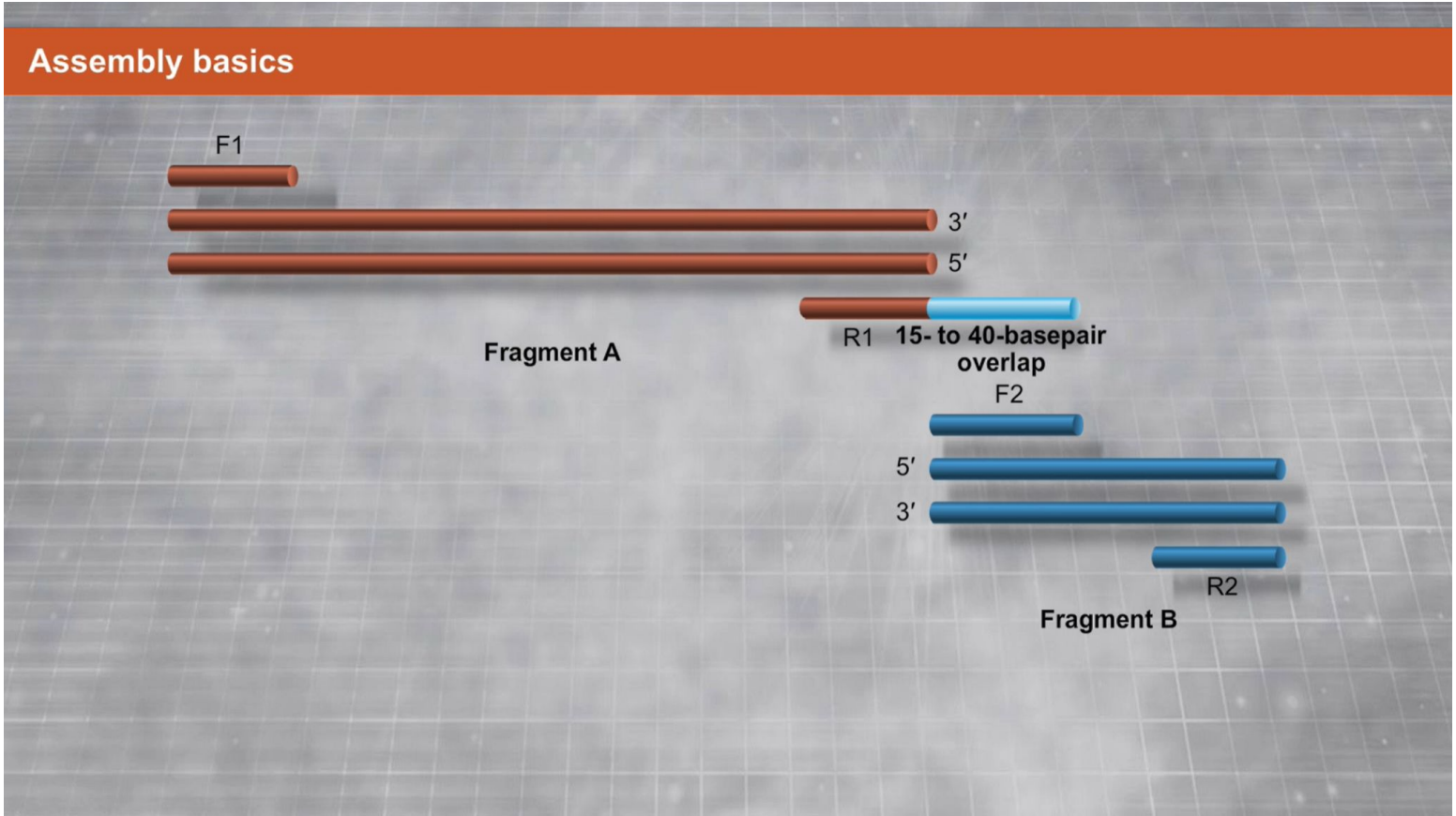
Gibson Assembly

The components that make up Gibson assembly primers.

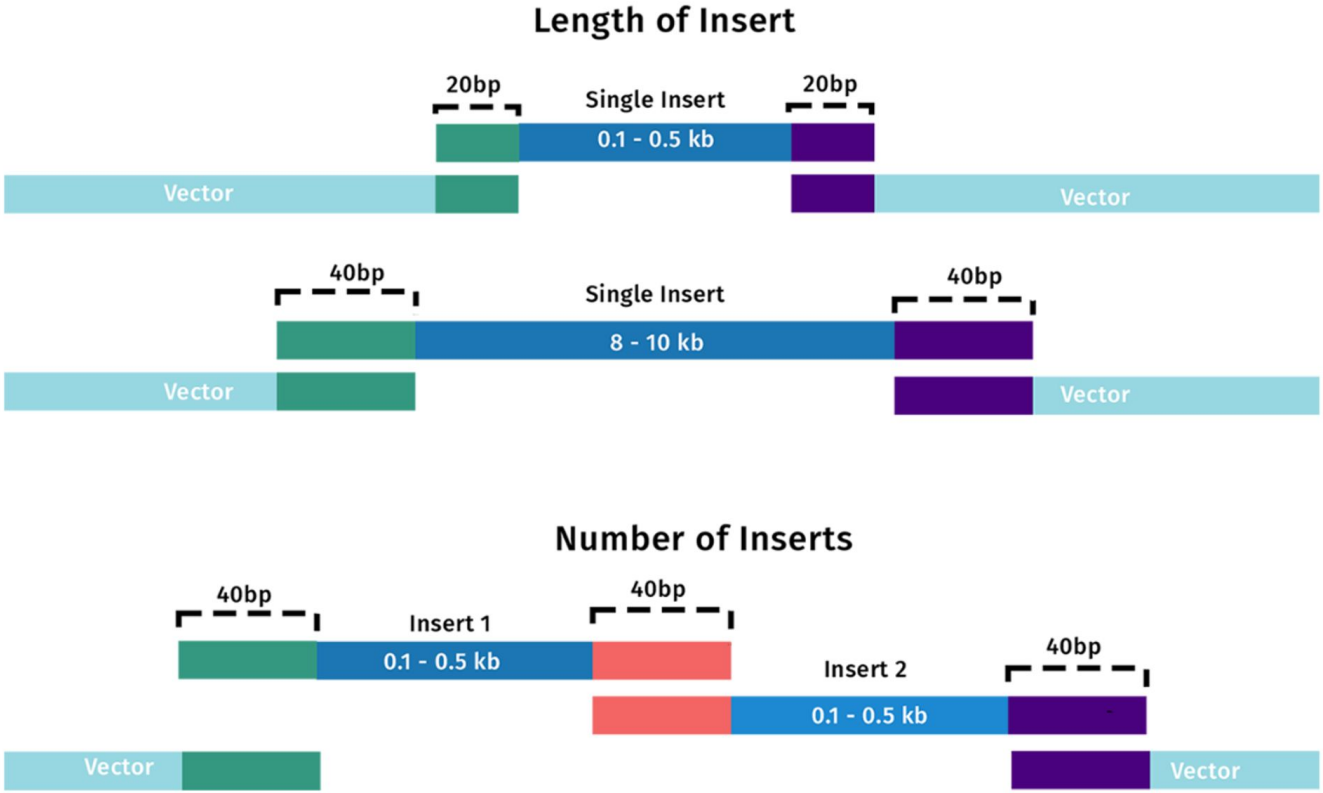
Gibson Assembly



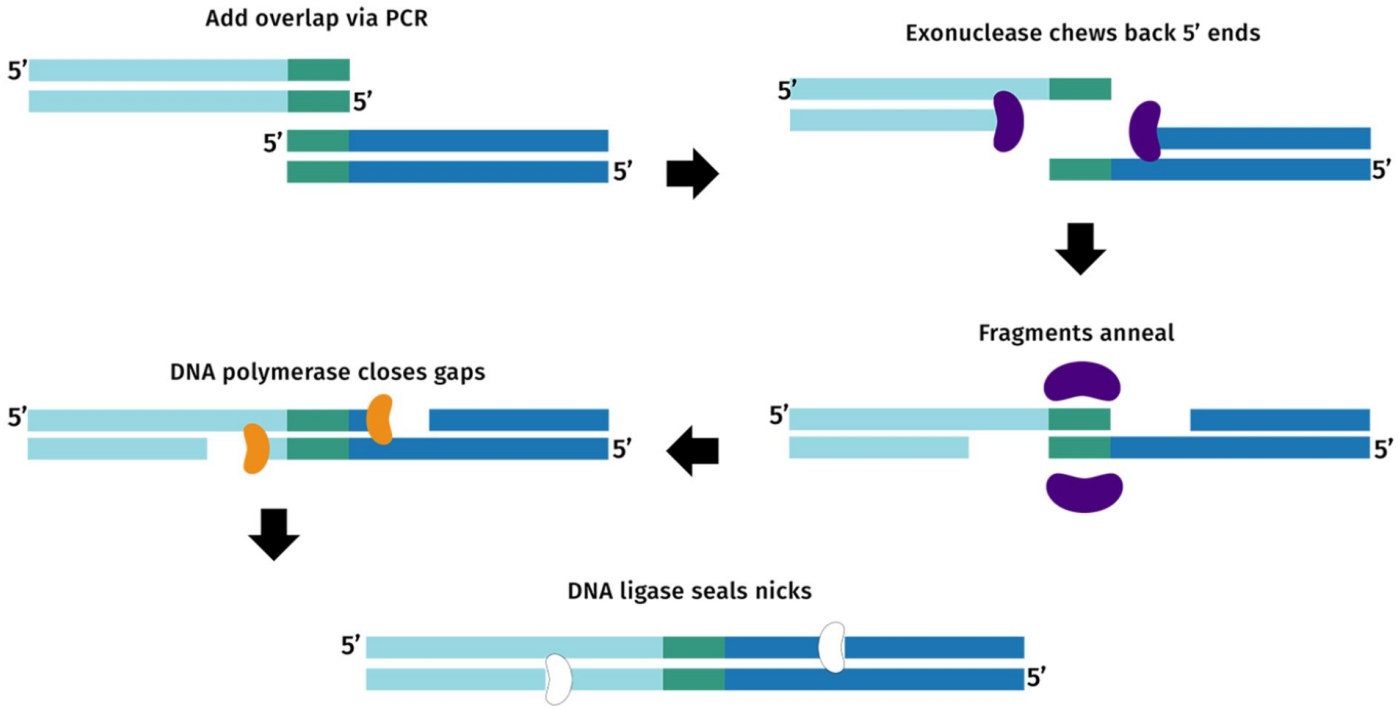
Gibson Assembly



Gibson Assembly

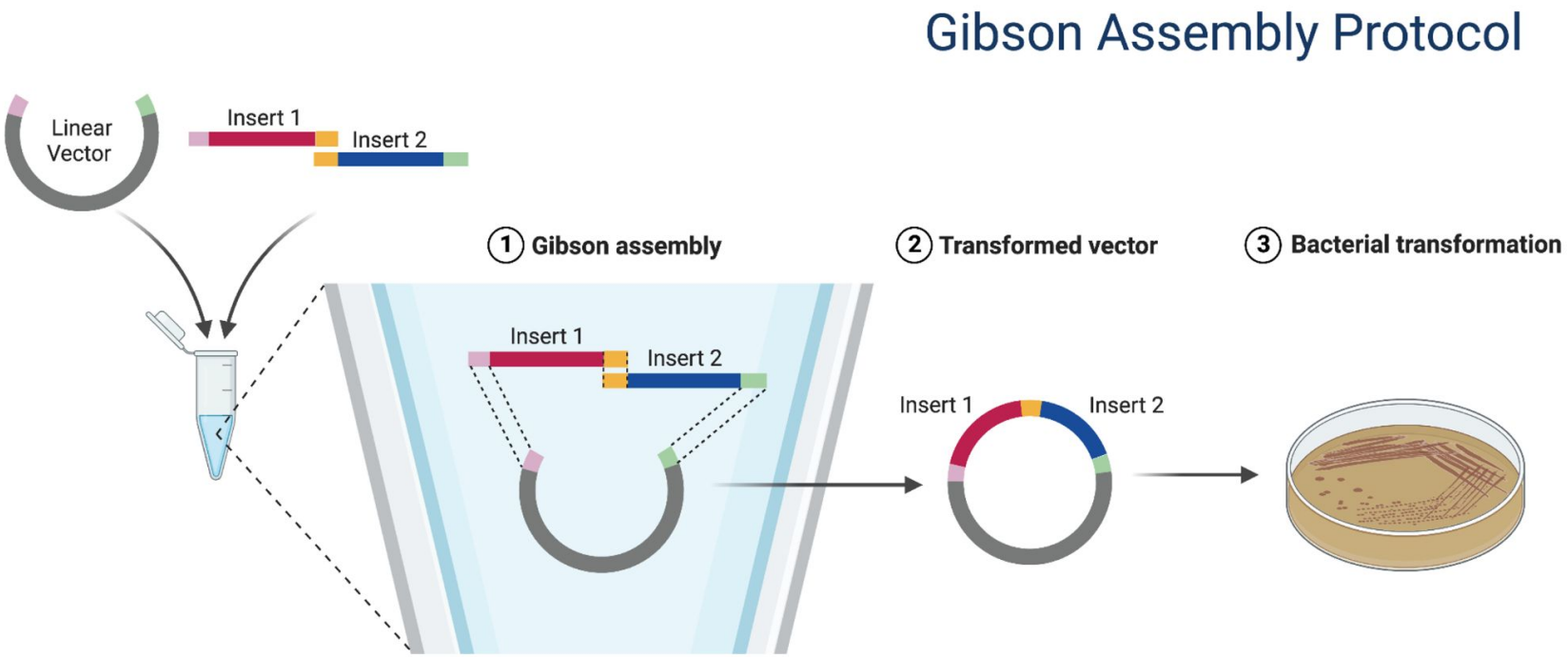


Gibson Assembly



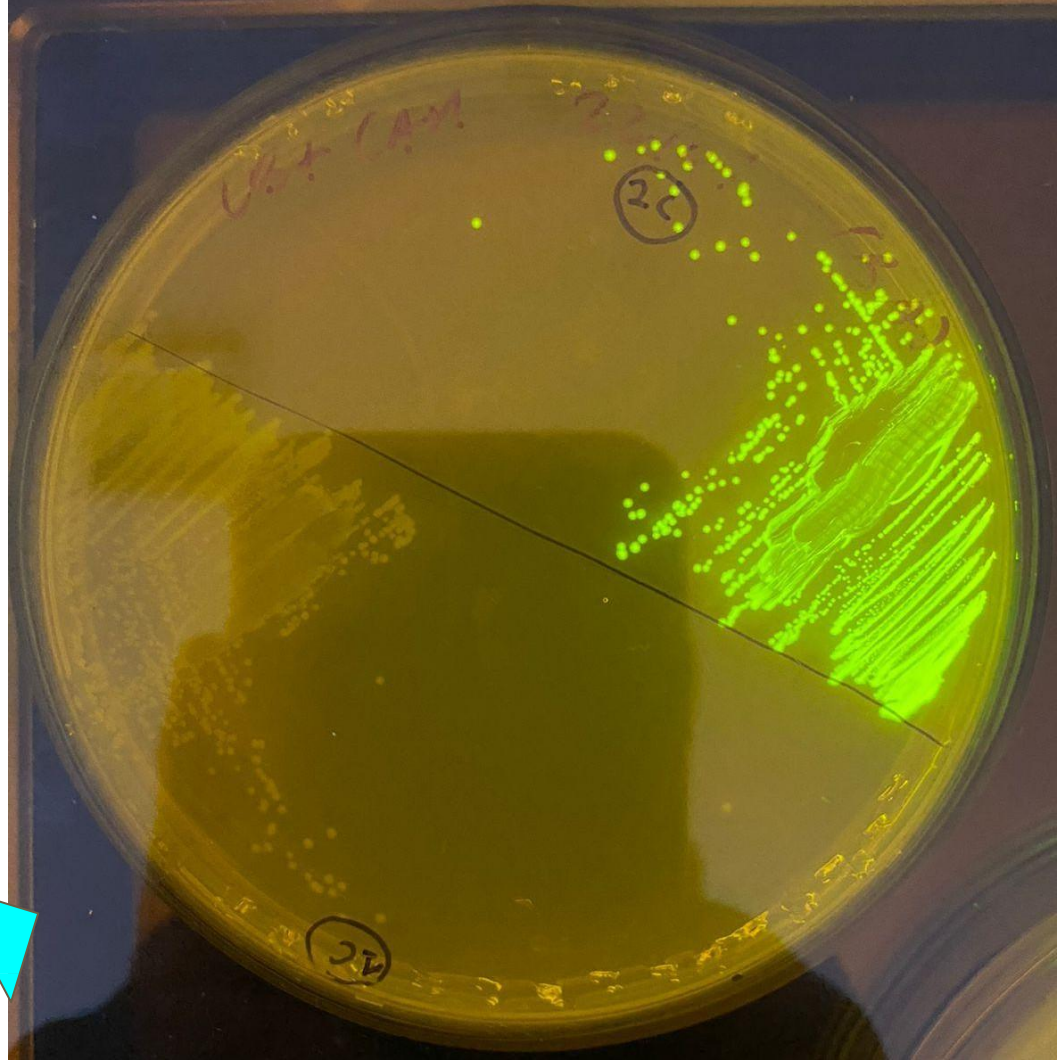
Gibson assembly cloning technique

Gibson Assembly

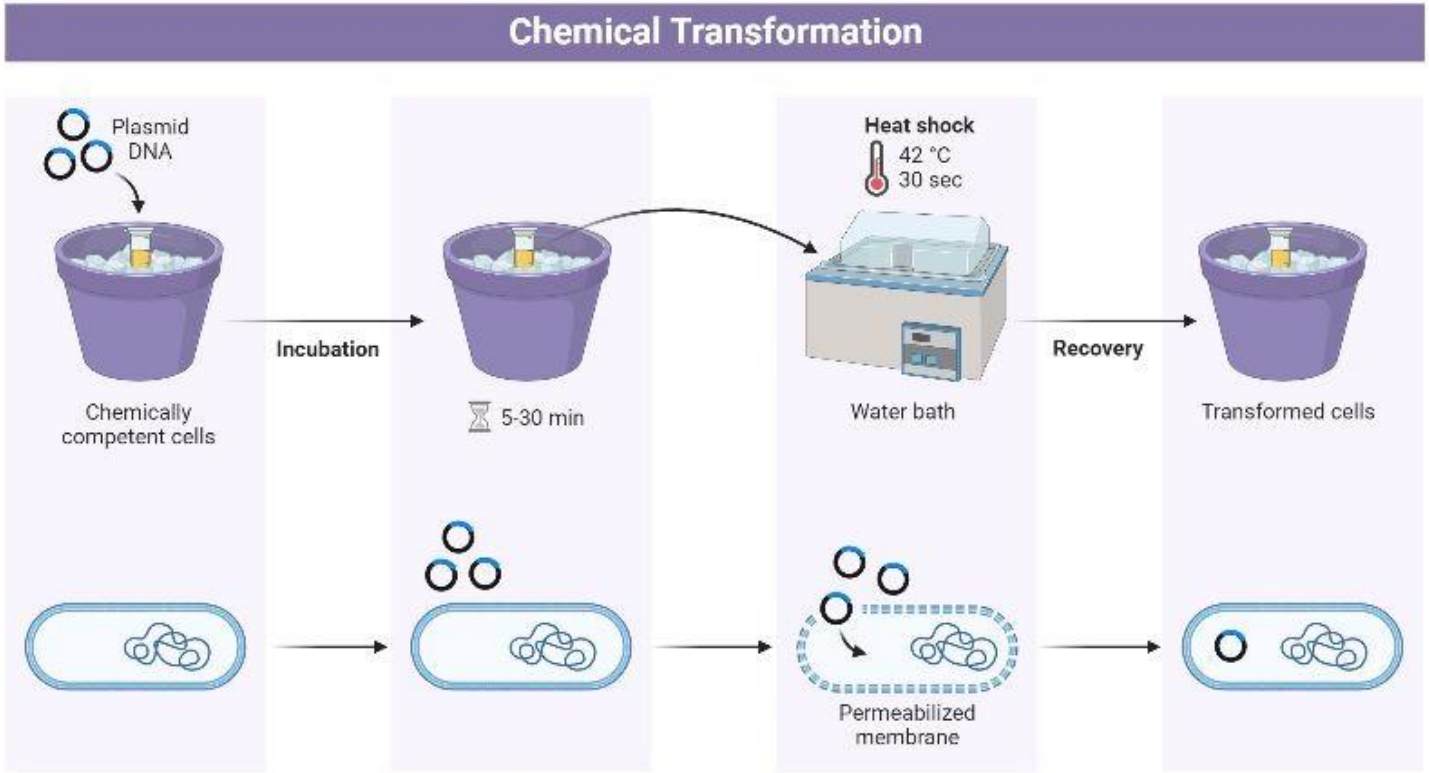


Cell transformation

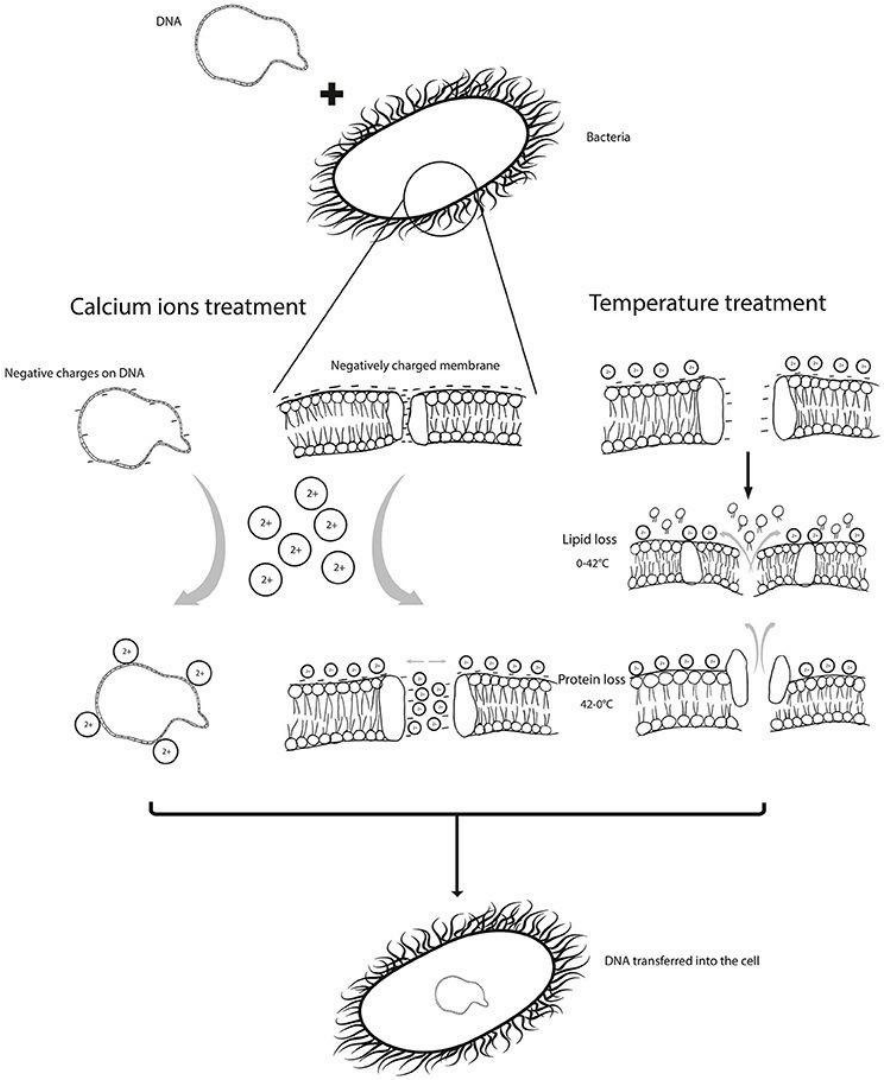
Process for incorporating exogenous DNA molecules inside bacterial cells



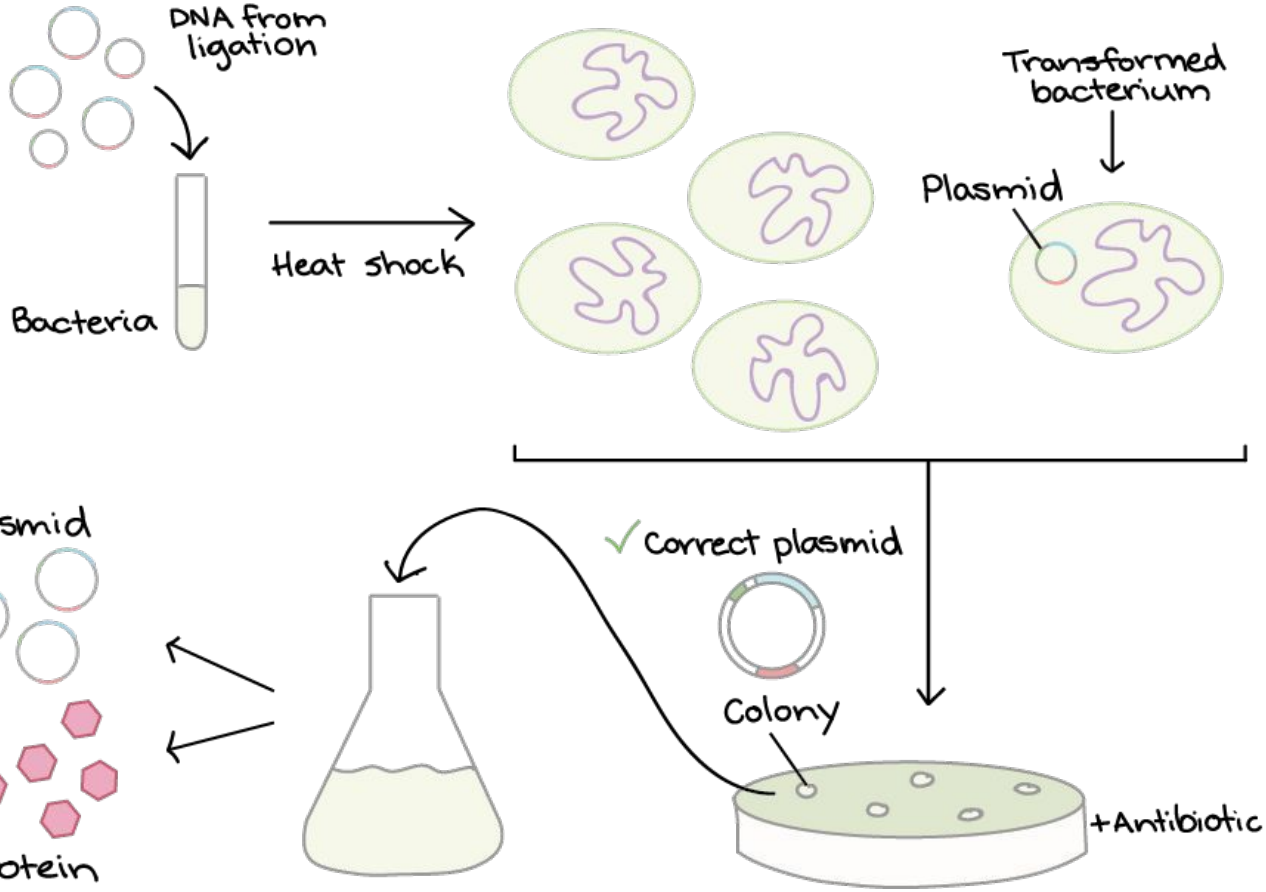
Cell transformation: chemical transformation



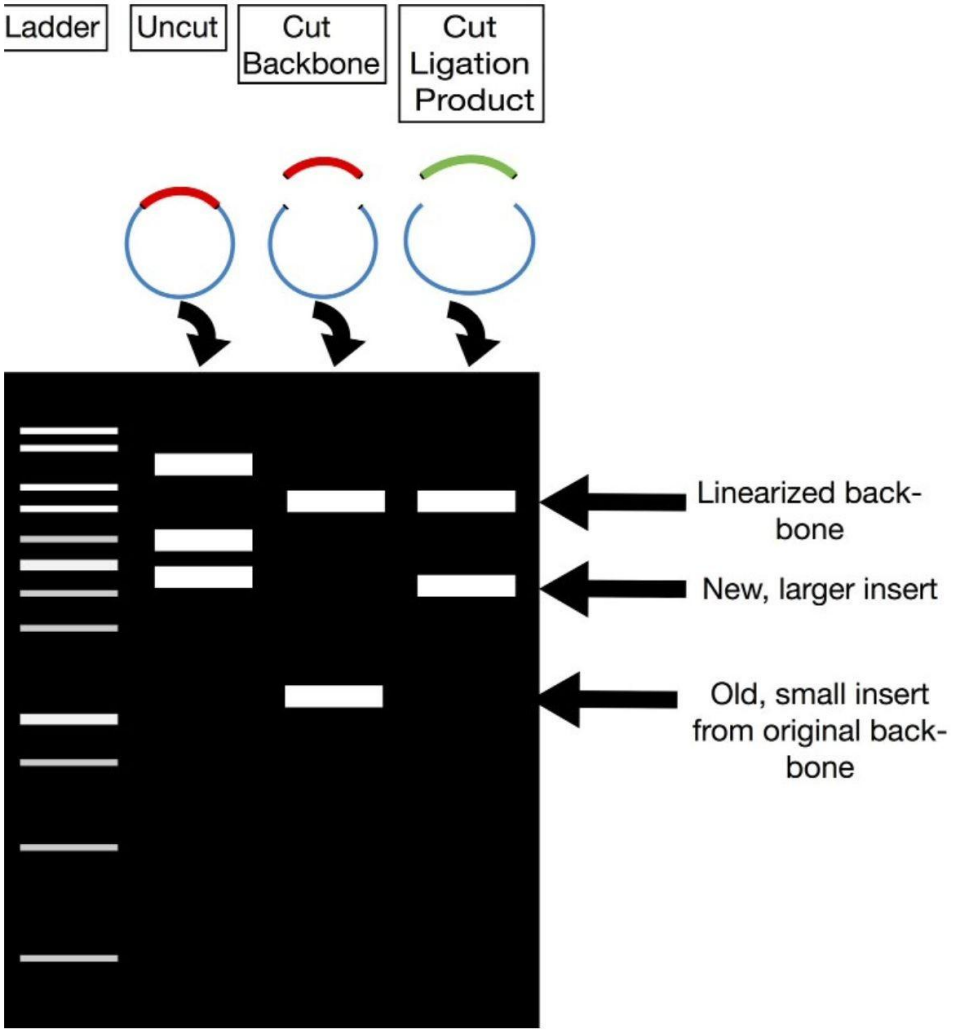
Cell transformation: chemical transformation



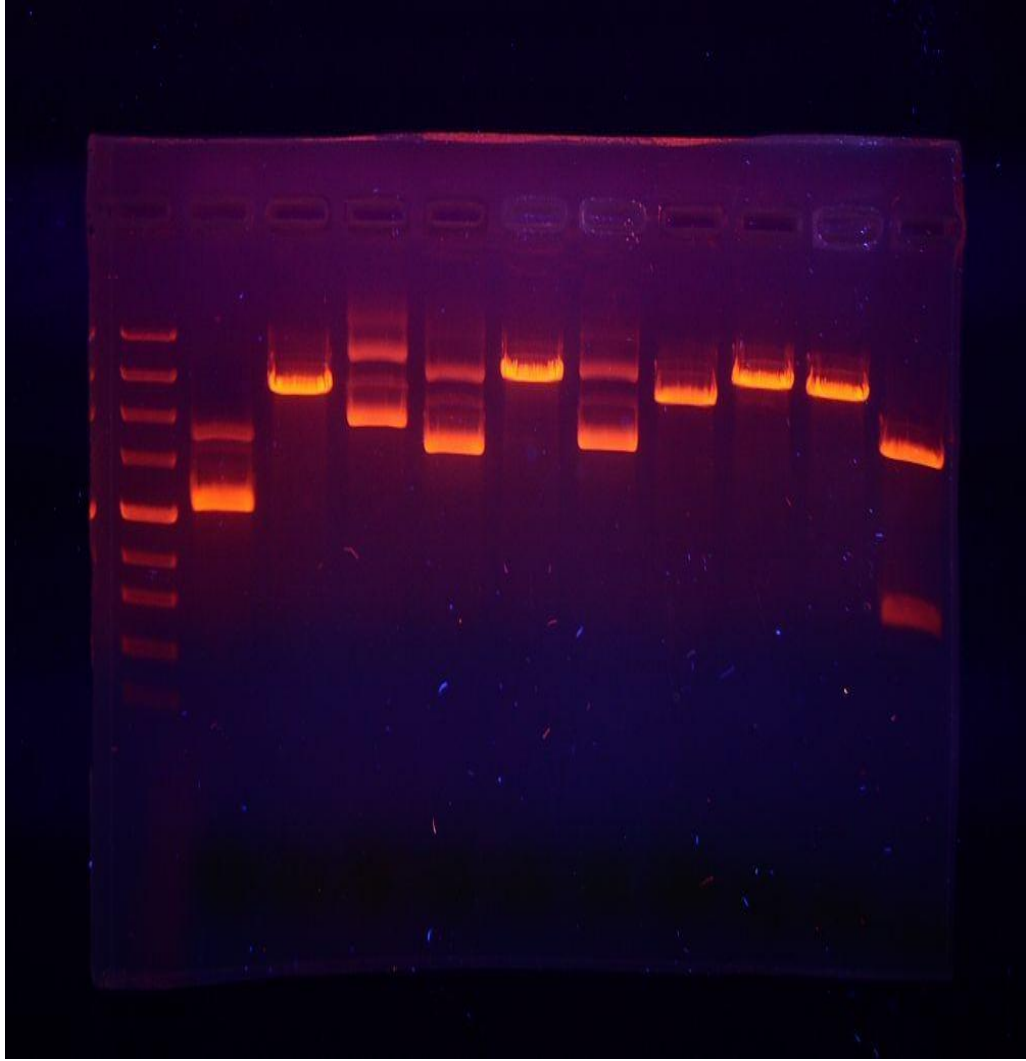
Cell transformation: chemical transformation



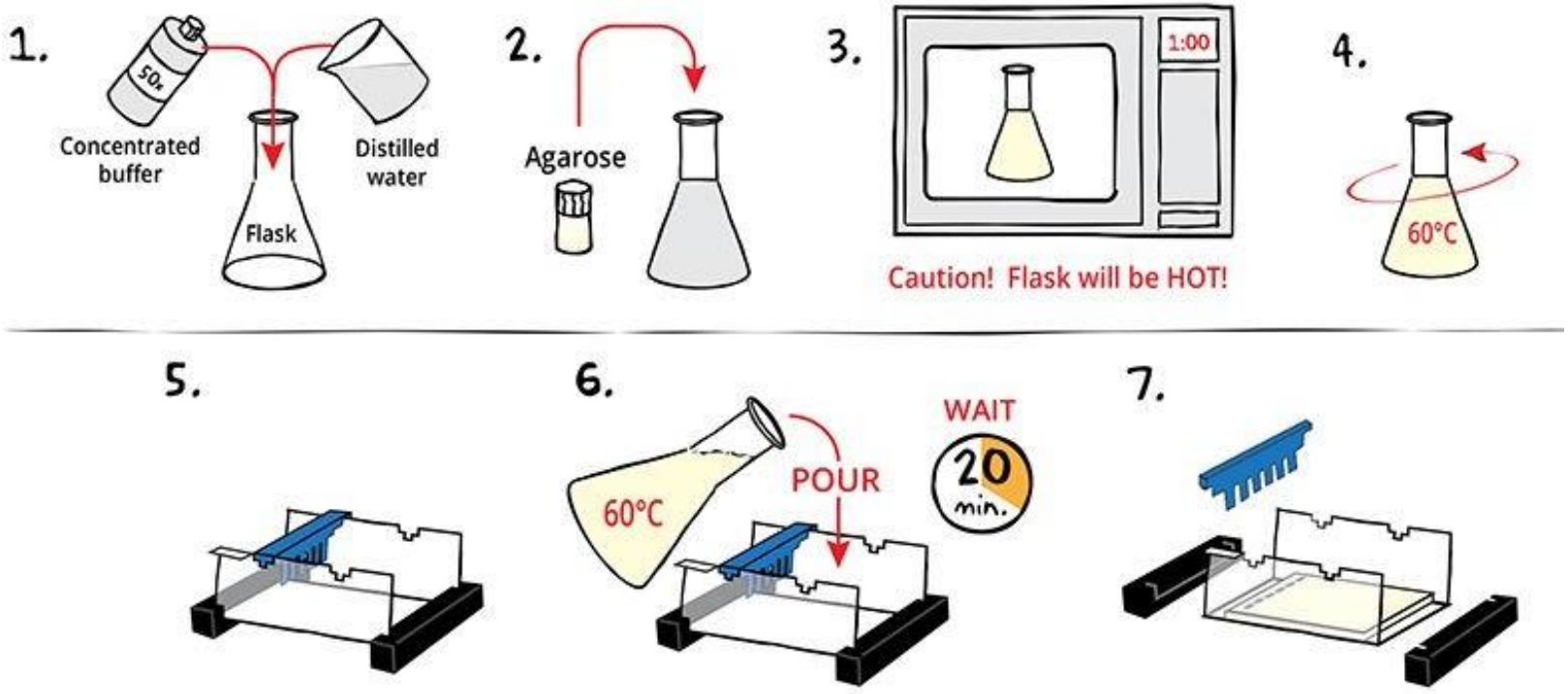
DNA sequence analysis: Gel electrophoresis



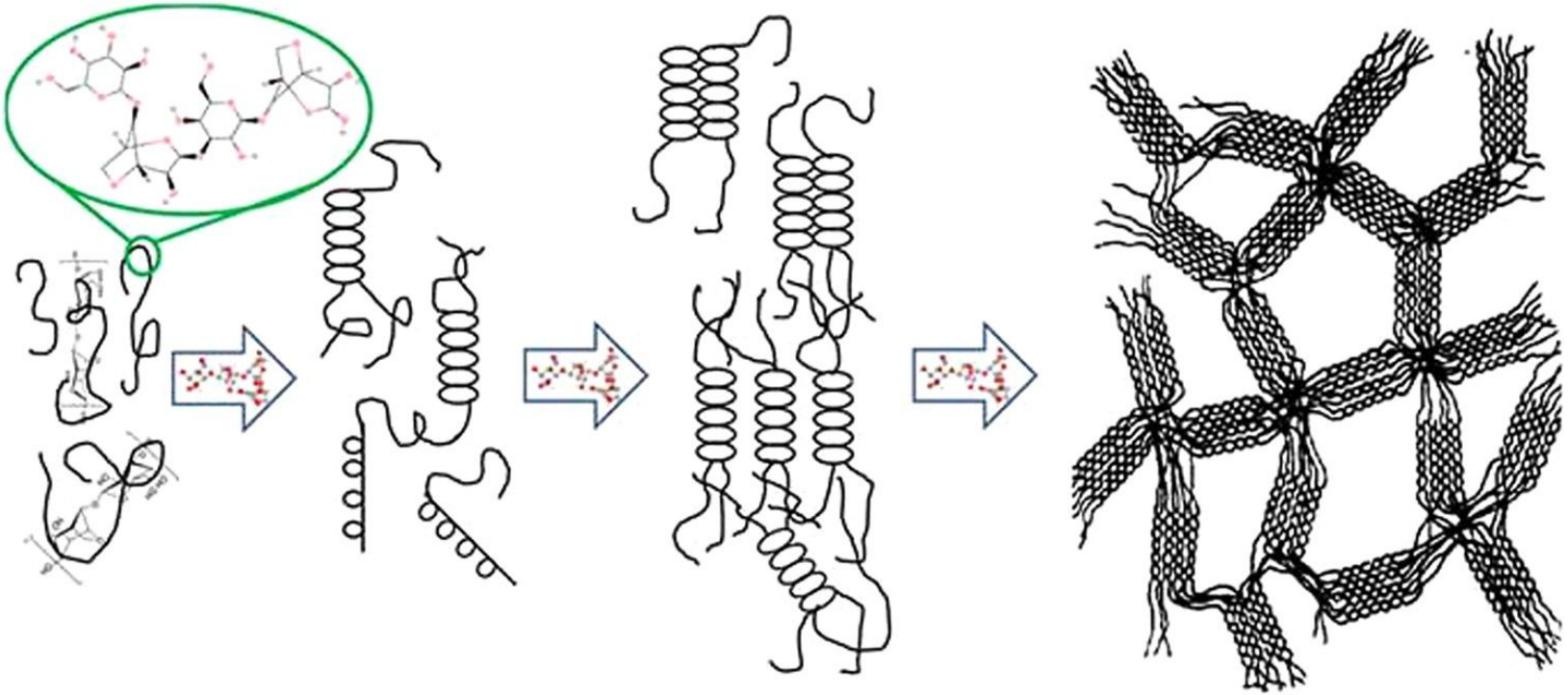
DNA sequence analysis: Gel electrophoresis



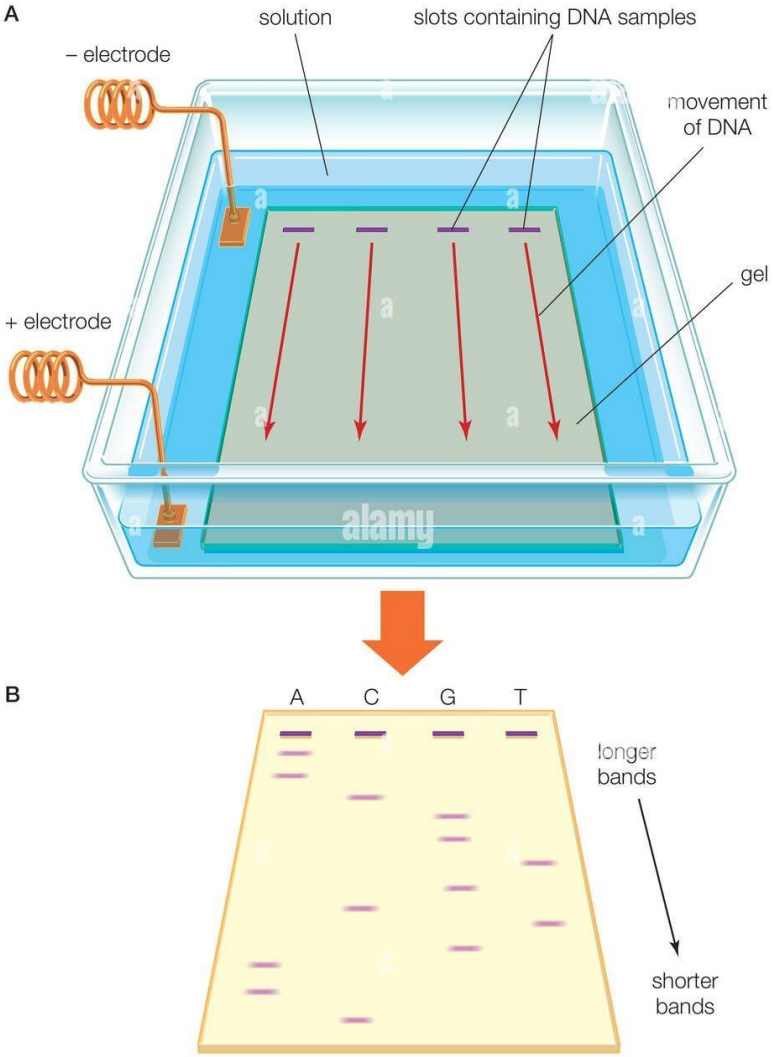
DNA sequence analysis: Gel electrophoresis



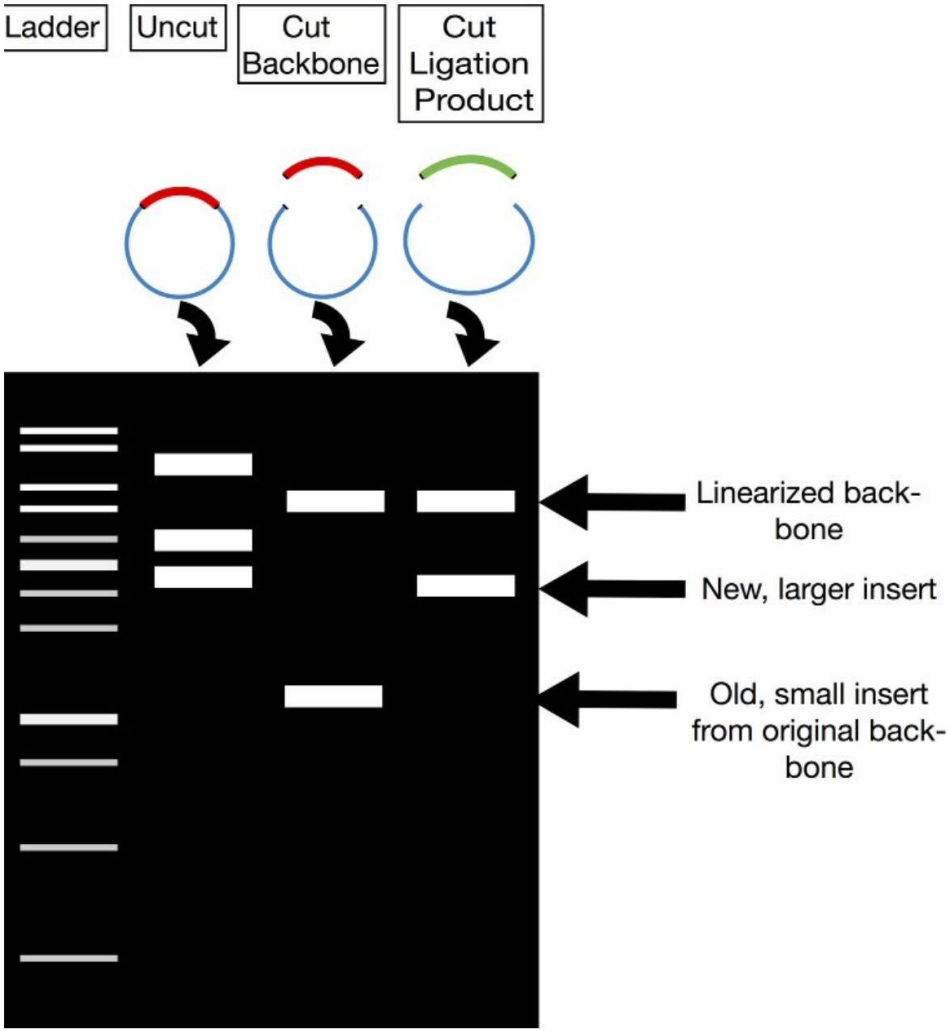
DNA sequence analysis: Gel electrophoresis



DNA sequence analysis: Gel electrophoresis



DNA sequence analysis: Gel electrophoresis



BioCalculators

NEBioCalculator®

NEW ENGLAND Biolabs 50 YEARS

version 1.15.8

ABOUT HELP

DNA

- Ligation
- ds: Mass ⇄ Moles
- ds: Mass → Ends
- ss: Mass ⇄ Moles

RNA

- ss: Mass ⇄ Moles

Protein

- Mass ⇄ Moles

General

- OD_{260}
- Dilution
- Molarity

Genome Editing

- sgRNA Designer

Ligation Calculator

This tool will calculate the mass of insert required at several molar insert:vector ratios in the range needed for typical ligation reactions.

Ligation | Tutorials

Insert DNA length

kb

Vector DNA length

kb

Vector DNA mass

ng

Required insert DNA mass

--- (1:1)

--- (2:1)

--- (3:1)

--- (5:1)

--- (7:1)

Formula

required mass insert (g) = desired insert/vector molar ratio x mass of vector (g) x ratio of insert to vector lengths

BioCalculators

version 1.15.8

ABOUT HELP

DNA
- Ligation
- ds: Mass ⇄ Moles
- ds: Mass → Ends
- ss: Mass ⇄ Moles

RNA
- ss: Mass ⇄ Moles

Protein
- Mass ⇄ Moles

General
- OD_{260}
- Dilution
- Molarity

Genome Editing
- sgRNA Designer

Ligation Calculator

This tool will calculate the mass of insert required at several molar insert:vector ratios in the range needed for typical ligation reactions.

Ligation | Tutorials

Insert DNA length
300 bp

Vector DNA length
2000 bp

Vector DNA mass
50 ng

Required insert DNA mass
- 7.500 ng (1:1)
- 15.00 ng (2:1)
- 22.50 ng (3:1)
- 37.50 ng (5:1)
- 52.50 ng (7:1)

Formula

required mass insert (g) = desired insert/vector molar ratio x mass of vector (g) x ratio of insert to vector lengths

BioCalculators

Tm Calculator

version 1.16.6

ABOUT HELP

Use the NEB Tm Calculator to estimate an appropriate annealing temperature when using NEB PCR products.

Instructions

- Select the product group of the polymerase or kit you plan to use.
- Select the polymerase or kit from the list of products.
- If needed, modify the recommended primer concentration.
- Enter primer sequences (with up to 3 ambiguous bases). Spaces allowed.

Note that an anealing temperature will only be displayed if both primer sequences are entered.

Product Group

Q5

Polymerase/Kit

Q5 High-Fidelity DNA Polymerase

Primer Concentration (nM)

500

Reset concentration

Primer 1

PRIMER 1 SEQUENCE

Primer 2

PRIMER 2 SEQUENCE

Switch to batch mode

Clear

Use example input

Anneal at

--- °C

Primer 1
--- nt
---% GC
Tm: ---°C

Primer 2
--- nt
---% GC
Tm: ---°C

BioCalculators

NEBuilder® Assembly Tool

version 2.10.3

NEW ENGLAND Biolabs 50 YEARS

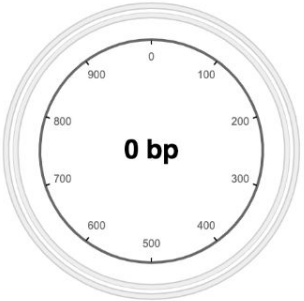
ABOUT HELP

Build | Summary | Settings | Load/Save | Resources

Get started designing primers.

NEBuilder Assembly Tool can be used to design primers for your NEBuilder® HiFi DNA Assembly or Gibson Assembly® reactions.

+ New Fragment + Load Project



Need assistance setting up your NEBuilder protocol and assembly reaction?

Use NEBuilder Protocol Calculator to generate your customized protocol.

Performing multi site-directed mutagenesis?

Use the updated NEBaseChanger tool to design primers.

Additional resources

- **Biocalculators**
  - NEBioCalculator: https://nebiocalculator.neb.com/#!/ligation
  - NEBuilder: https://nebuilder.neb.com/#!/
  - Tm Calculators:
    - NEB: https://tmcalculator.neb.com/#!/main
  - Primer Check - Primer3: https://primer3.ut.ee/
- **More about weblabs**
  - Lab Protocols: https://www.neb.com/en/tools-and-resources/search?type=Protocol
  - Plasmids 101: https://blog.addgene.org/plasmids-101-what-is-a-plasmid
  - Virtual lab: https://www.labster.com/simulation-courses/molecular-biology

Thanks!
and please
leave
questions
on chat!