Biotechnology of drugs – Basics of genetic engineering II.

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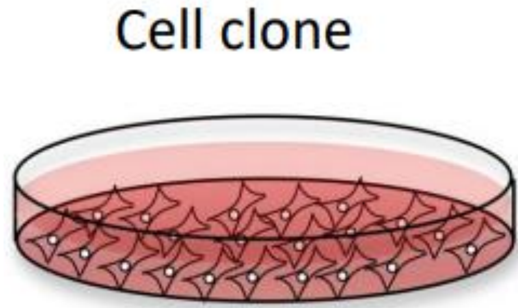
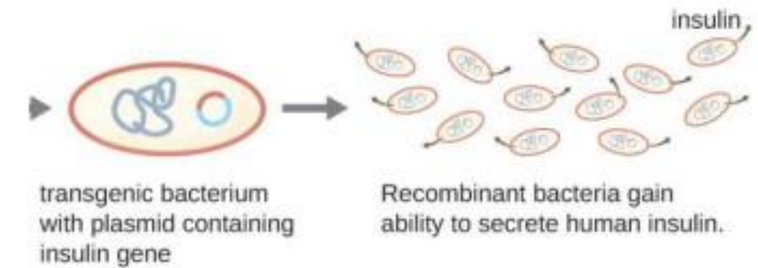
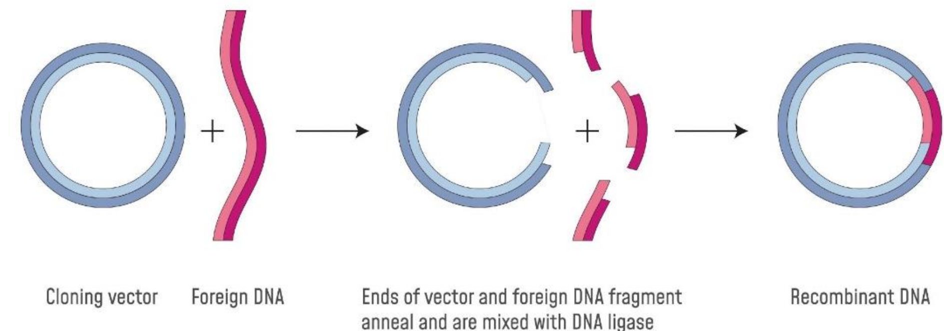
Cloning

- **Classic definition**
  - creating a new individual genetically identical to the original
- **Biotechnology definition**
  - fusion of vector with gene → **creation of genetically identical cells/organisms carrying vector with insert**



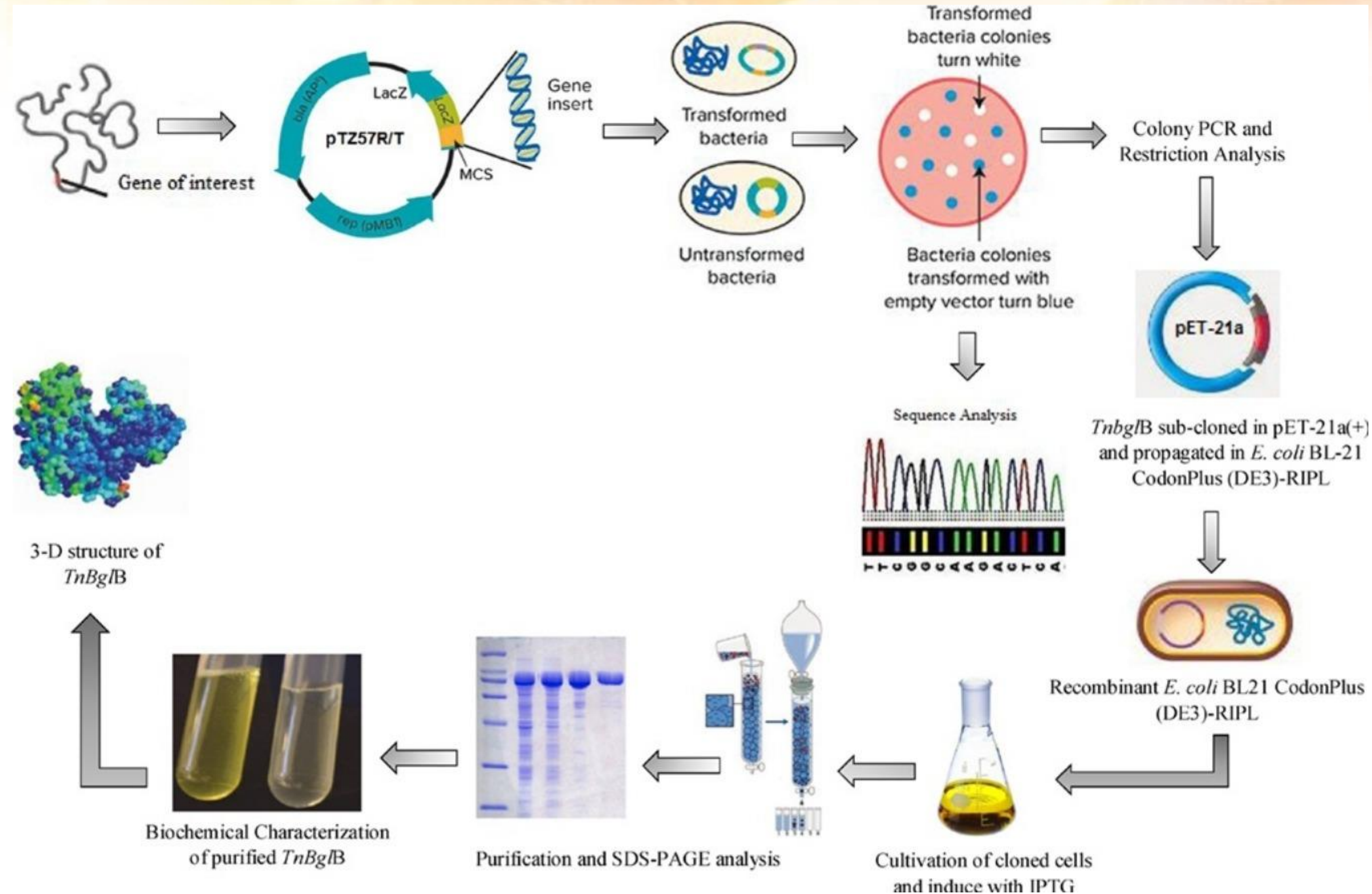




https://www.news-medical.net/life-sciences/The-Biochemistry-of-Cloning.aspx

Basic steps in gene cloning

1) **Cleavage** of DNA at desired sites
2) **Recombination** - connection of DNA fragments
3) **Transformation** – insertion of recombined DNA into a cell
4) **Selection** of cells containing a foreign gene
5) **Analysis** of cloned DNA

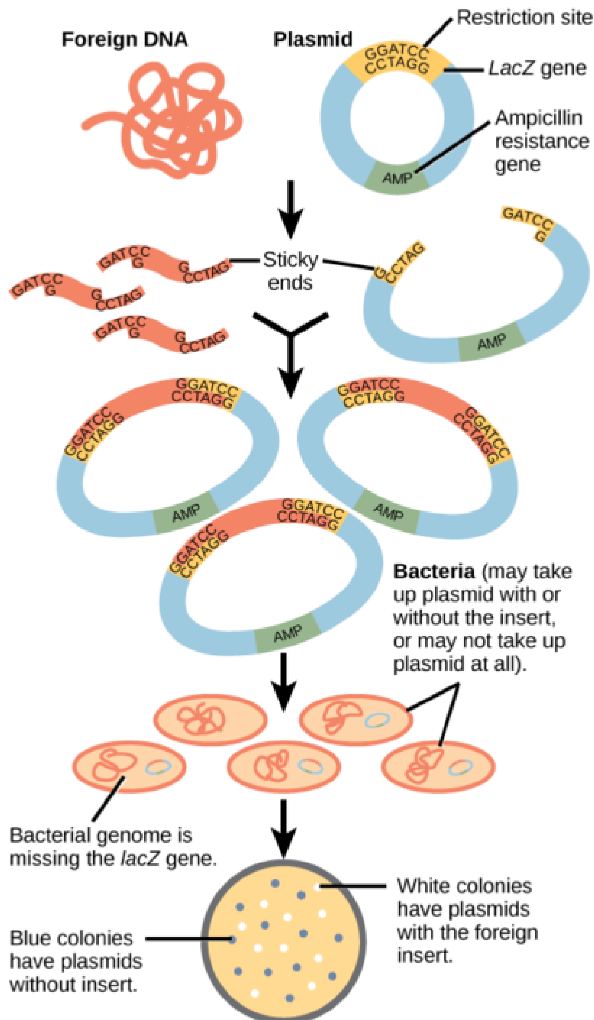


https://doi.org/10.1016/j.procbio.2017.12.007

Cleavage of DNA by restrictases

Non-oriented cloning - we connect the DNA fragments with the vector after they have been cleaved by the same restrictase **= the same overhanging ends on both sides**

Oriented cloning - different restrictases are used to cut the vector and the cloned DNA **= different overhangs on each side**



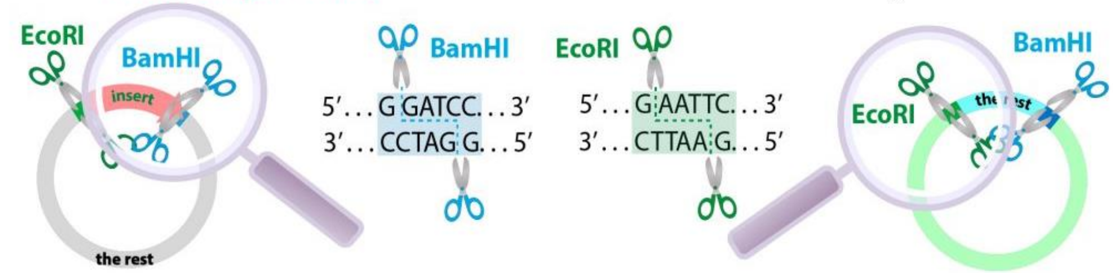
The foreign DNA and plasmid are cut with the same **restriction enzyme**, which recognizes a particular sequence of DNA called a *restriction site*. The restriction site occurs only once in the plasmid, and is located within the *lacZ* gene, a gene necessary for metabolizing lactose.

The restriction enzyme creates sticky ends that allow the foreign DNA and cloning vector to anneal. An enzyme called ligase glues the annealed fragments together.

The ligated cloning vector is transformed into a bacterial host strain that is ampicillin sensitive and is missing the *lacZ* gene from its genome.

Bacteria are grown on media containing ampicillin and X-gal, a chemical that is metabolized by the same pathway as lactose. The ampicillin kills bacteria without plasmid. Plasmids lacking the foreign insert have an intact *lacZ* gene and are able to metabolize X-gal, releasing a dye that turns the colony blue. Plasmids with an insert have a disrupted *lacZ* gene and produce white colonies.

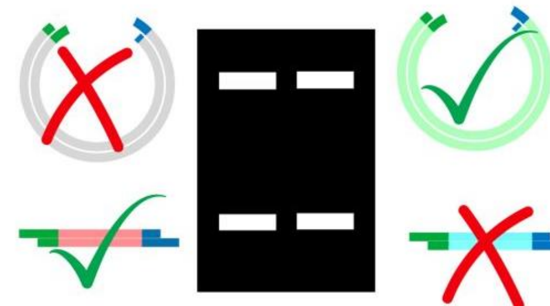
cut insert (what you want to put in) and **vector** (home you want to put it in) with the same 2 restriction enzymes



This generates DNA pieces with complementary "sticky ends" you can mix & match (once you separate them)

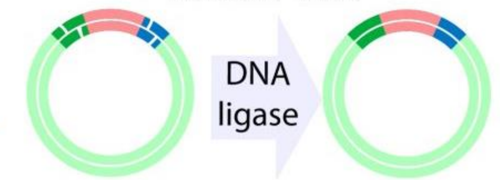
purify the pieces you want

run an agarose gel to separate by size, then cut out & purify those you want



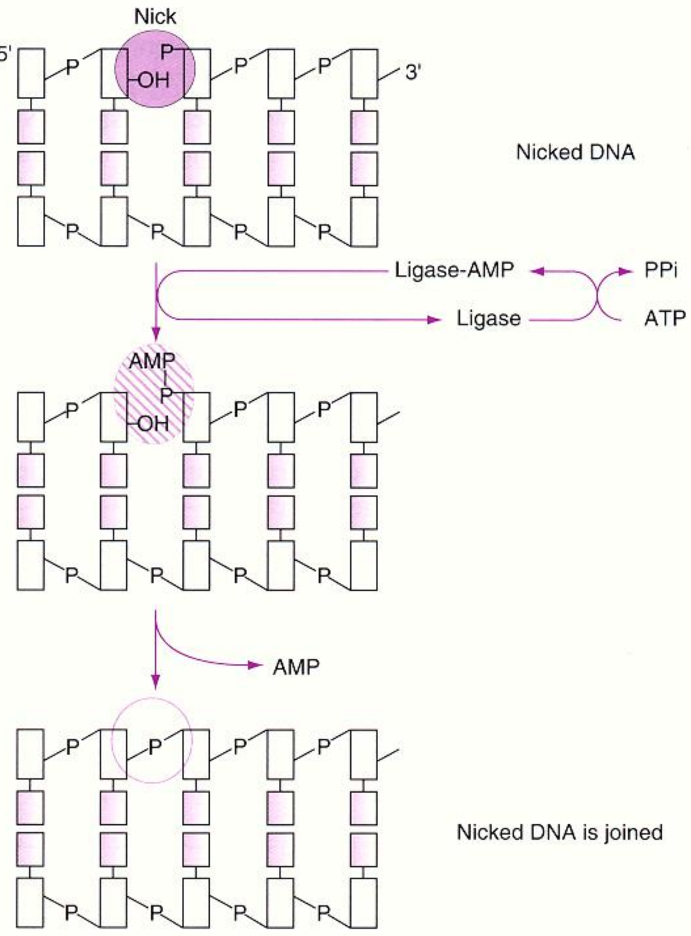
mix 'em

stitch 'em

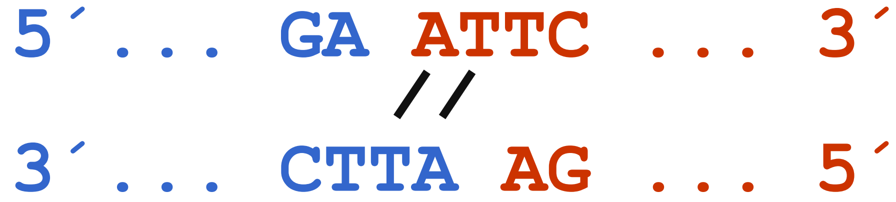


https://thebumblingbiochemist.com/365-days-of-science/molecular-cloning-using-restriction-enzymes/

Ligation = covalent joining of a vector to a fragment

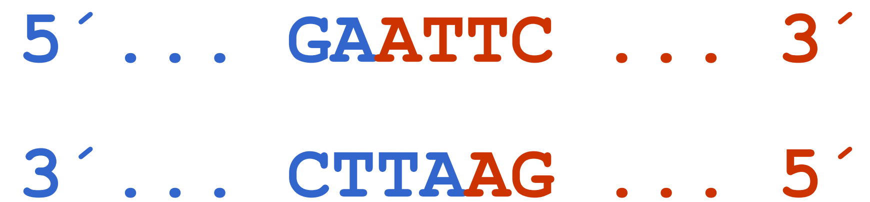


spontaneous connection



joining by ligase

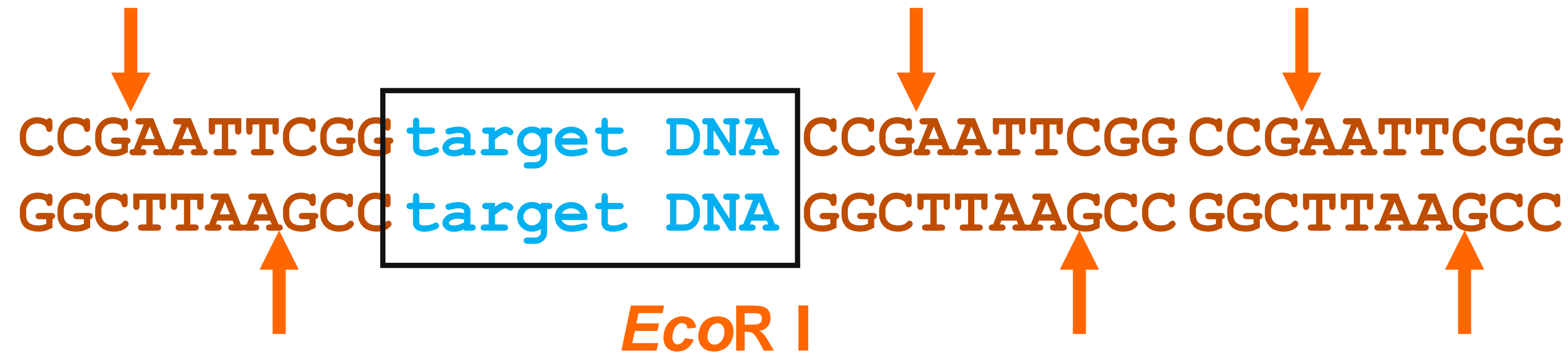
+ 2 x ATP



Creation of overhanging ends - linkers

ligation

5´... CCGAATTCGG ... 3´ + target DNA
3´... GGCTTAAGCC ... 5´ target DNA



5´... AATTCGG target DNA CCG ... 3´
3´... GCC target DNA GGCTTAA ... 5´

Cloning of PCR products – I → attachment of restriction sites and restriction cleavage

GCNNNGAATTCTACGTCCATC

ATGCAGGTAG

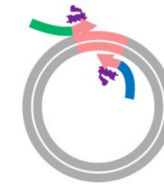
GCTAGTGTCA

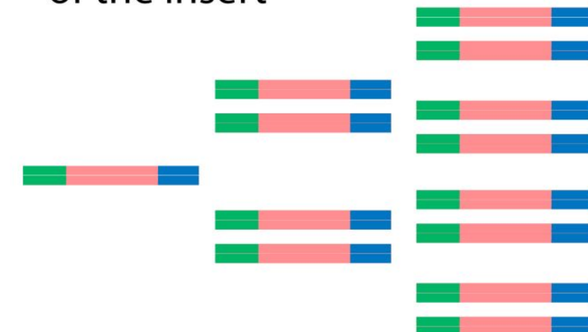
CGATCACAGTCTTAAGNNNCG

amplification

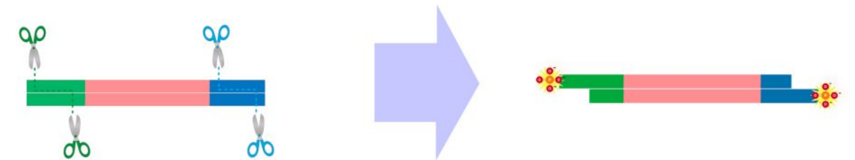
restriction cleavage

you can use use PCR to make lots of copies of the insert





and you can use the primers to add on cut sites you want

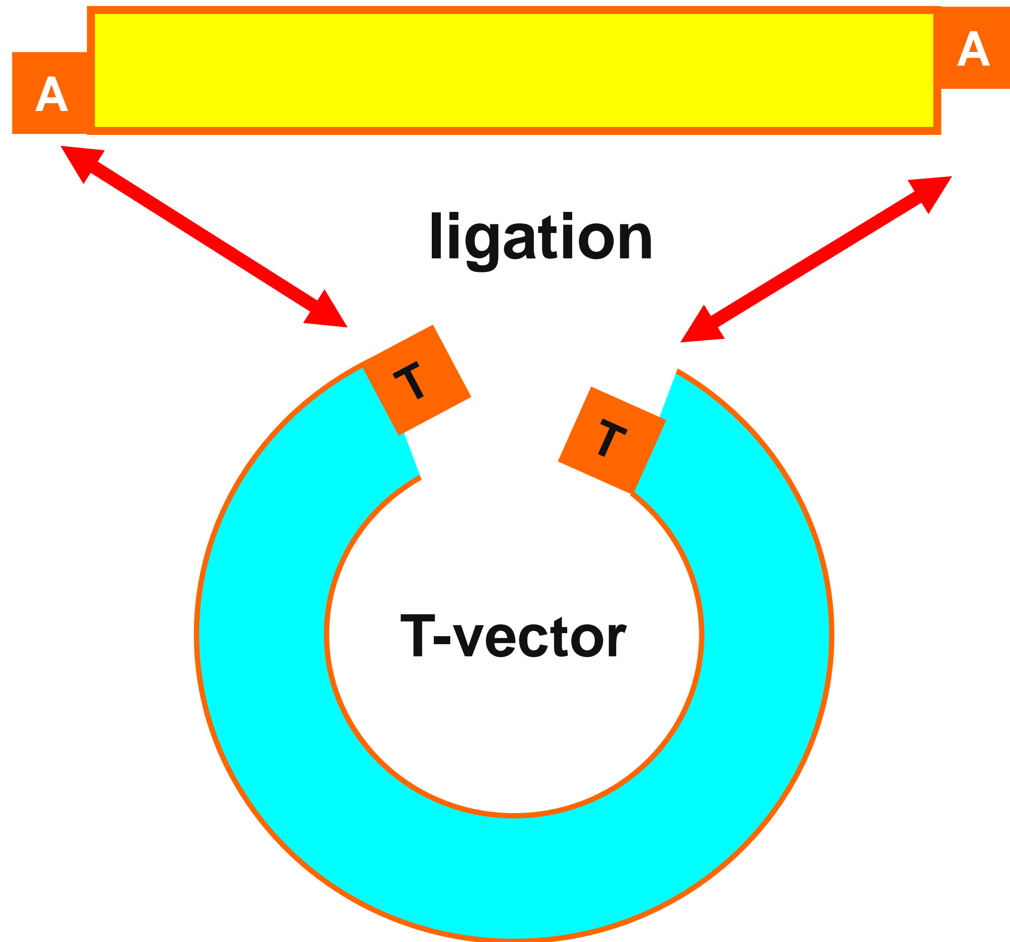


this cutting leaves you with phosphorylated ends but the primers are usually synthesized without phosphates - this only comes into play if your vector is dephosphorylated

https://thebumblingbiochemist.com/365-days-of-science/molecular-cloning-using-restriction-enzymes/

Cloning of PCR products – II → TA cloning

Taq polymerase creates single-nucleotide 3'-overhangs, most often A

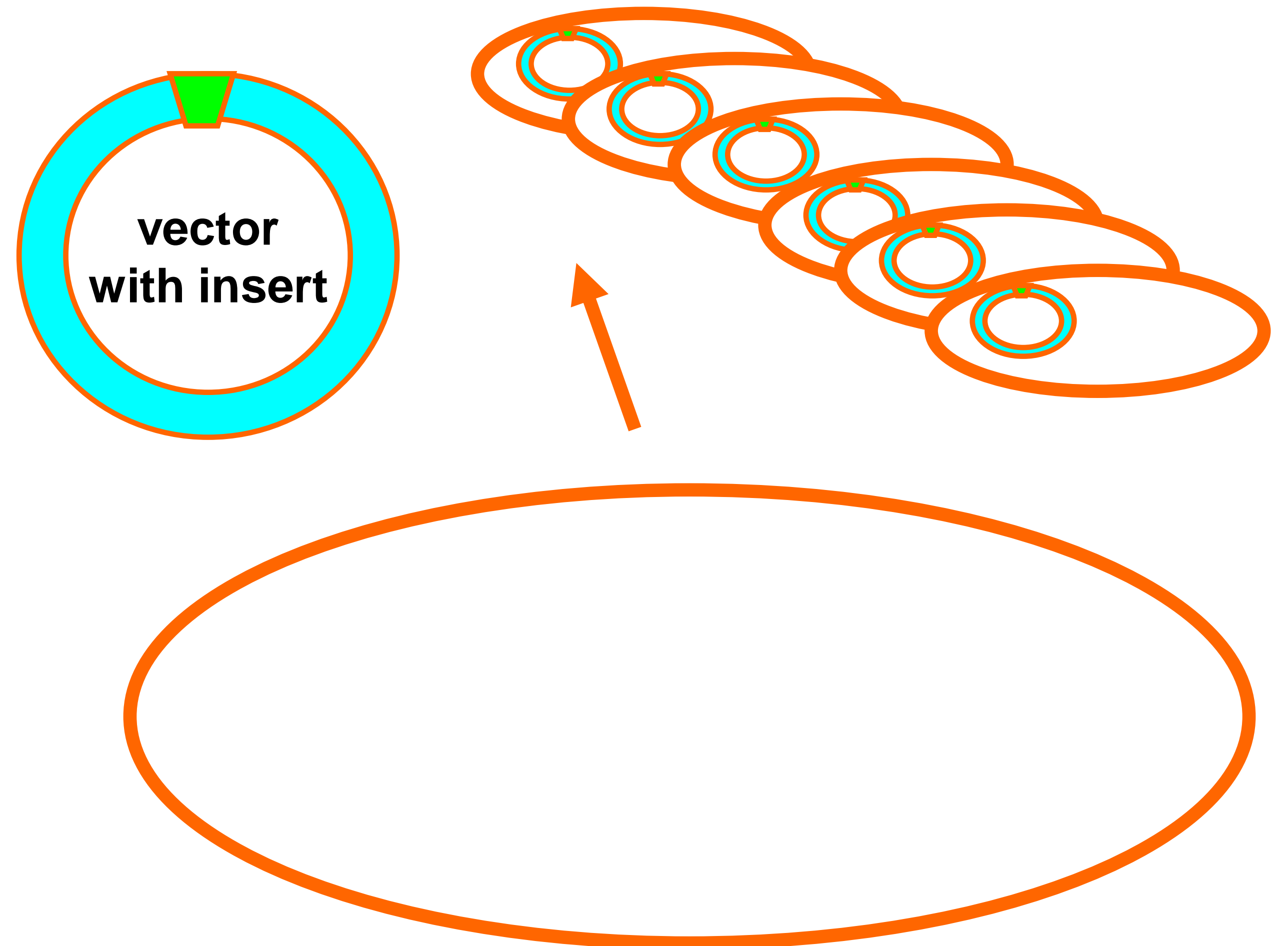


CCT AAG
GGA TTC
NLaz-Prom
Plac
lacZ
pCRII-TOPO
Amp
DH5α™ *Escherichia coli*
pCRII-TOPO *NLaz*-Prom
pCRII-TOPO

D. Pérez Torres et al. / Revista Clínica (2011)

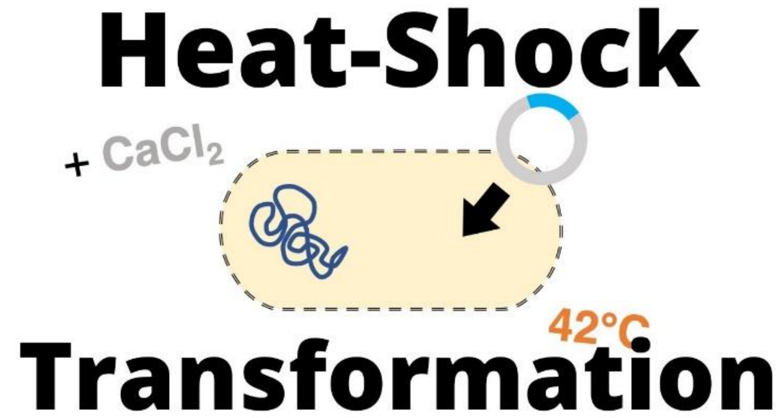
Introduction the construct into the host

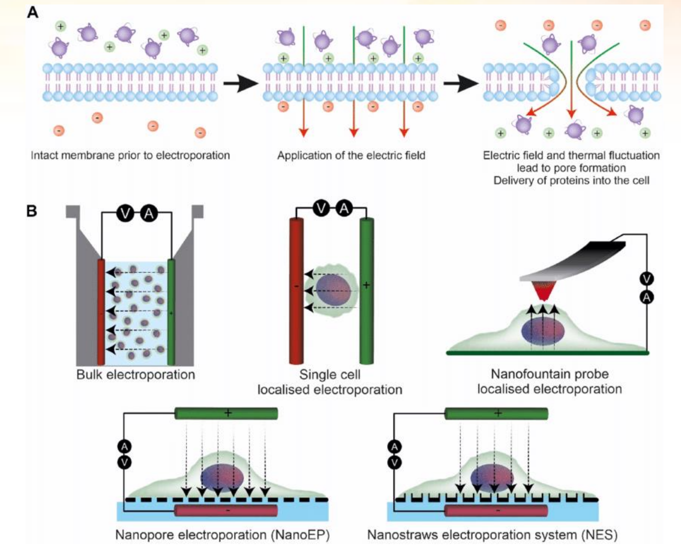
- **Transformation =** introduction of non-viral DNA into **prokaryotes** and non-animal eukaryotes
- **Transfection =** introduction of DNA into **a euraryotic cell**
- **Transduction** = transfer of DNA using **viral vectors**

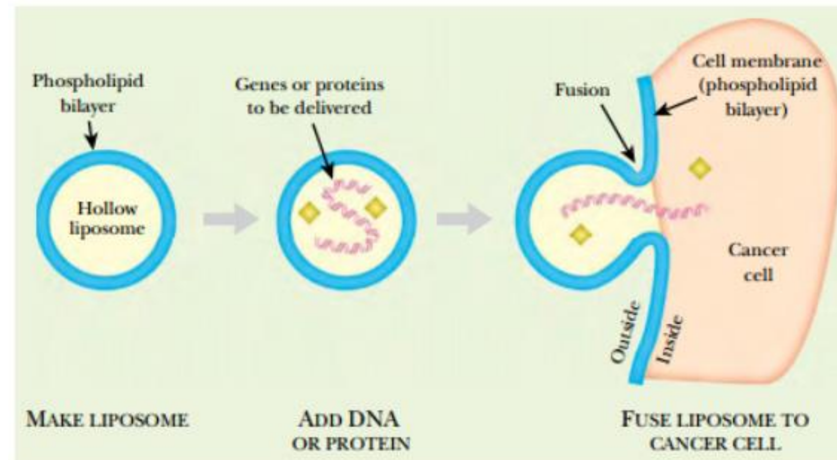


Methods of transformation/transfection

- Heat shock + $CaCl_2$
- Electroporation
- Lipofection
- Microinjection and „gene gun“

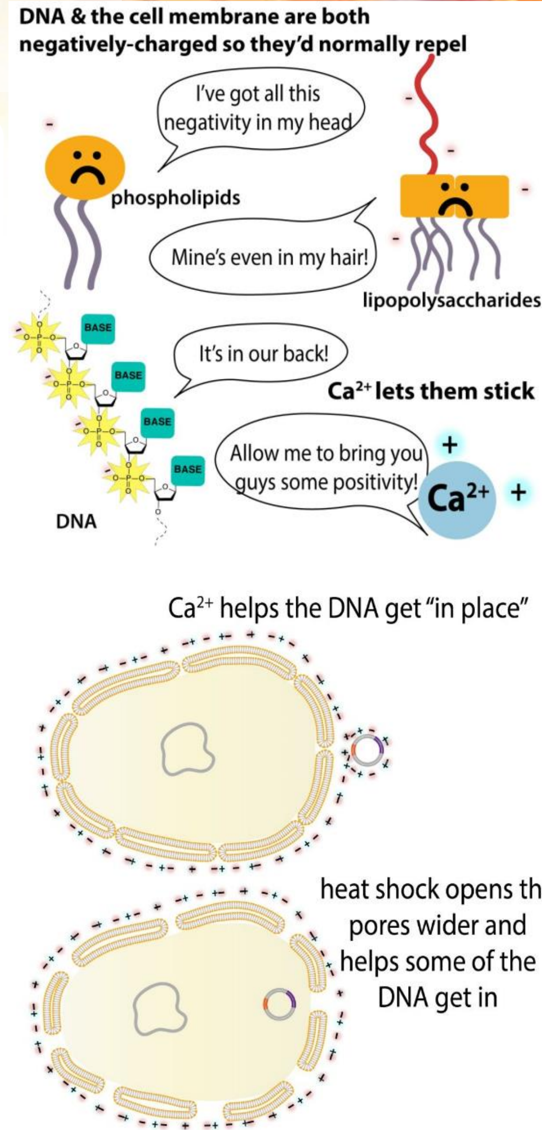






„Heat shock“

Chemocompetent cells with $CaCl_2$ → Add DNA, 4° C, 30 min → Heat shock, 42° C, 30 s → Entry of foreign DNA into cells → Cell incubation → Selection of transformants



https://thebumblingbiochemist.com/365-days-of-science/bacterial-transformation-heat-shock-chemically-competent-cells/

DNA

Bacteria

Calcium ions treatment

Temperature treatment

Negative charges on DNA

Negatively charged membrane

Lipid loss 0-42°C

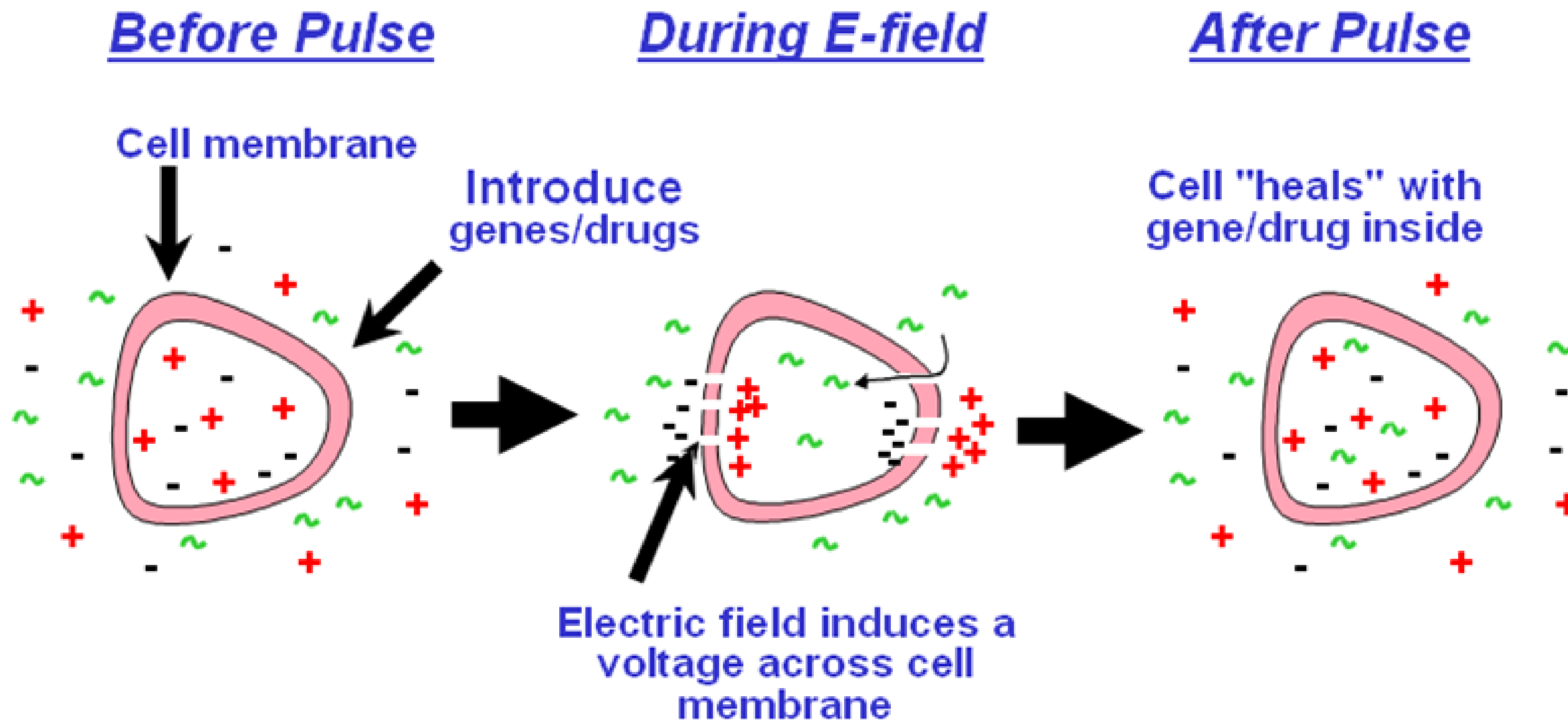
Protein loss 42-0°C

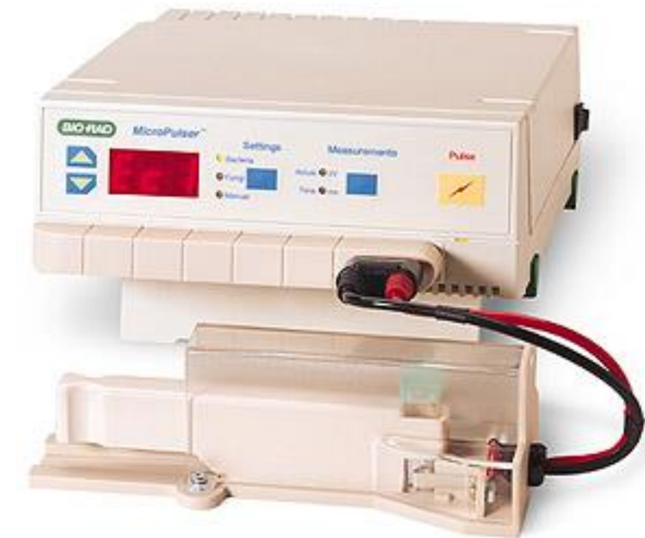
DNA transferred into the cell

https://doi.org/10.3389/fmicb.2017.02169

Electroporation

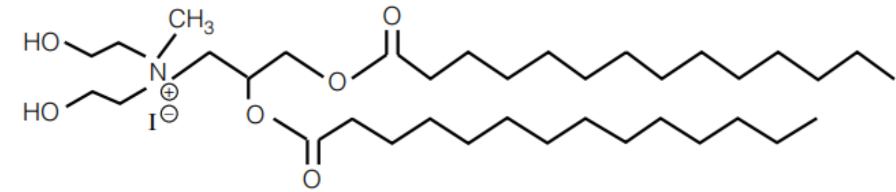
- Electrical impulses cause the formation of pores in the cell membrane and the entry of exogenous DNA into the cell





https://www.socmucimm.org/news-media/bacterial-transformation-electroporation/

Lipofection



- Formation of liposomes with encapsulated DNA
- Possibility of transfection of oligo DNA, RNA, siRNA, YAC
- Widely used in eukaryotes
- Possibility of transfection *in vivo*

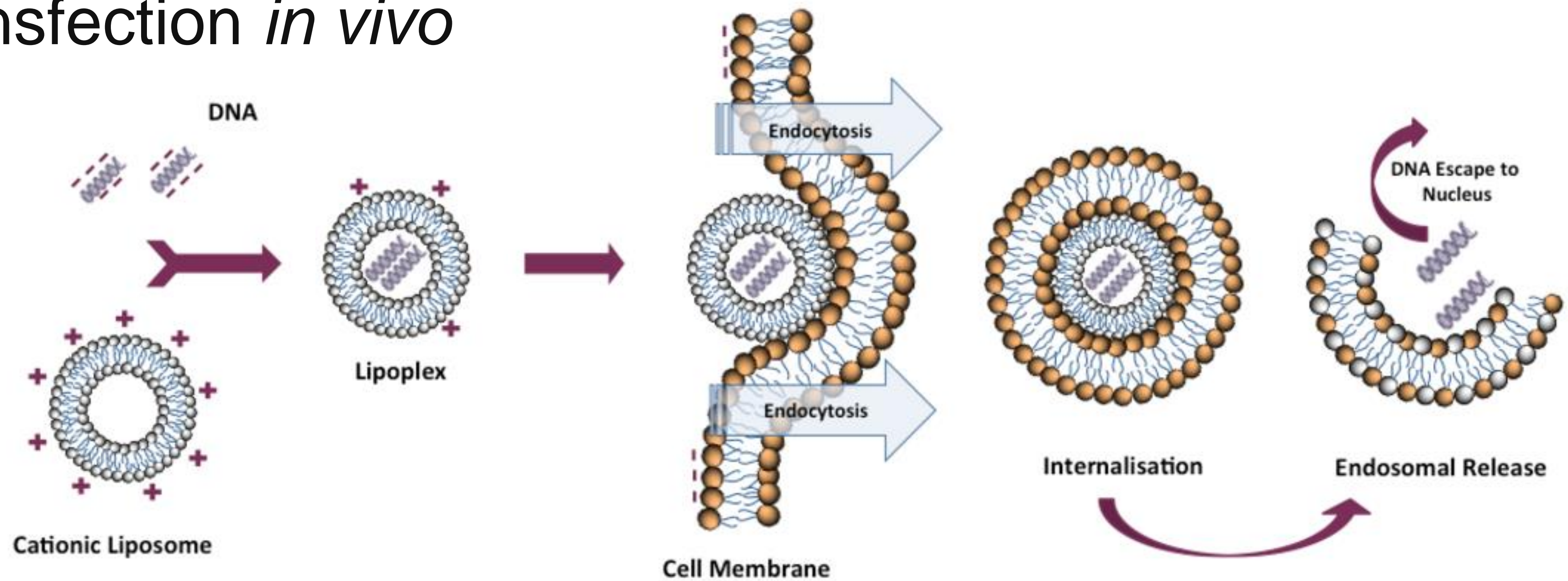


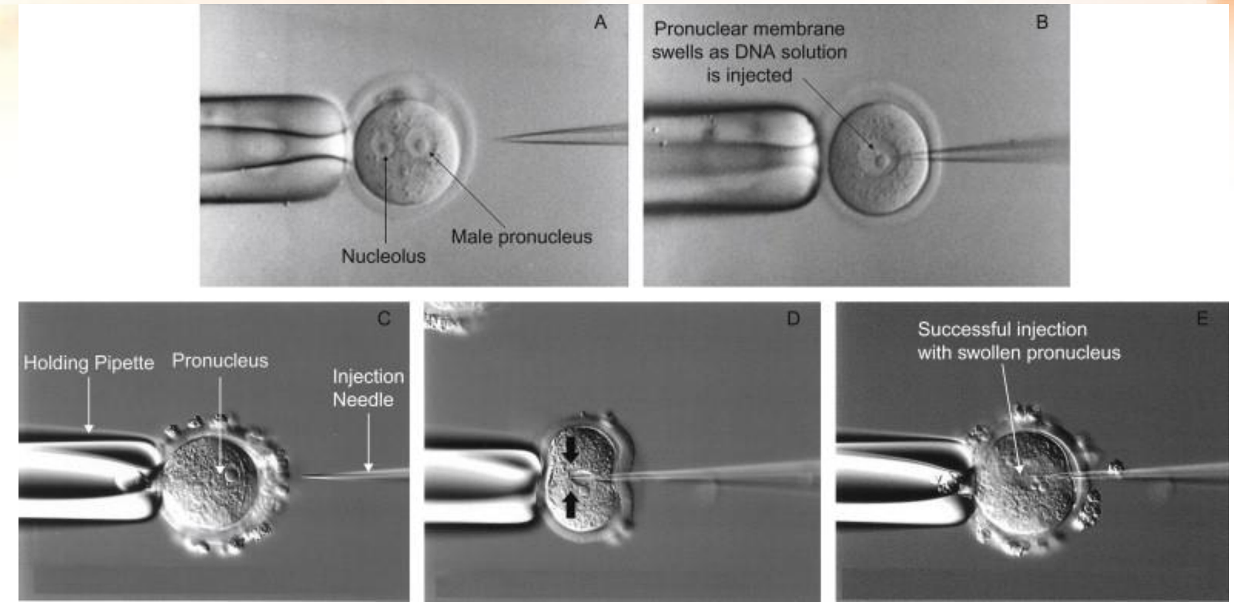
Figure 1.5: Proposed mechanisms of cationic lipoplex condensation and uptake. In brief, cationic liposomes are attracted by electrostatic interactions to the negative charges of DNA forming a lipoplex. Lipoplex binding to the cell surface followed by internalisation and then release of DNA from the lipoplex. DNA enters the nucleus and in the nucleus, RNA will be transcribed.

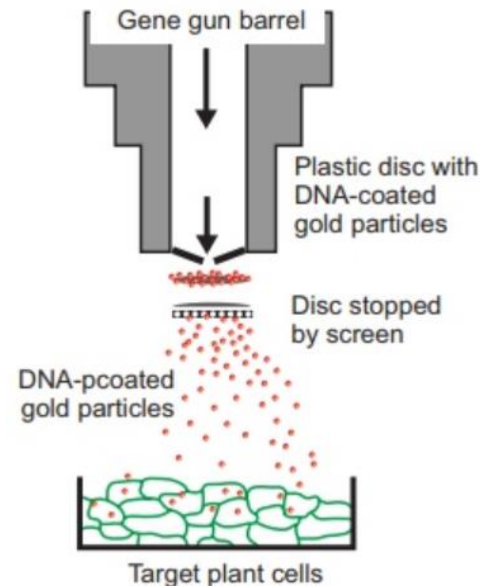
Moghaddam, B. (2013). Design and development of cationic liposomes as DNA vaccine adjuvants.

„Gene gun“ and microinjection

https://doi.org/10.1016/C2011-0-05817-9

- Mechanical introduction of DNA into the cell - mainly suitable for eukaryotes due to the size of the "vectors".
- Microinjection – introduction of DNA directly into the nucleus of a cell (eggs, embryonic stem cells...)
- "Gene gun" (bioballistic technique) - injecting nano-/microparticles coated with DNA into cells





Gene gun Helios^{TM} by BioRad is used to transfect cells in cultures and plant leaves

Selection of cells with a recombinant gene

1) Restriction analysis of plasmid DNA after mini-preparation

2) Inactivation by the insert

3) α-complementation

4) Hybridization of colonies

5) PCR test

6) Sequencing

Selection is primarily based on antibiotic resistance

The host cell is sensitive to the antibiotic

The vector carries resistance genes

The transformed cell is resistant to the antibiotic

Cloning foreign DNA using E. coli, cont.

Cloning site

Determine colonies with insert using selection on antibiotic plates

https://library.uams.edu/assets/COM/BioChem/MolecularTools/MolecularToolsSDL10.html

Inactivation by the insert – TET^S selection

Bsu15I 23
HindIII 29
EcoRI, XapI 4359
Eco32I 185
AatII 4284
NheI 229
SspI 4168
BamHI 375
SgrAI 409
PaeI 562
ScaI 3844
XagI 622
PvuI 3733
SalI 651
PstI 3607
BoxI 712
VspI 3537
Eco31I 3433
Eco52I 939
Eam1105I 3361
Bsp68I 972
BspMI 1063
pBR322
4361 bp
bla (Ap^R)
tet (Tc^R)
rep (pMB1)
rop
4153
86
3293
3133
1276
2519
2106
1915
Mva1269I 1353
Eco130I 1369
CaiI 2884
Eco88I 1425
MlsI 1444
Bpu10I 1580
BsgI 1650
AflIII, BspLU11I 2473
Kpn2I 1664
SapI 2350
BseJI 1668
PvuII 2064
NdeI 2295
Esp3I 2122
Bst1107I 2244
PsyI 2217
BsaAI 2225

colonies of transformed cells

TET

AMP

picking colonies by a sterile toothpick

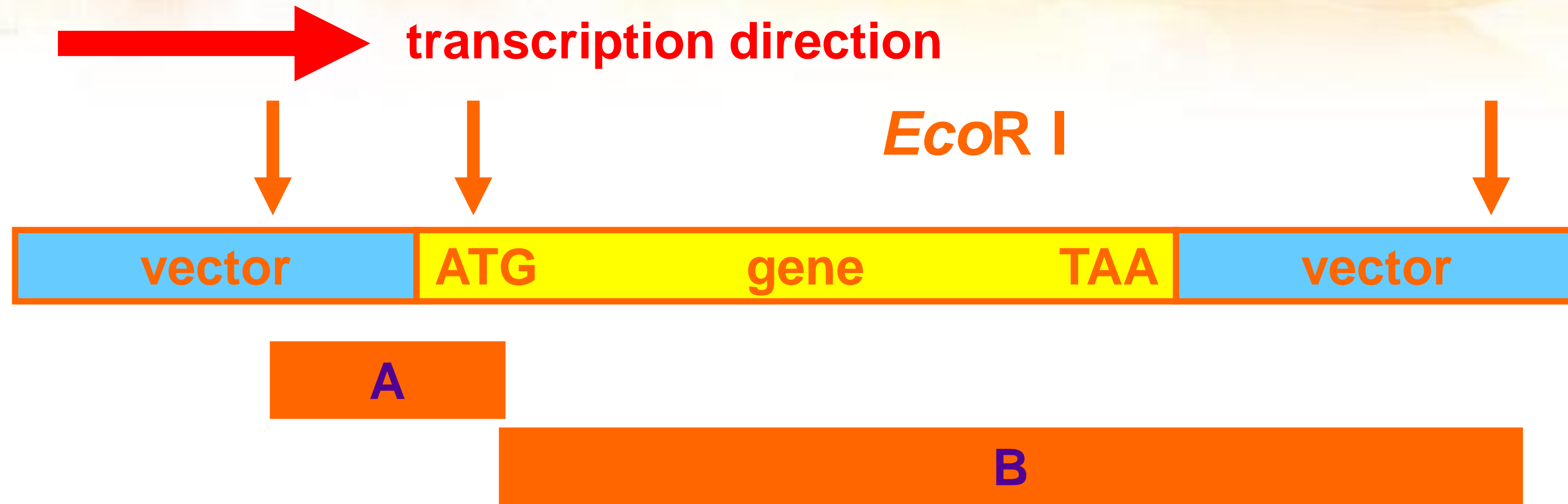
TET

AMP

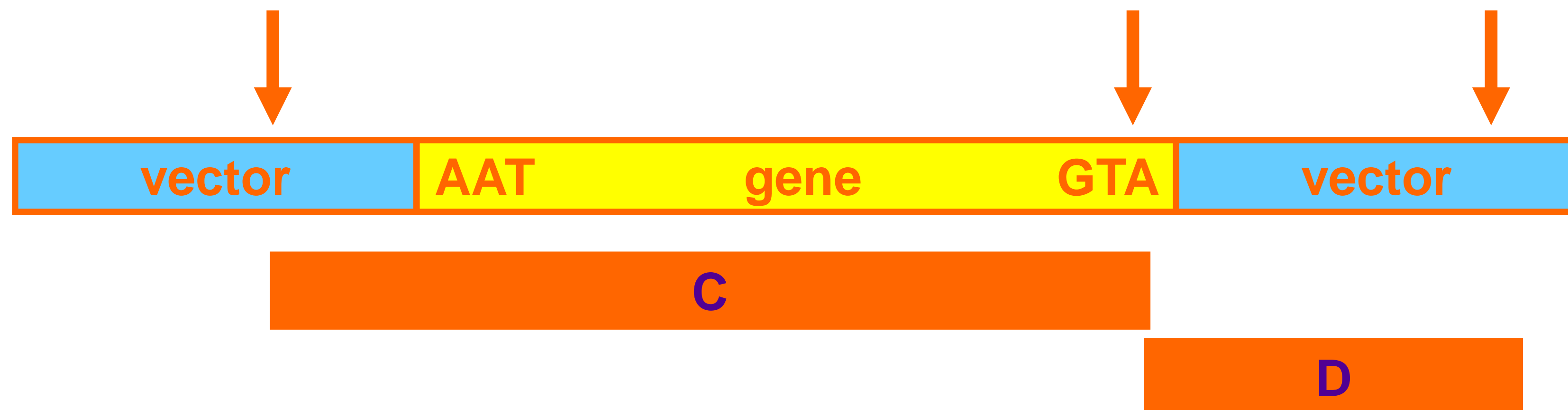
overnight incubation at 37°C

AMP^R, TET^S colonies

Plasmid restriction digestion

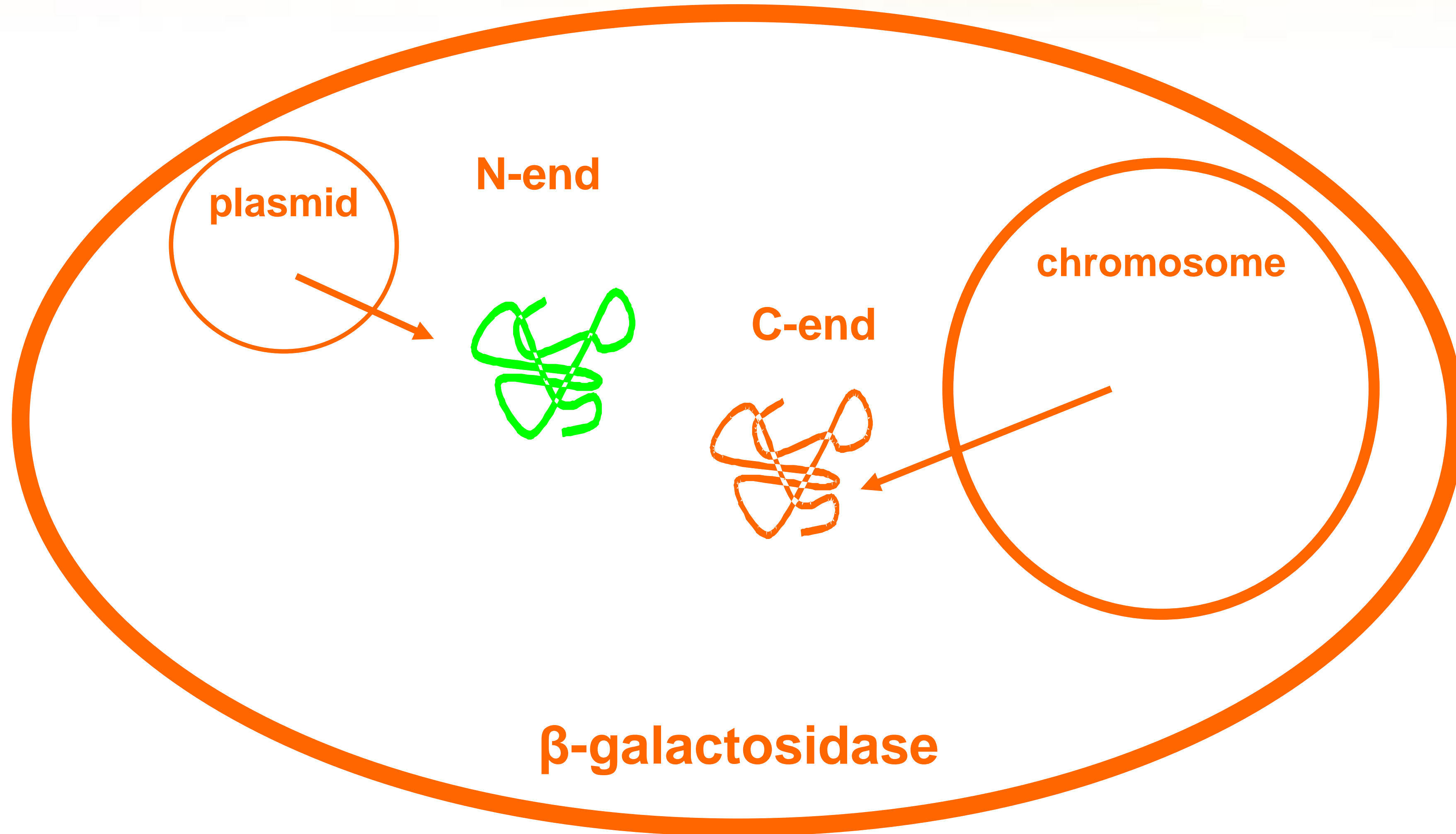


Right orientation - Insert with ORF



Wrong orientation - Insert without ORF

β-galactosidase function in α-complementation



β-galactosidase function

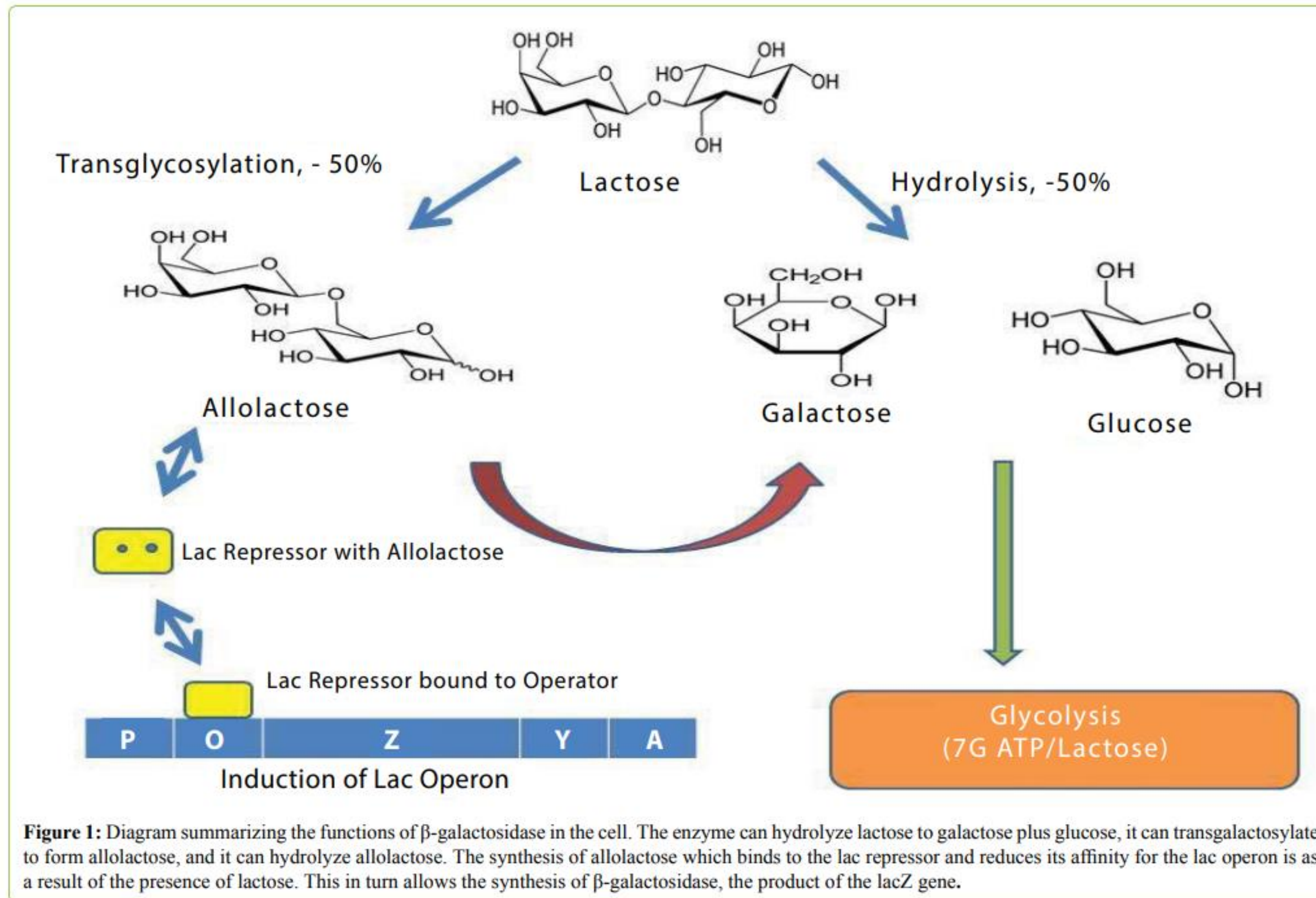
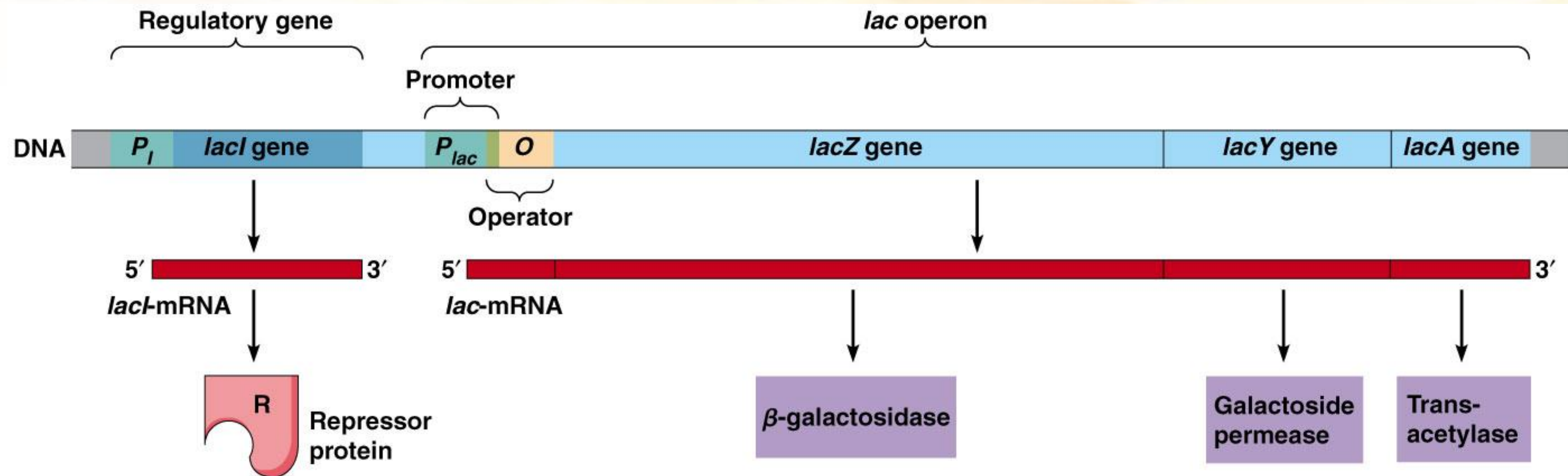


Figure 1: Diagram summarizing the functions of β-galactosidase in the cell. The enzyme can hydrolyze lactose to galactose plus glucose, it can transgalactosylate to form allolactose, and it can hydrolyze allolactose. The synthesis of allolactose which binds to the lac repressor and reduces its affinity for the lac operon is as a result of the presence of lactose. This in turn allows the synthesis of β-galactosidase, the product of the lacZ gene.

J Multidis Res Rev 2018

lac operon of Escherichia coli

enzymes for lactose metabolism



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I: code represor

Z: code enzyme beta-galactosidase (lac → glu + gal)

Y: code enzyme permease

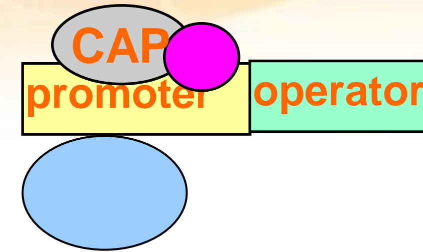
A: code enzyme thiogalactoidtransacetylase

inductor - laktose

Regulation of *lac* operon

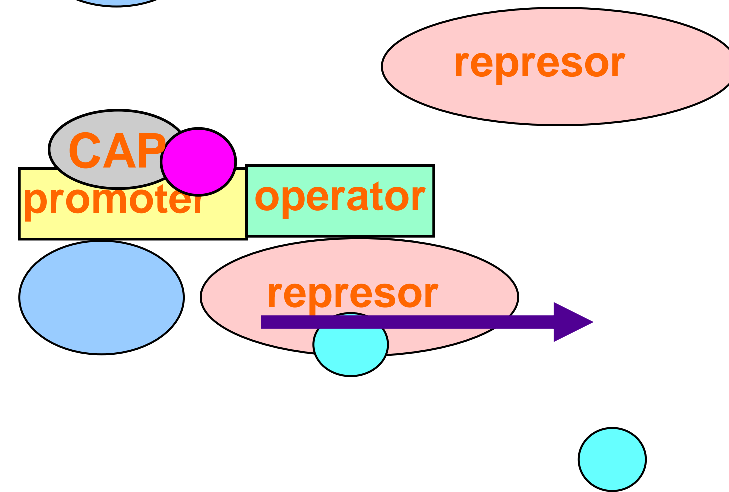
a) glc is NOT present, lac is NOT present

→ binding of the repressor to the operator



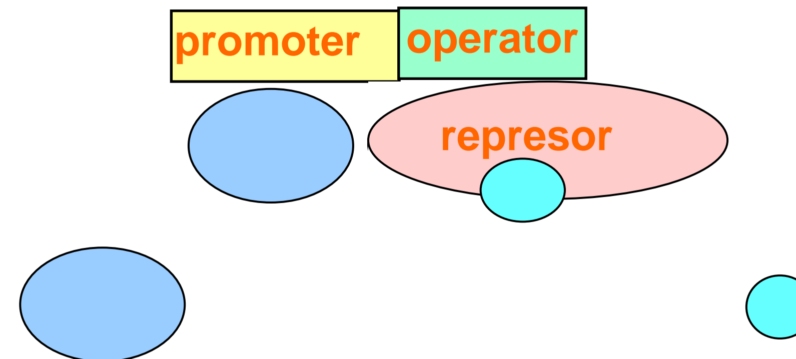
b) glc is NOT present, lac IS present

→ enzyme induction



c) glc IS present, lac IS present

→ catabolic represion



Regulation of *lac* operon

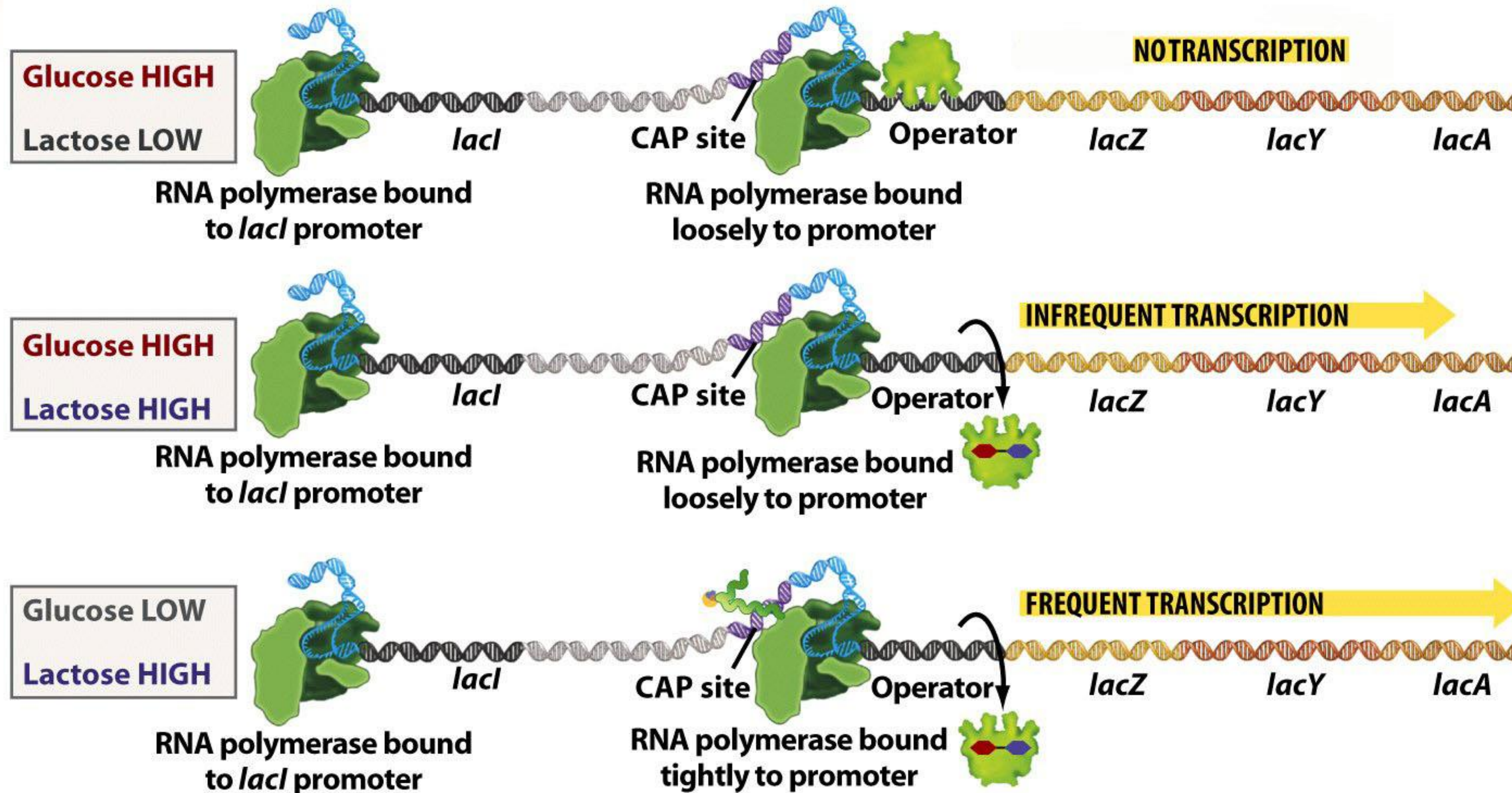


Figure 17-10 Biological Science, 2/e

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Regulation of β-galactosidase expression

polylinker (MCS)

promoter	operator	lacZ	another genes

repressor

IPTG

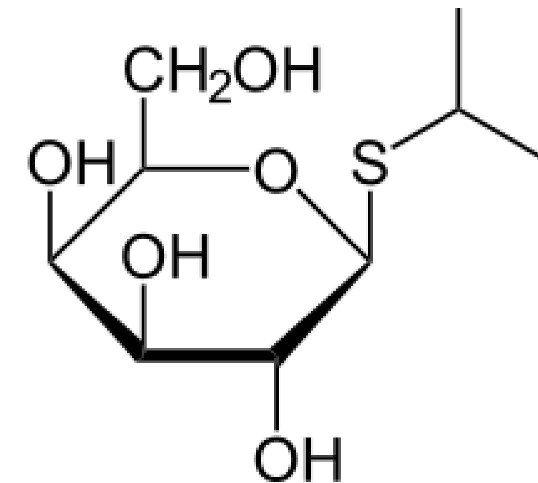
β-galactosidase

IPTG

X-gal

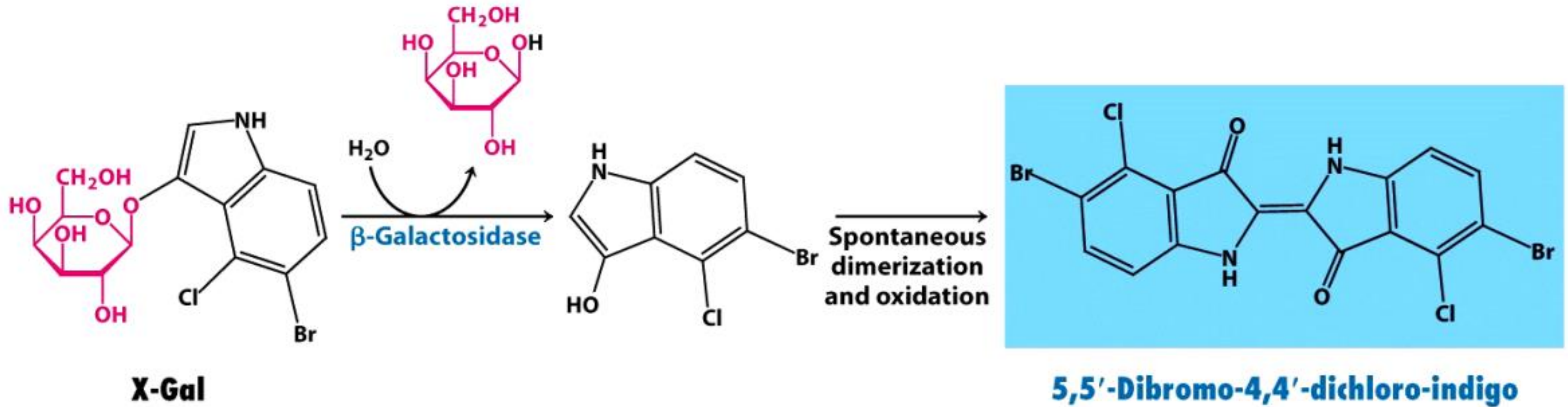
IPTG

Isopropyl β-D-1-thiogalactopyranoside



The medium must not contain glucose!

Formation of blue coloration

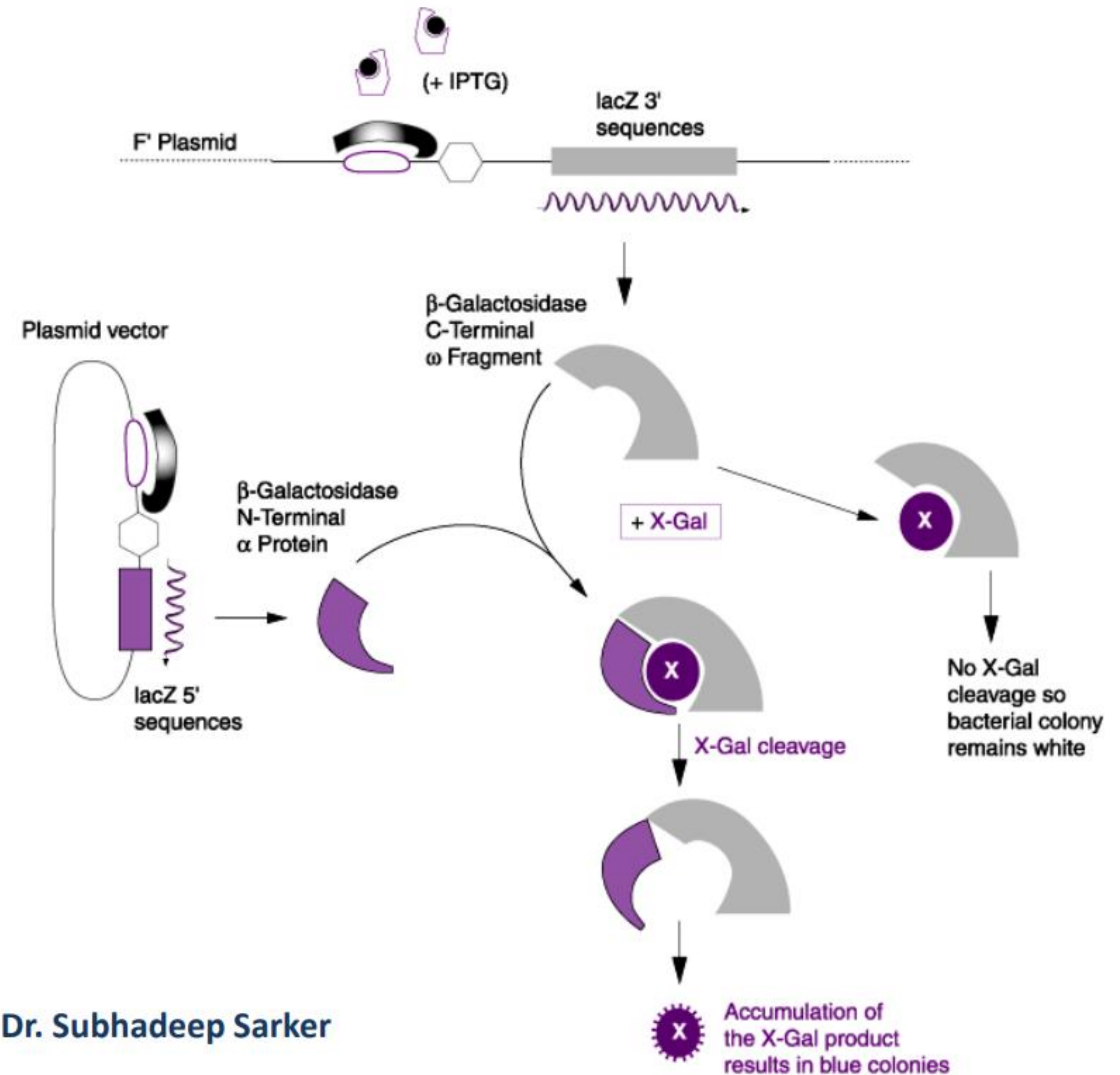


Dr. Subhadeep Sarker

http://seramporecollege.org/a-s-c/wp-content/uploads/2020/03/Blue_White_Screening_ss.pdf

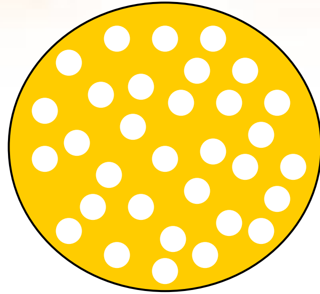
Selection based on α-complementation

- Part of the gene for β-galactosidase is present on the chromosome, the other on the vector
- A cell must express both subunits simultaneously to make a functional enzyme

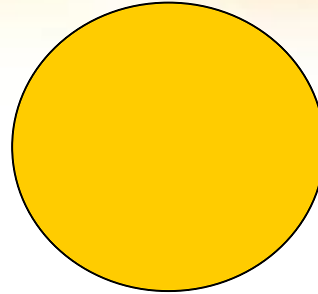


http://seramporecollege.org/a-s-c/wp-content/uploads/2020/03/Blue_White_Screening_ss.pdf

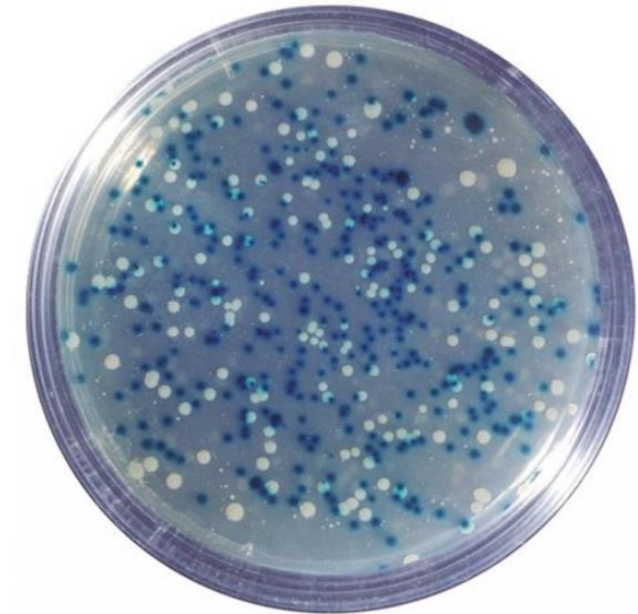
Selection based on α-complementation

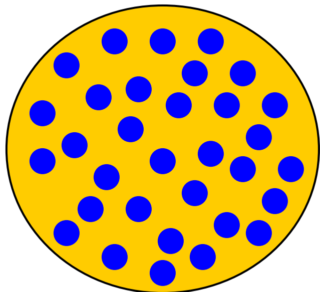


Cell w/o plasmid
LB medium

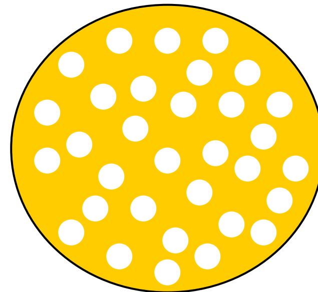


Cell w/o plasmid
LB medium, ampicilin, X-gal, IPTG

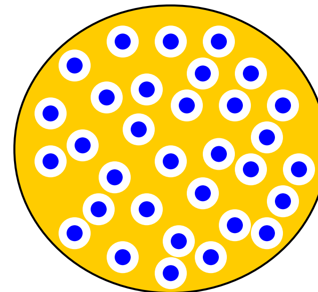




cells with plasmid
w/o insert



cells with plasmid
with long insert

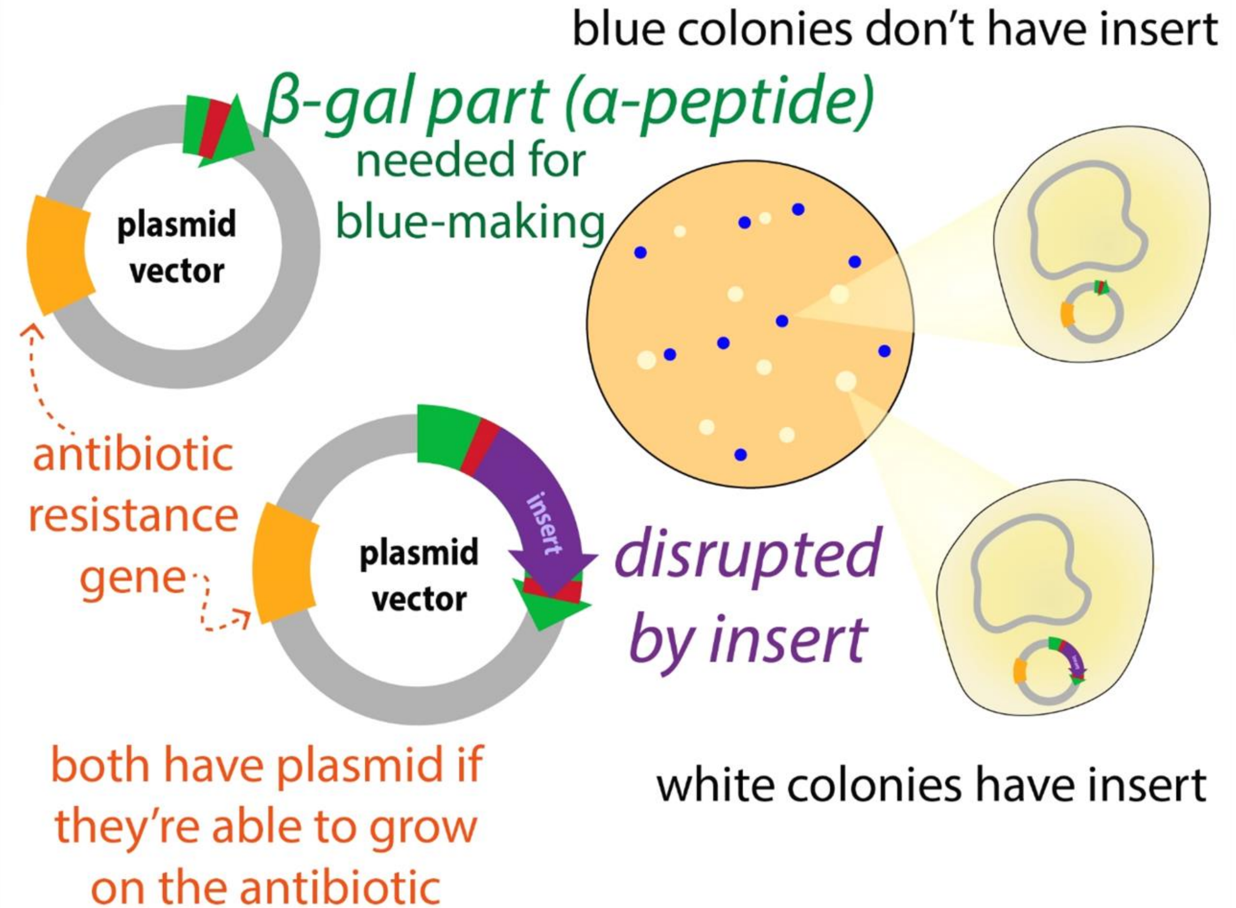


cells with plasmid
with short insert

LB medium, ampicilin, X-gal, IPTG

blue-white screening

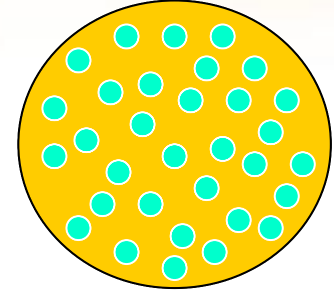
IPTG
derepression
ω-fragment
host bacterial DNA
lacZΔm15
dimer
empty vector
antibiotic resistance gene
α-peptide gene
vector with insert
insert
α-peptide
α-complementation
functional β-gal
interrupted α-peptide gene
H_2O
X-gal
hydrolysis
galactose
5-bromo-4-chloro-3-hydroxyindole
5,5'-dibromo-4,4'-dichloro-indigo
white colonies have your insert
blue ones don't



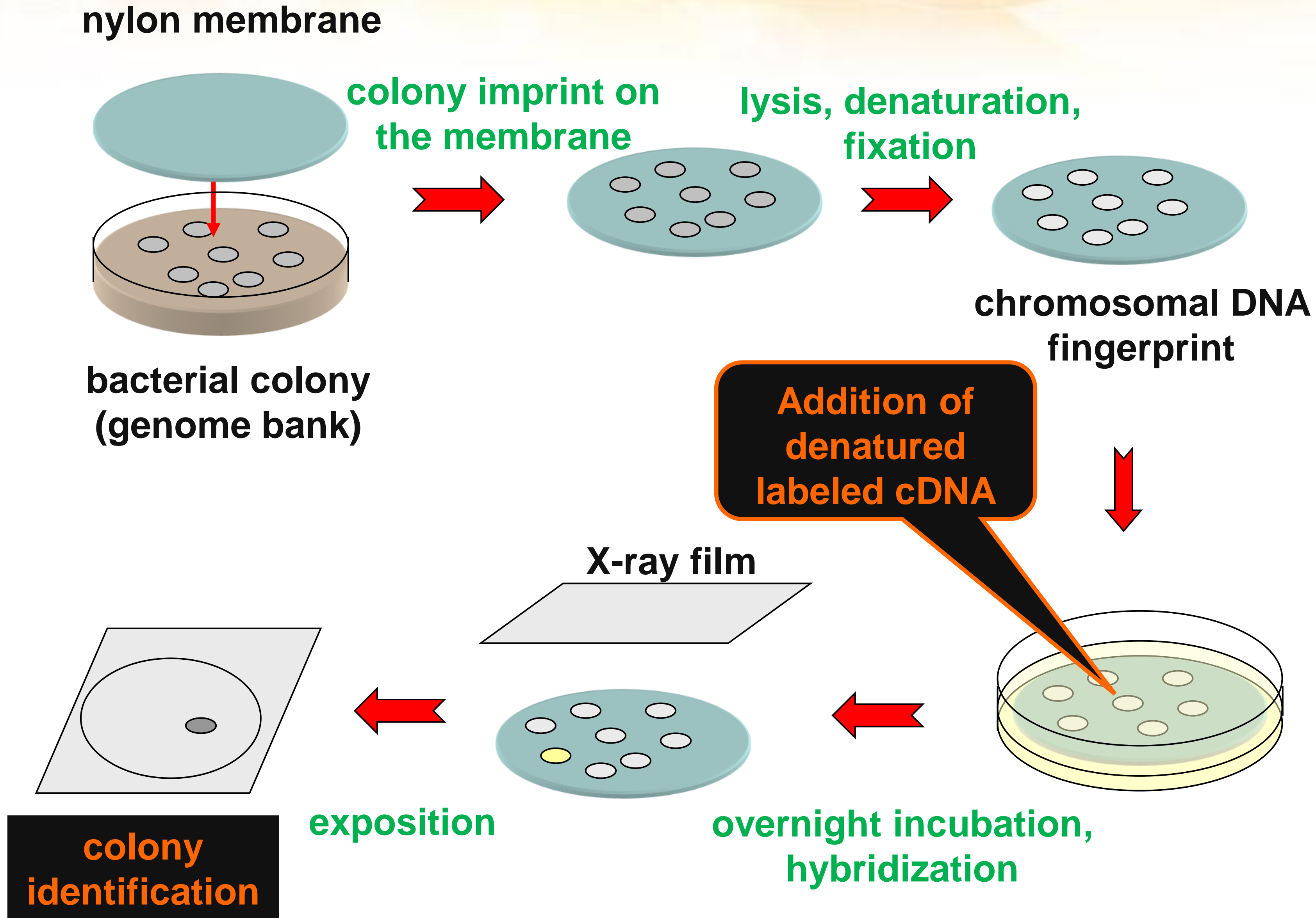
https://thebumblingbiochemist.com/365-days-of-science/blue-white-screening-2/

Cloning into bacteriophage λ

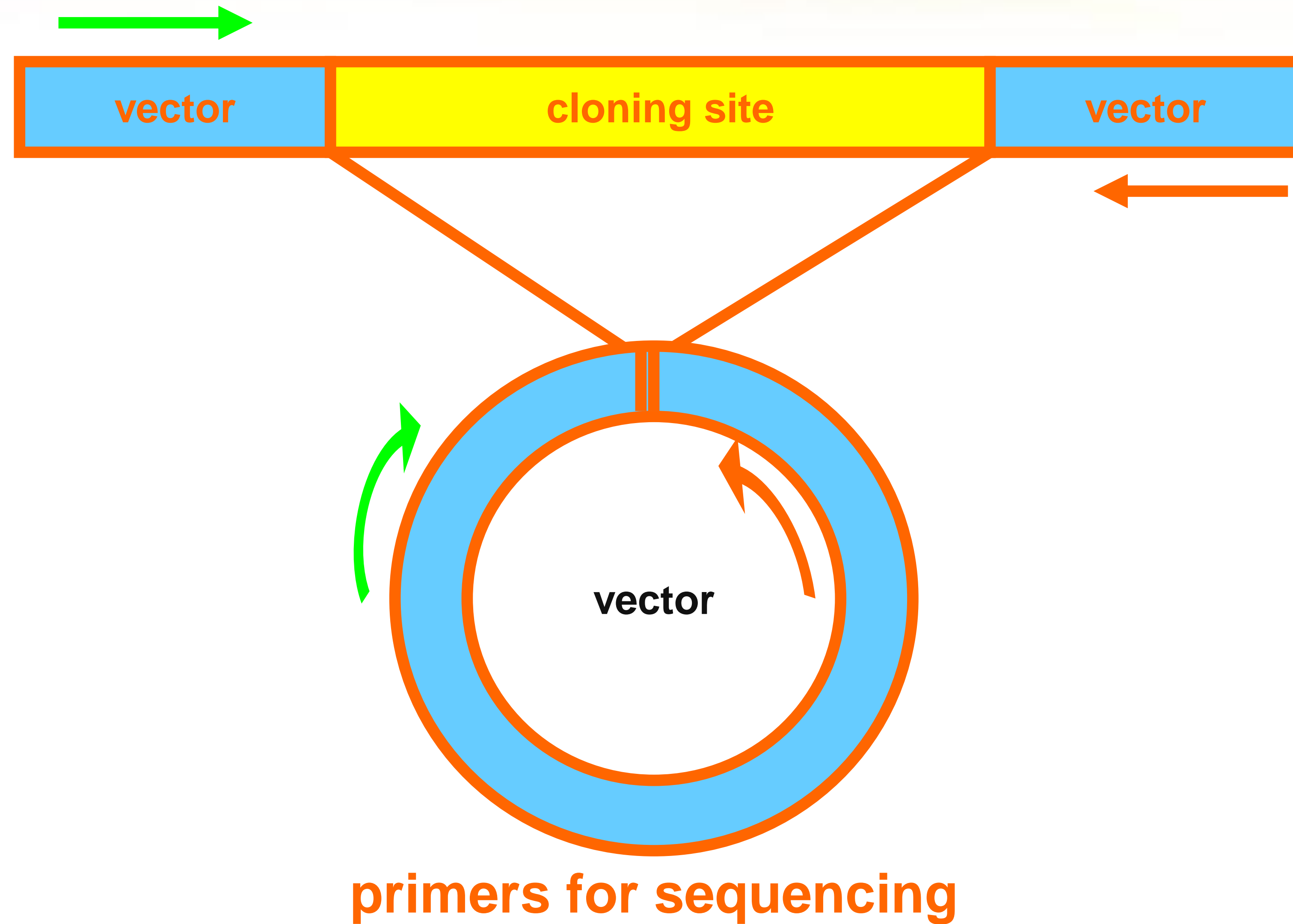
- grows on bacteria in the form of plaques
- it only needs about 2/3 of the genome to infect
- up to <u>20 kbp</u> of DNA can be cloned
- 78-105% length DNA can be packaged efficiently



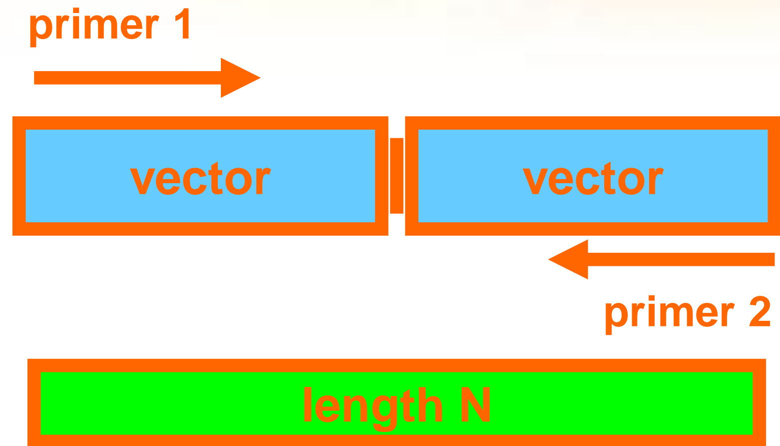
Hybridization of colonies

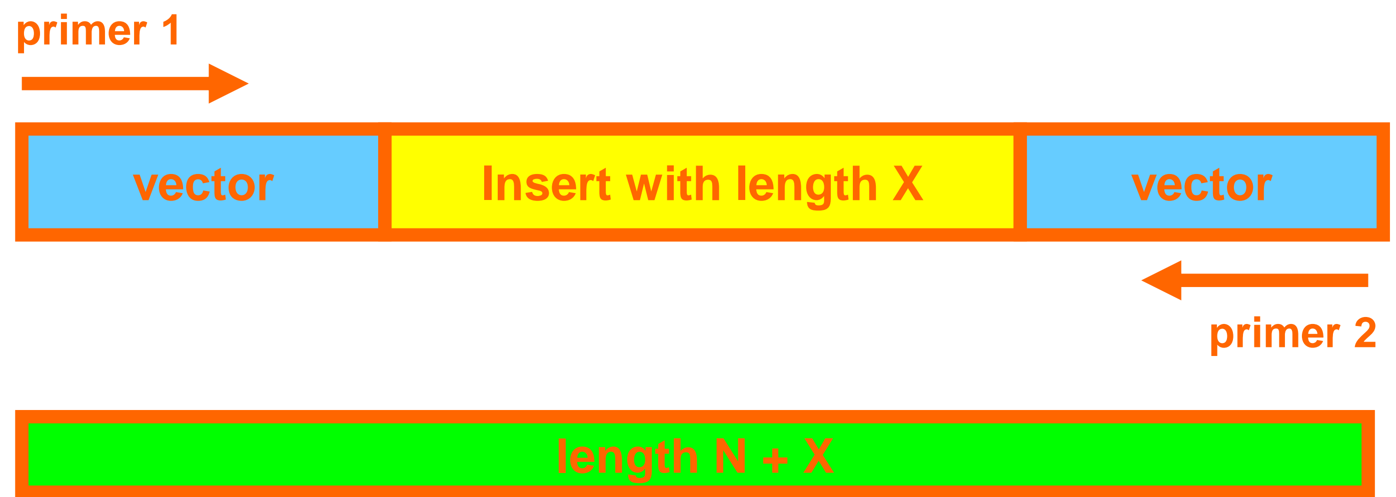


Testing of recombinant plasmids by the PCR method

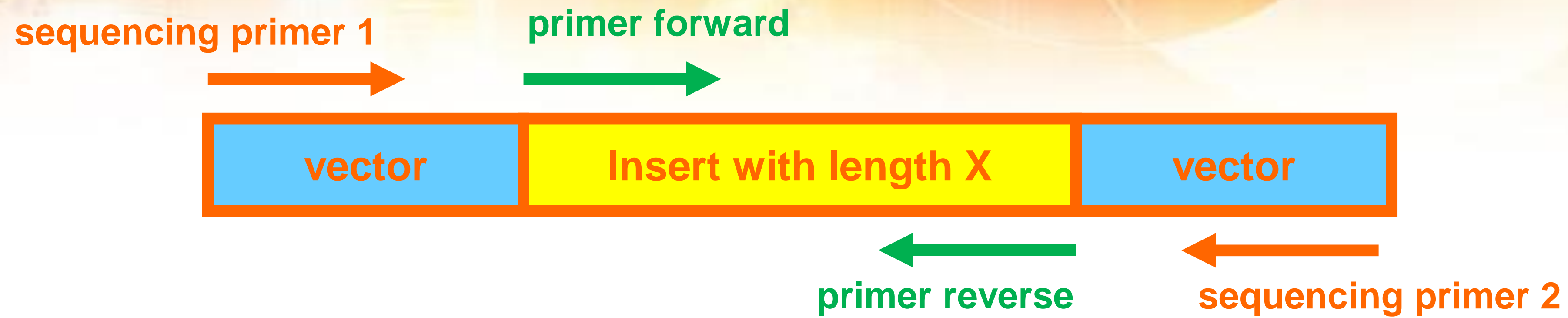


Confirmation of the presence of the insert using primers for sequencing



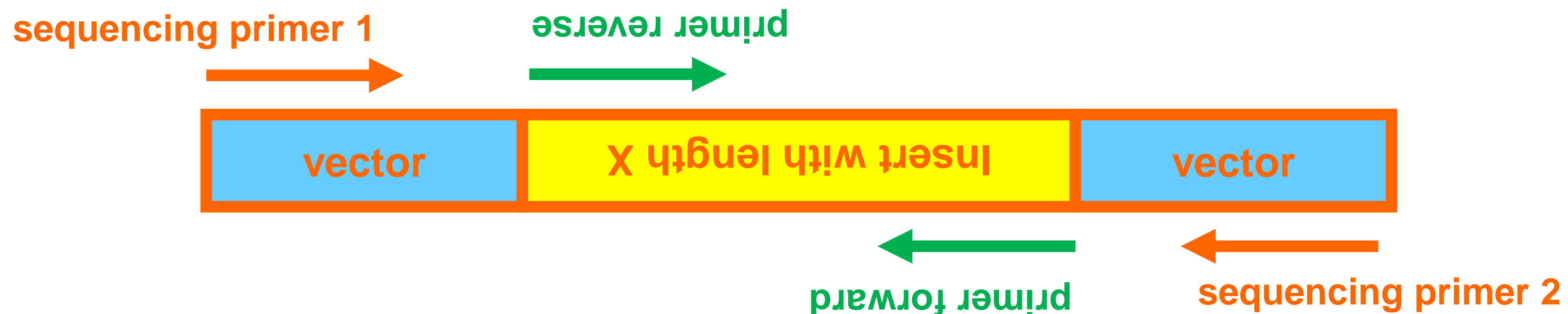


Determining the orientation of the insert in the vector



Amplicons are created by combining primers

- sequencing primer 1 + sequencing primer 2 (amplicon length = N + X)
- forward primer + reverse primer (amplicon length = X)
- sequencing primer 1 + reverse primer
- sequencing primer 2 + forward primer



Sequencing

- decisive method
- each insert needs to be sequenced