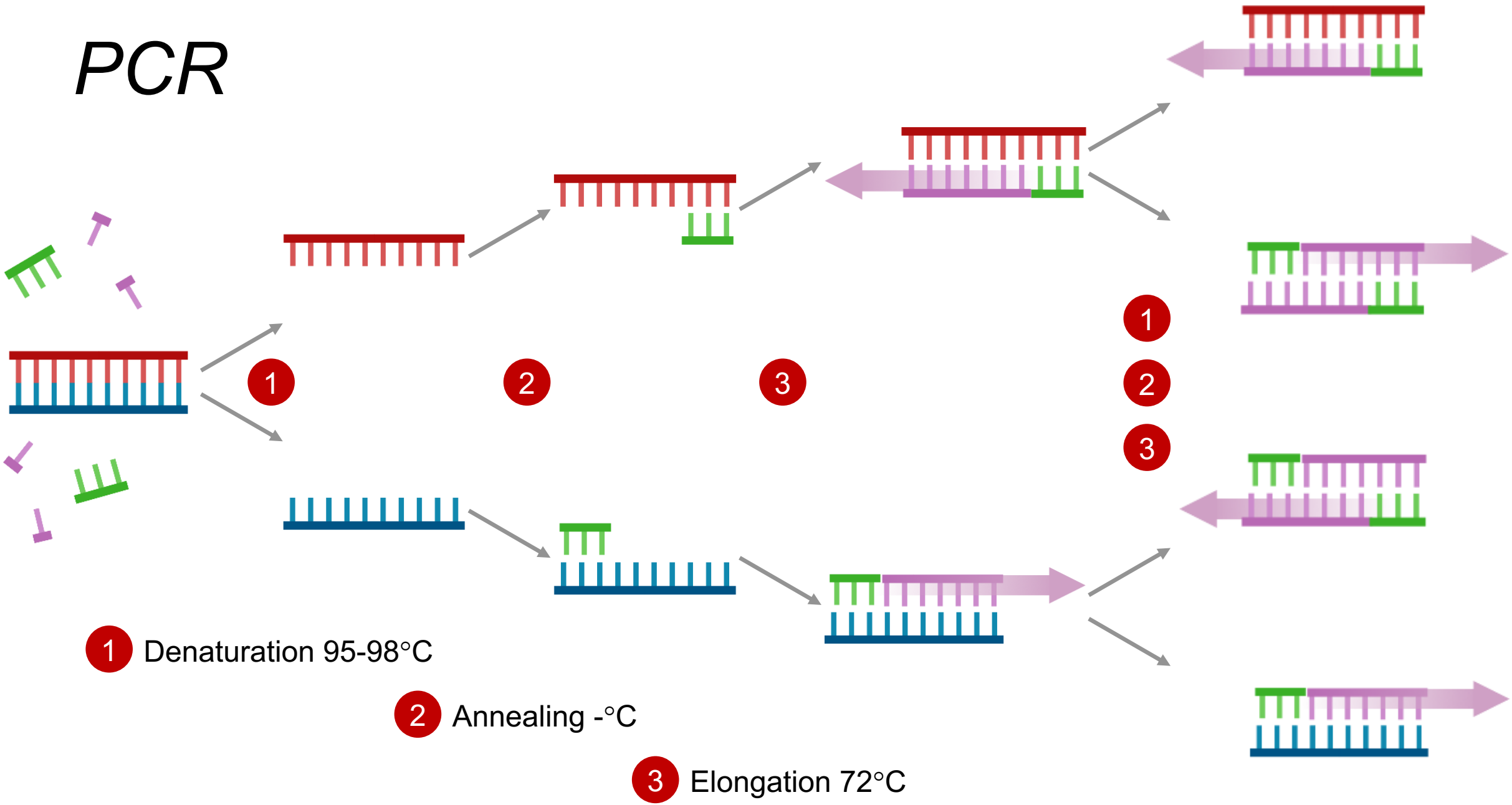
PCR



1 Denaturation 95-98°C

2 Annealing -°C

3 Elongation 72°C

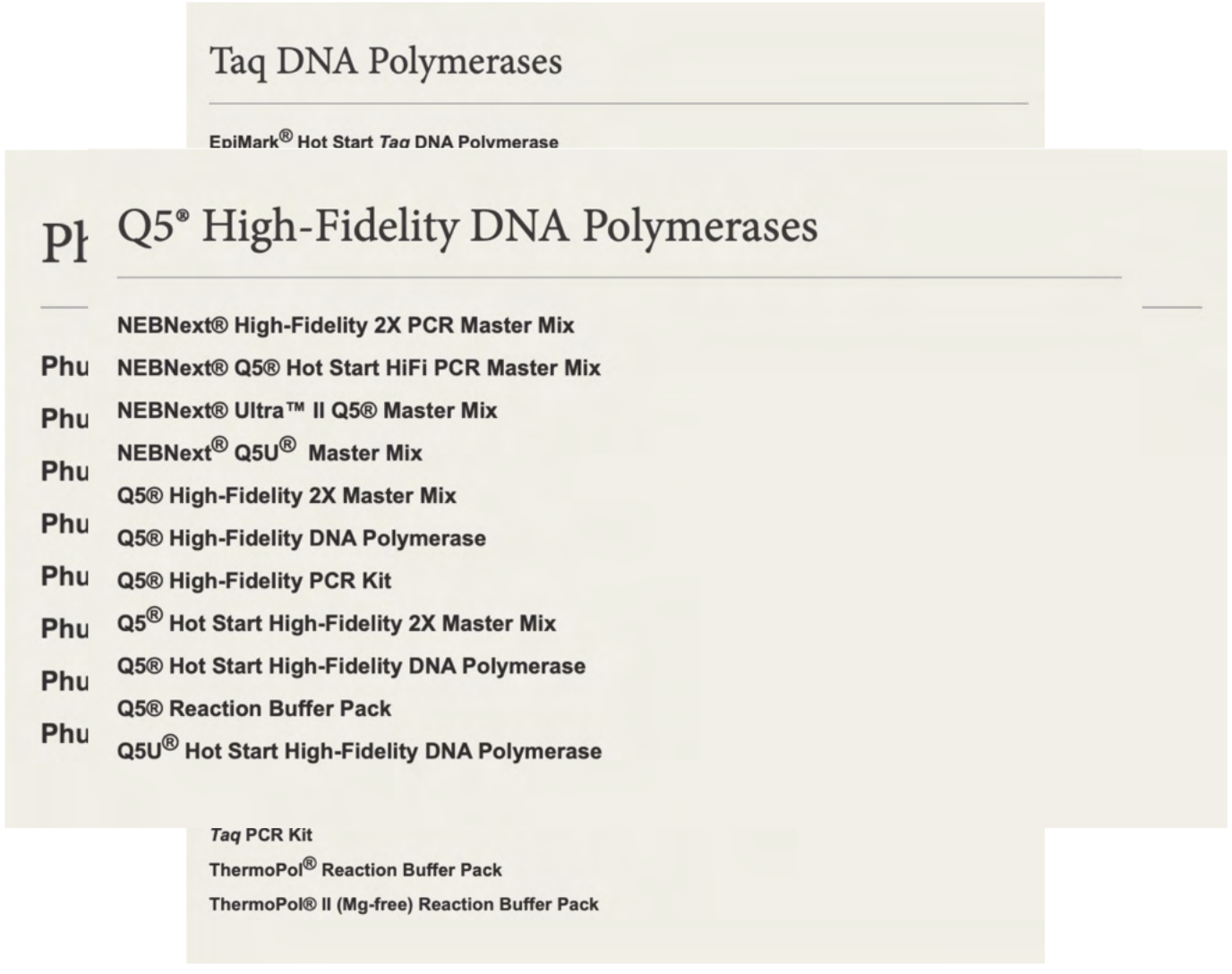
Choosing the polymerase

1. Thermal stability
2. Extension rate
3. Fidelity
4. Processivity

Choosing the polymerase

- Standard thermostable DNA polymerases
- Hot-start (HS) polymerases
- High-fidelity polymerases (Hi-Fi)
- Polymerases for amplification of long amplicons

Commercial polymerases



Taq DNA Polymerases

EpiMark® Hot Start *Taq* DNA Polymerase

Q5® High-Fidelity DNA Polymerases

NEBNext® High-Fidelity 2X PCR Master Mix

NEBNext® Q5® Hot Start HiFi PCR Master Mix

NEBNext® Ultra™ II Q5® Master Mix

NEBNext® Q5U® Master Mix

Q5® High-Fidelity 2X Master Mix

Q5® High-Fidelity DNA Polymerase

Q5® High-Fidelity PCR Kit

Q5® Hot Start High-Fidelity 2X Master Mix

Q5® Hot Start High-Fidelity DNA Polymerase

Q5® Reaction Buffer Pack

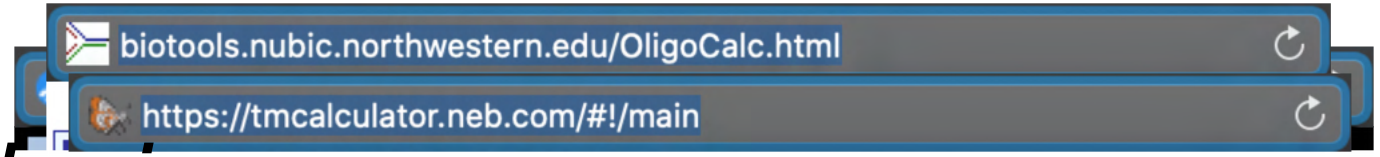
Q5U® Hot Start High-Fidelity DNA Polymerase

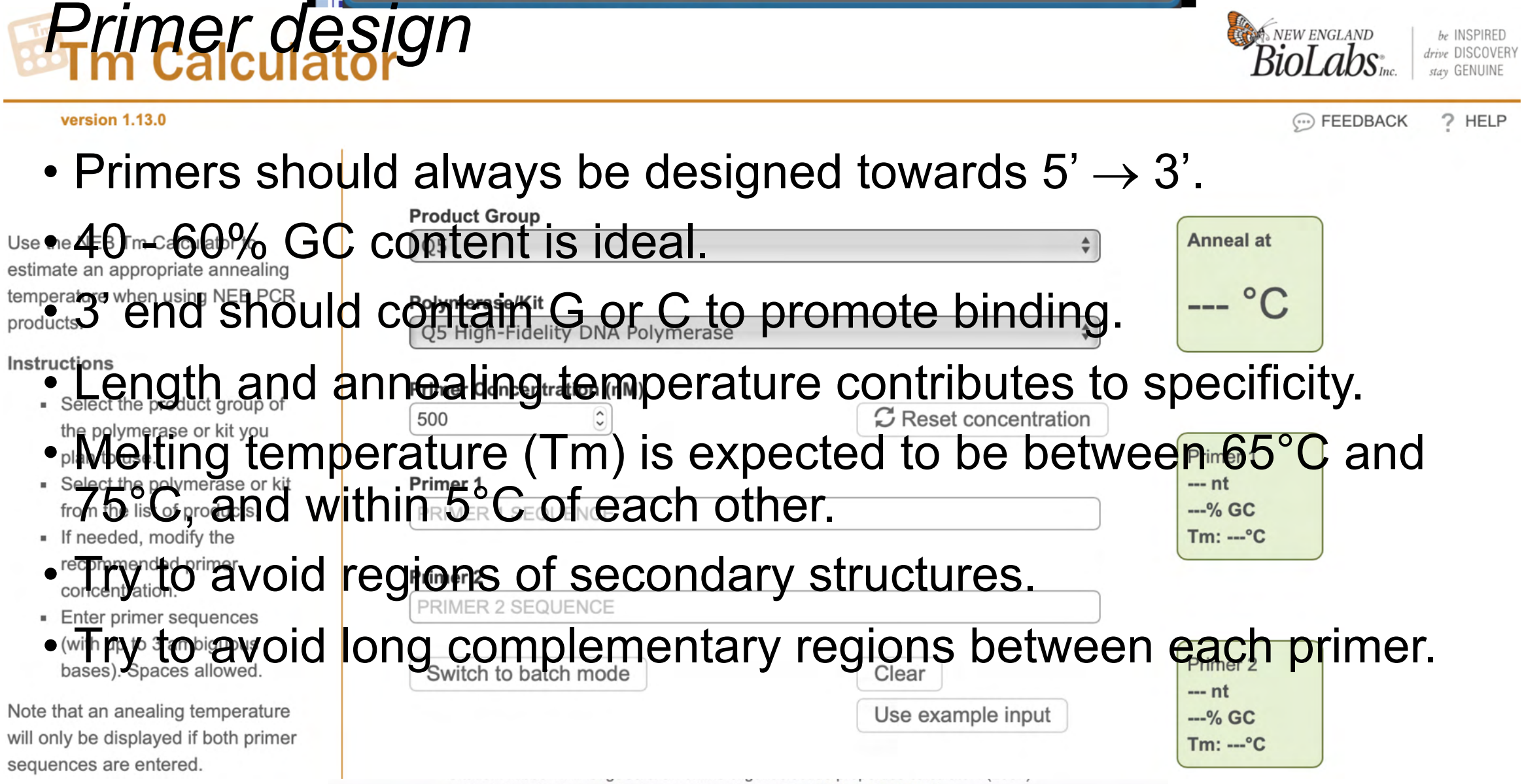
Taq PCR Kit

ThermoPol® Reaction Buffer Pack

ThermoPol® II (Mg-free) Reaction Buffer Pack

Primer design





- Primers should always be designed towards 5’ → 3’.
- 40 – 60% GC content is ideal.
- 3’ end should contain G or C to promote binding.
- Length and annealing temperature contributes to specificity.
- Melting temperature (Tm) is expected to be between 65°C and 75°C, and within 5°C of each other.
- Try to avoid regions of secondary structures.
- Try to avoid long complementary regions between each primer.

Primer3 & Primer3Plus

Primer3Plus

pick primers from a DNA sequence

Primer3Manager | Help | About | Source Code

Task: Detection

Select primer pairs to detect the given template sequence. Optionally targets and included/excluded regions can be specified.

Pick Primers | Reset Form

Main | General Settings | Advanced Settings | Internal Oligo | Penalty Weights | Sequence Quality

Sequence Id:

Paste source sequence below

Or upload sequence file: Choose File no file selected Upload File

Mark selected region: < > [ ] { } Clear Save Sequence

Excluded Regions: < >

Targets: [ ]

Included Region: { }

Pick left primer or use left primer below.	Pick hybridization probe (internal oligo) or use oligo below.	Pick right primer or use right primer below (5'->3' on opposite strand).

U.S. National Library of Medicine
National Center for Biotechnology Information

ocevheroglu

BLAST® » blastn suite

Home Recent Results Saved Strategies Help

Align Sequences Nucleotide BLAST

blastn | blastp | blastx | tblastn | tblastx

BLASTN programs search nucleotide subjects using a nucleotide query. more...

Reset page | Bookmark

Enter Query Sequence

Enter accession number(s), gi(s), or FASTA sequence(s) Clear

Query subrange

From

To

New columns added to the Description Table
Click 'Select Columns' or 'Manage Columns'.

Or, upload file Choose File no file selected

Job Title

Enter a descriptive title for your BLAST search

Align two or more sequences

Enter Subject Sequence

Enter accession number(s), gi(s), or FASTA sequence(s) Clear

Subject subrange

From

To

Or, upload file Choose File no file selected

Program Selection

Optimize for

- Highly similar sequences (megablast)
- More dissimilar sequences (discontiguous megablast)
- Somewhat similar sequences (blastn)

Choose a BLAST algorithm

BLAST

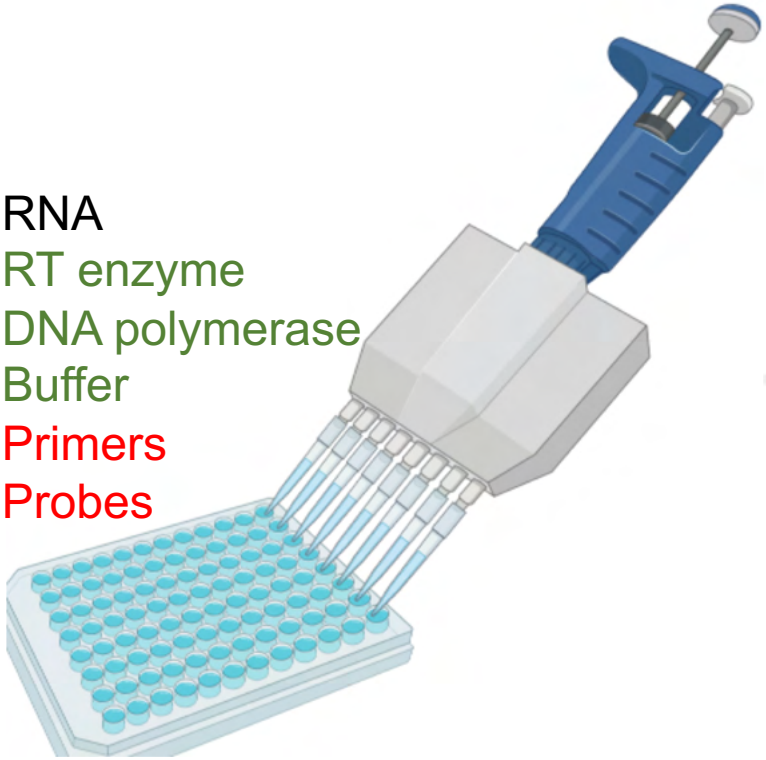
Search nucleotide sequence using Megablast (Optimize for highly similar sequences)

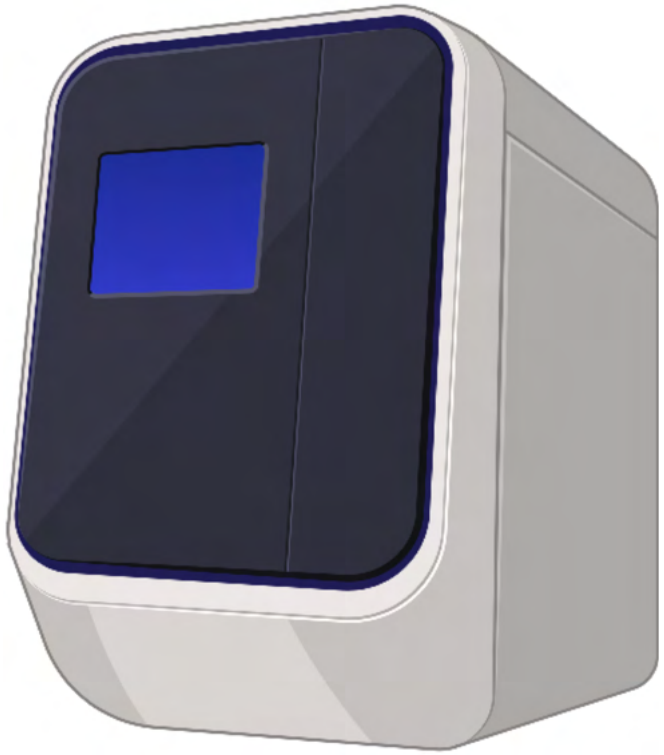
Show results in a new window

+ Algorithm parameters

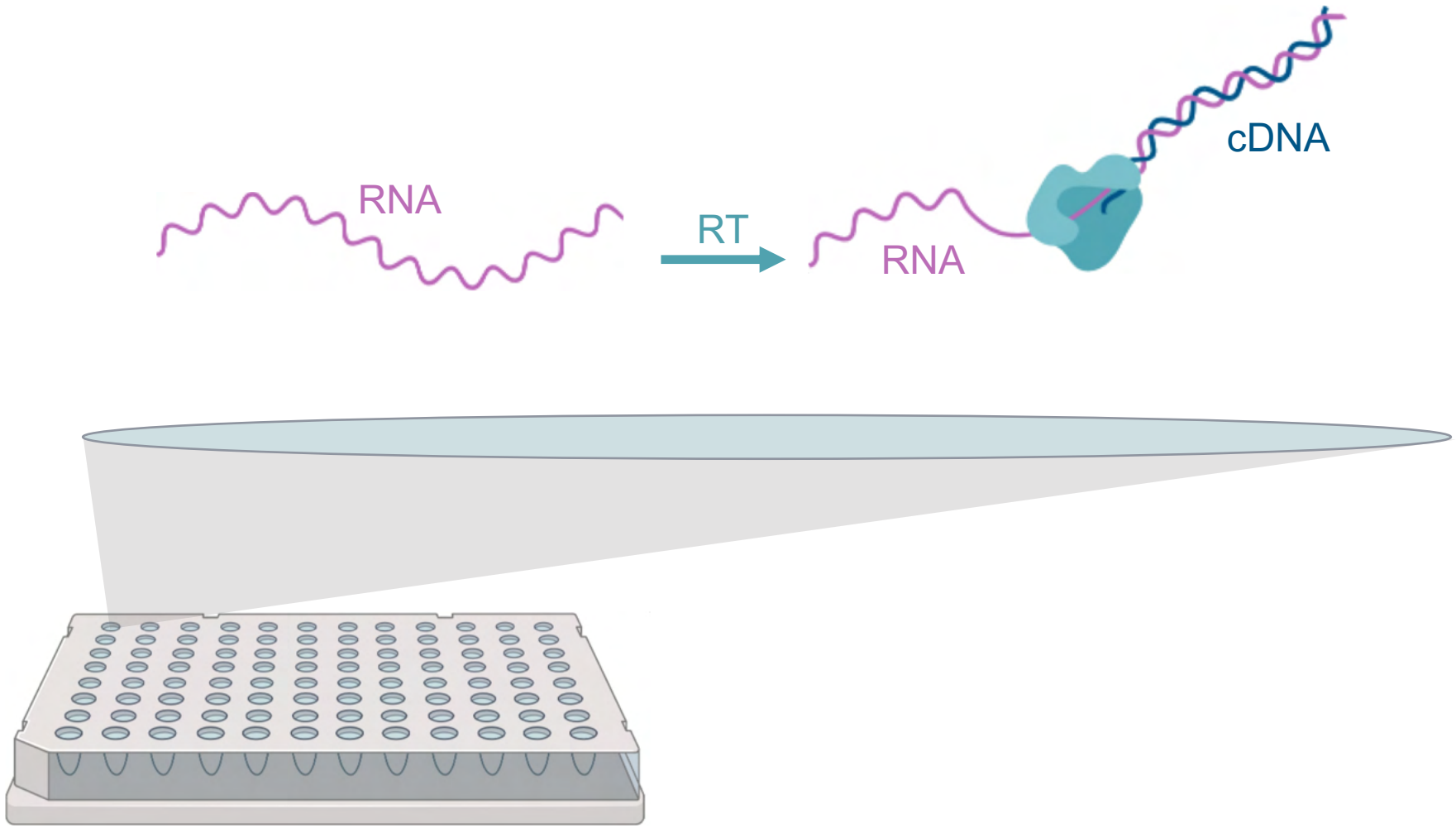
RT-PCR

RNA
RT enzyme
DNA polymerase
Buffer
Primers
Probes

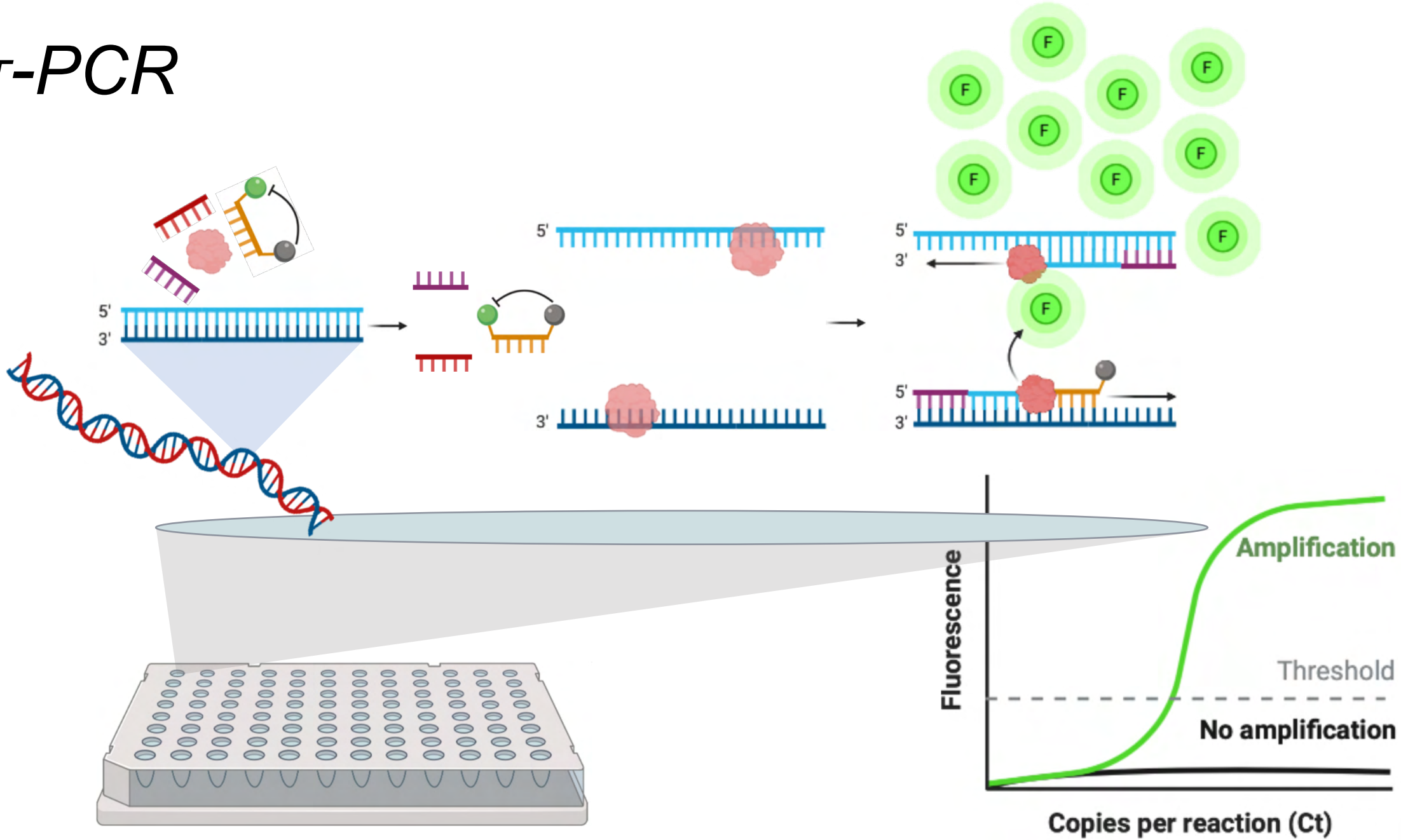




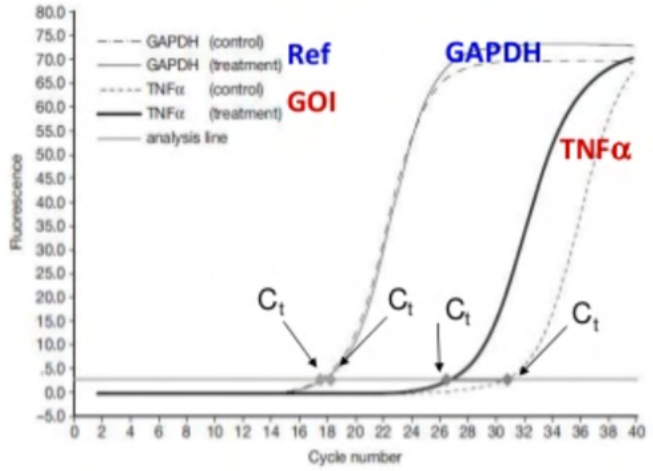
RT-PCR



RT-PCR


F
5'
3'
Fluorescence
Amplification
Threshold
No amplification
Copies per reaction (Ct)

RT-qPCR



$\Delta\Delta Ct = \Delta Ct\ (TNF\alpha_{treat}\text{-}GAPDH_{treat}) - \Delta ct\ (TNF\alpha_{control}\text{-}GAPDH_{control})$

The fold change = $2^{(-\Delta\Delta Ct)}$

single data (n = 1) e.g. array results:

$$\text{relative expression} = \frac{E_{target}^{\Delta CP_{target}\ (control\ -\ sample)}}{E_{ref}^{\Delta CP_{ref}\ (control\ -\ sample)}}$$

Pfaffl, Nucleic Acids Research 2001

multiple data (1 < n < 16) e.g. experimental groups:

$$\text{relative expression} = \frac{E_{target}^{\Delta CP_{target}\ (MEAN\ control\ -\ MEAN\ sample)}}{E_{ref}^{\Delta CP_{ref}\ (MEAN\ control\ -\ MEAN\ sample)}}$$

Pfaffl et al., Nucleic Acids Research 2002

MIQE guidelines for RT-qPCR

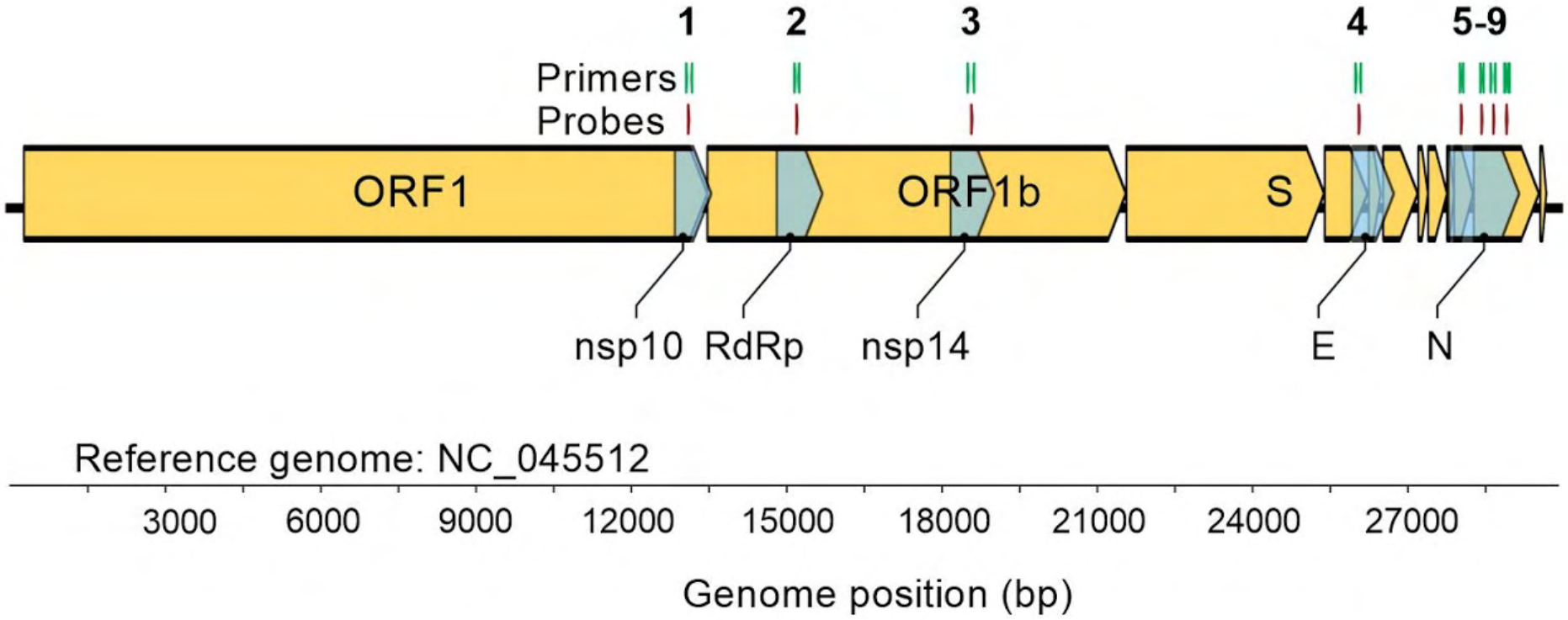
Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

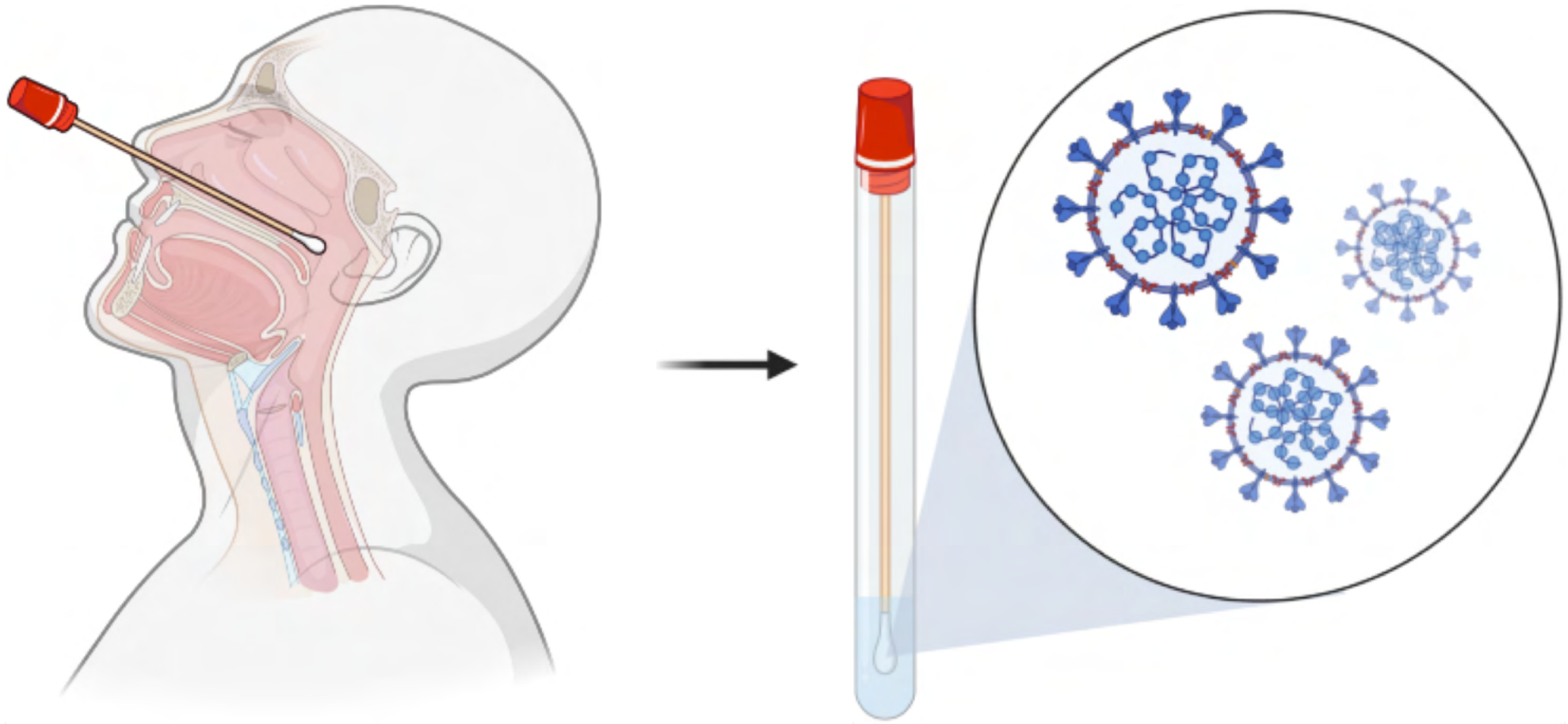
Applications: famous COVID19 tests



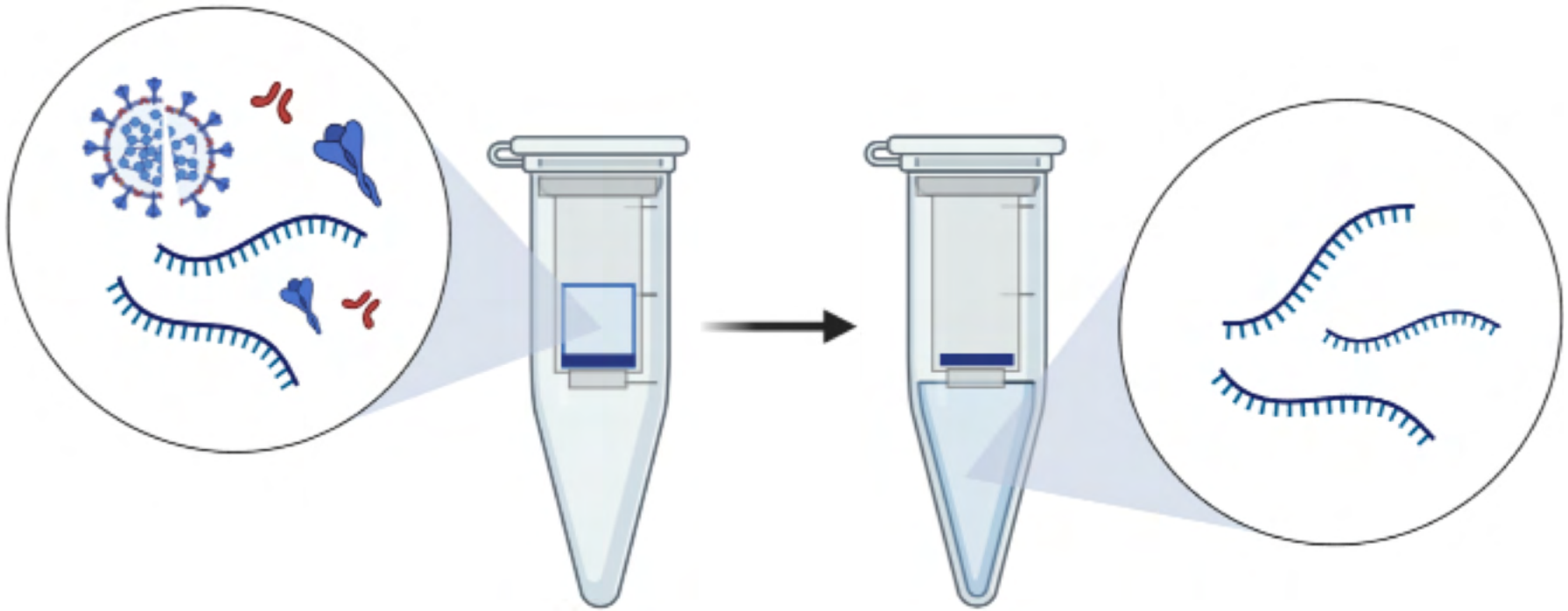
Design of Primers

- Internal control primers:
  - GAPDH, RNAseP, ACTB, eEF-1 etc.
- Viral DNA primers: N region
  - N gene (CDC Panel, Hong Kong Panel, Japan National Institute of Infectious Diseases Panel)
  - E gene (Berlin-Charité Panel, Institut Pasteur Panel)
  - ORFab primers (Berlin-Charité Panel, China CDC panel)
  - RdRp (Berlin-Charité Panel, Institut Pasteur Panel)
  - S gene

Nasopharyngeal & Oropharyngeal swabs

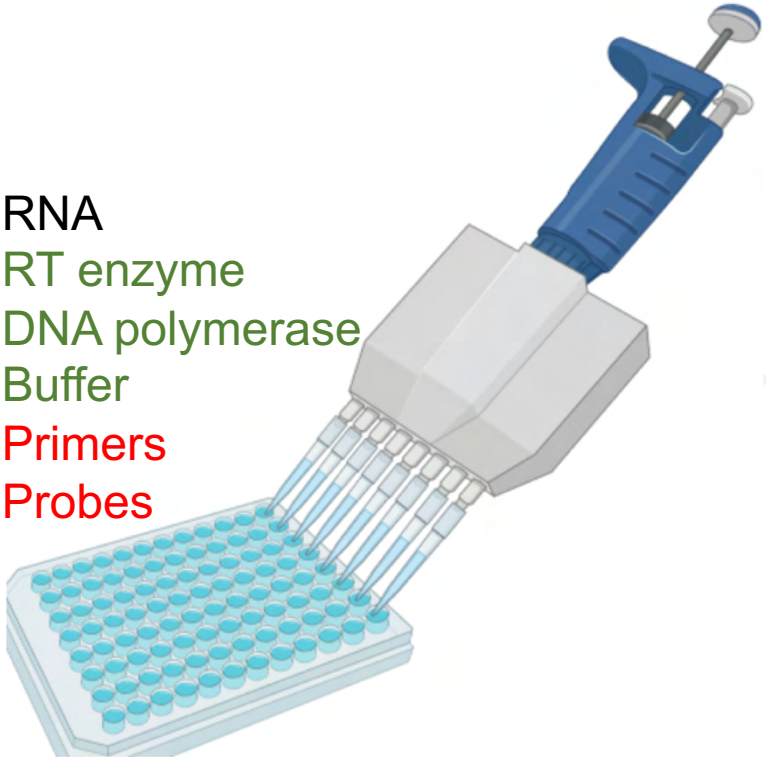


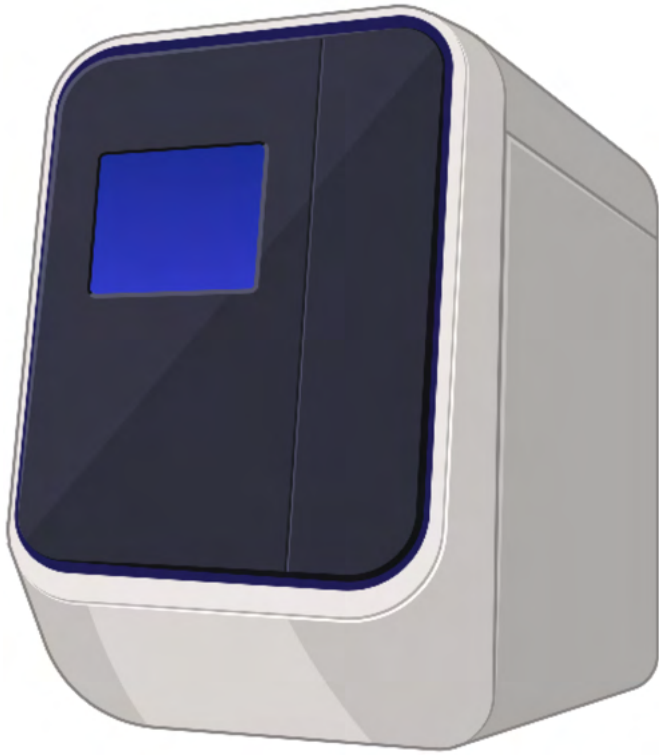
RNA isolation



RT-PCR

RNA
RT enzyme
DNA polymerase
Buffer
Primers
Probes

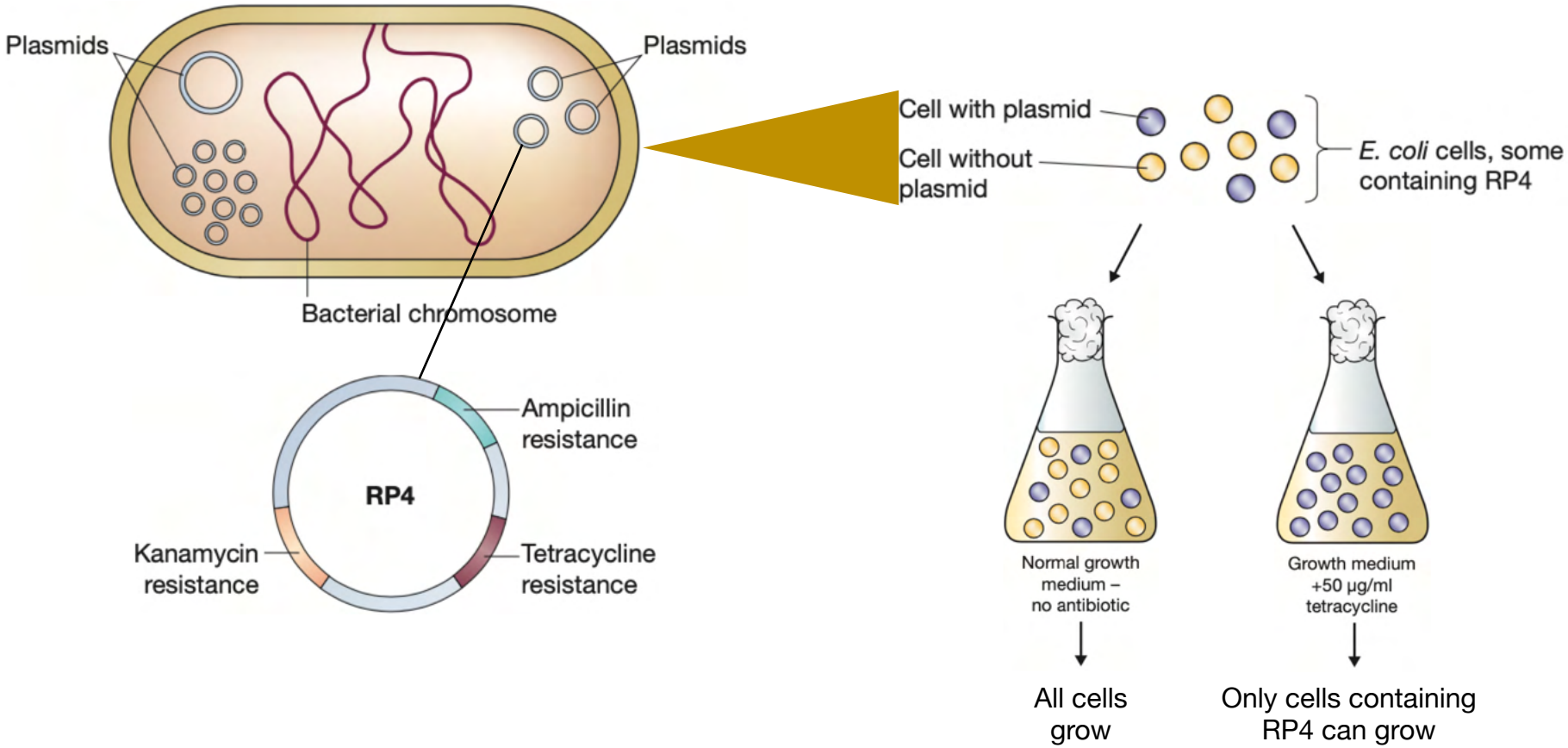


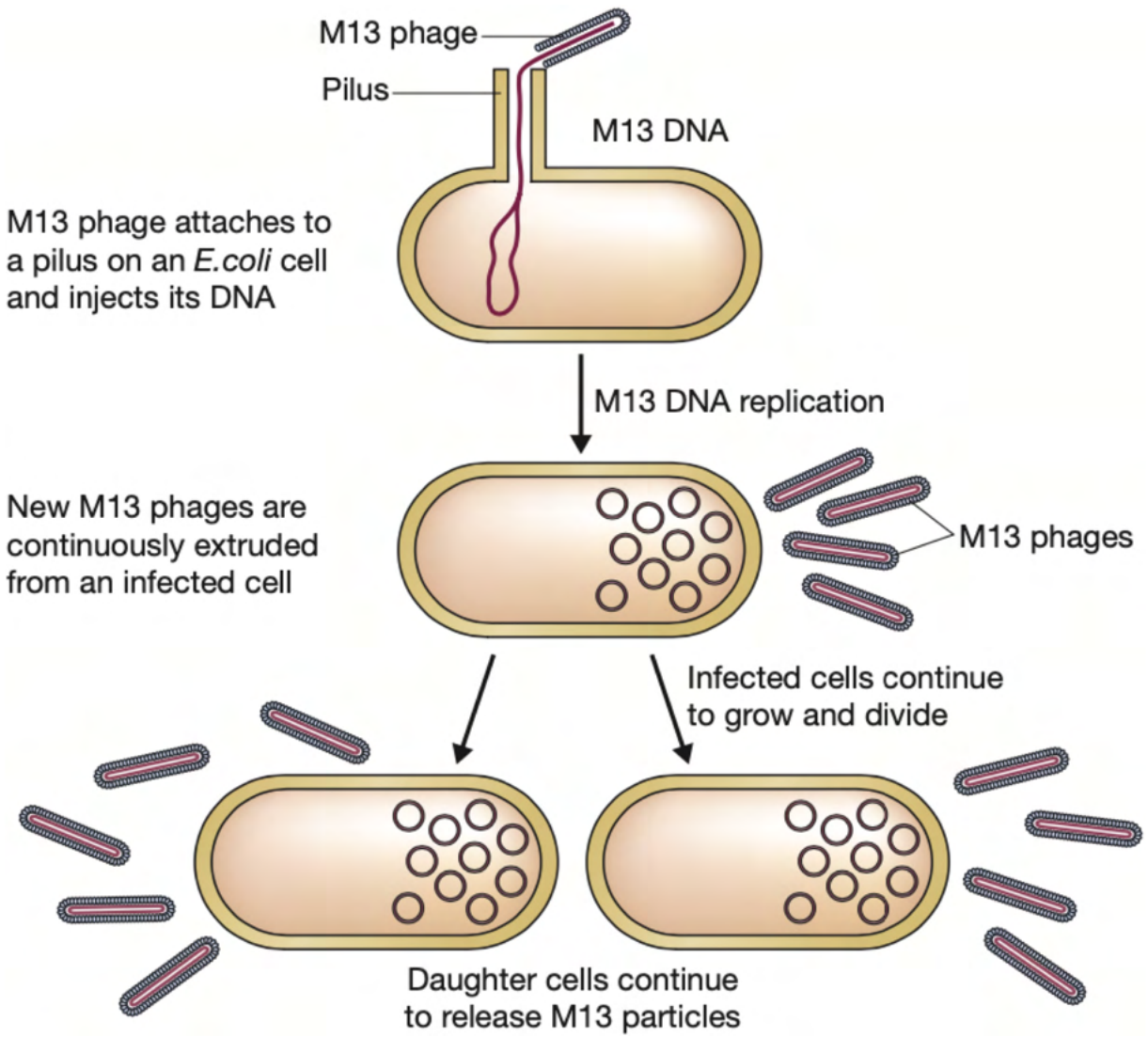


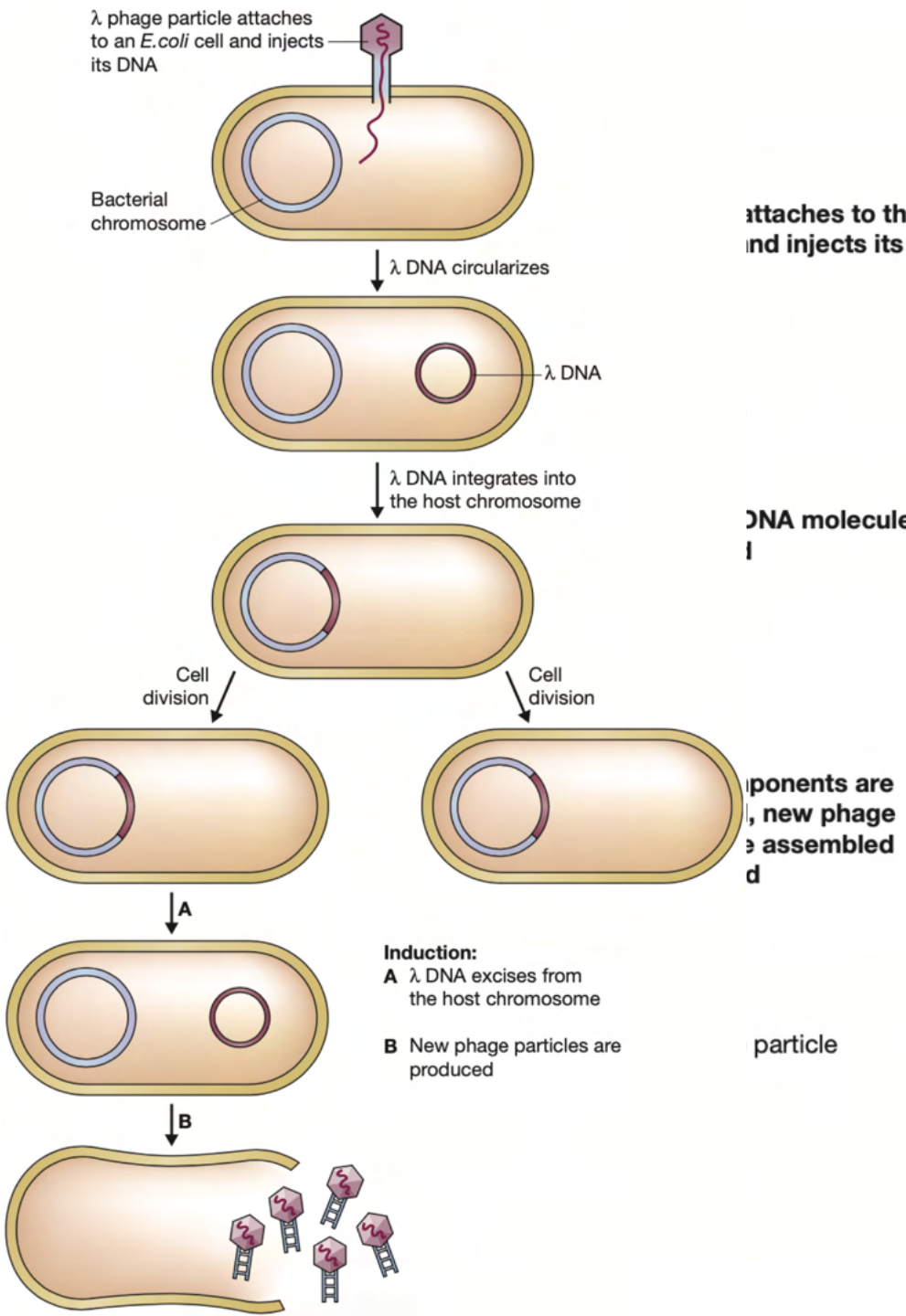
GENE CLONING

Part I: Vectors for Gene Cloning

Plasmids


Plasmids
Plasmids
Bacterial chromosome
Ampicillin resistance
RP4
Kanamycin resistance
Tetracycline resistance
Cell with plasmid
Cell without plasmid
E. coli cells, some containing RP4
Normal growth medium – no antibiotic
Growth medium +50 µg/ml tetracycline
All cells grow
Only cells containing RP4 can grow

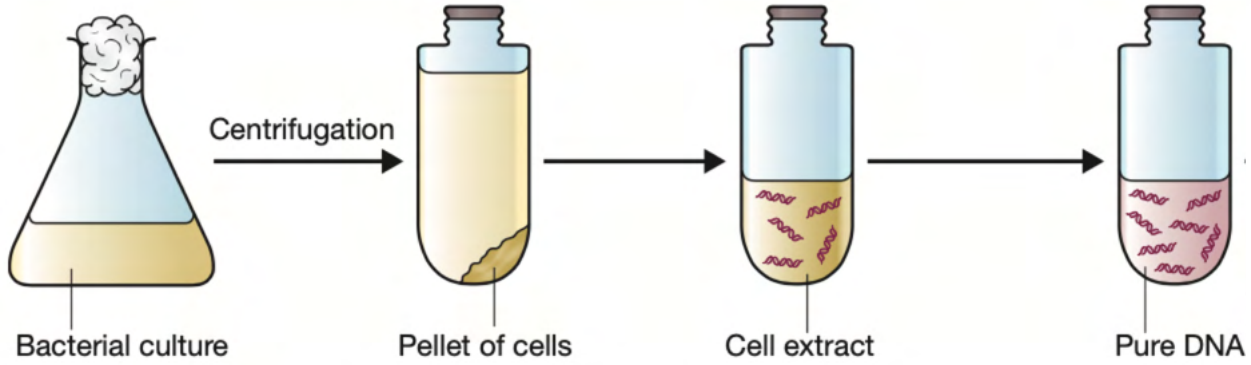

M13 phage
Pilus
M13 DNA
M13 phage attaches to a pilus on an *E.coli* cell and injects its DNA
M13 DNA replication
New M13 phages are continuously extruded from an infected cell
M13 phages
Infected cells continue to grow and divide
Daughter cells continue to release M13 particles

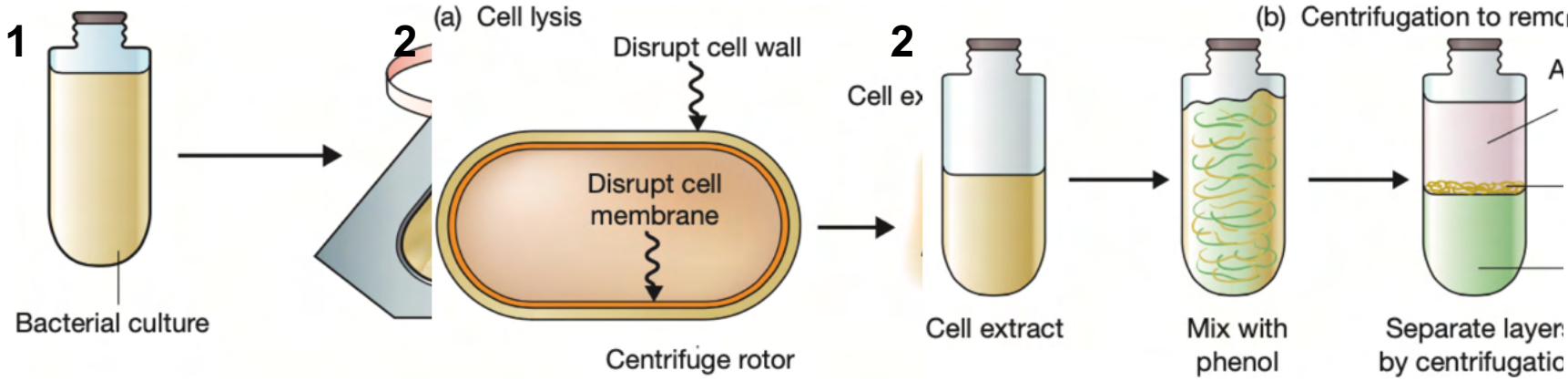

λ phage particle attaches to an *E.coli* cell and injects its DNA
Bacterial chromosome
λ DNA circularizes
λ DNA
λ DNA integrates into the host chromosome
Cell division
Cell division
A
B
Induction:
A λ DNA excises from the host chromosome
B New phage particles are produced

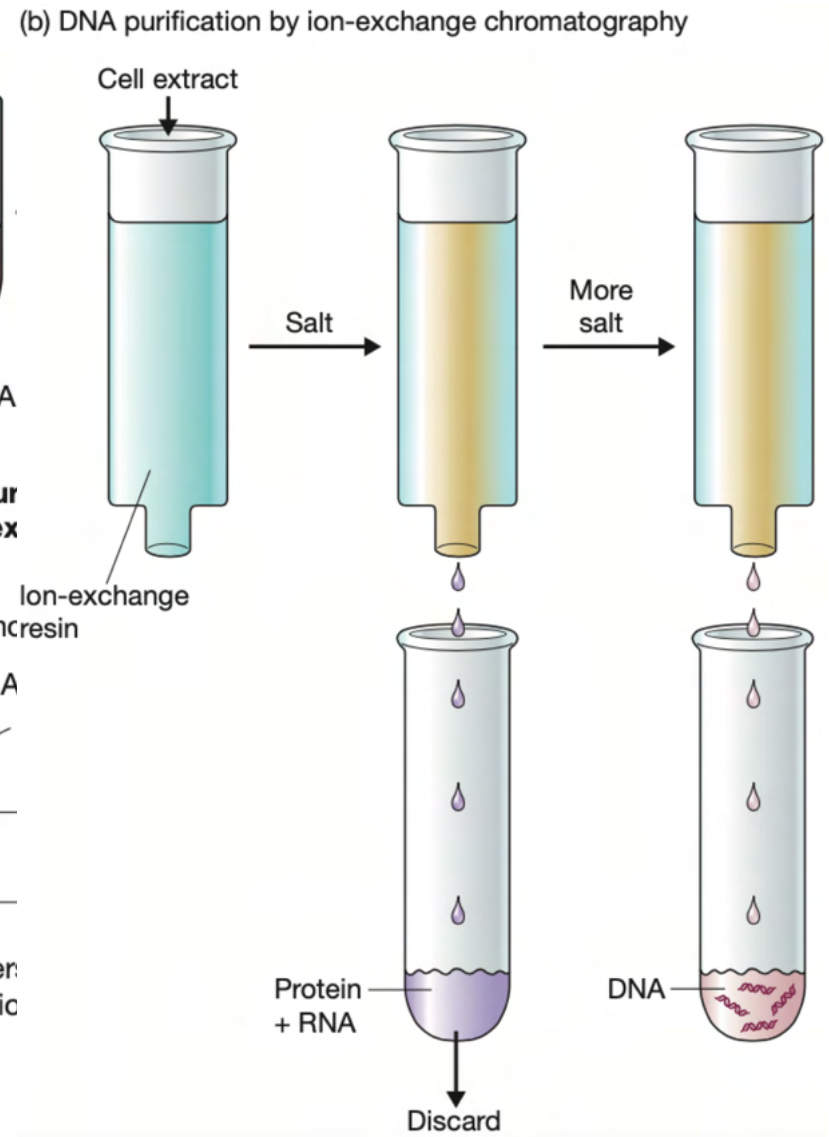
Viruses are cloning vectors for other organisms

- **adenoviruses** are used in **gene therapy.**
- **baculoviruses** are used to synthesize important pharmaceutical proteins in insect cells.
- **caulimoviruses** and **geminiviruses** have been used for cloning in plants.

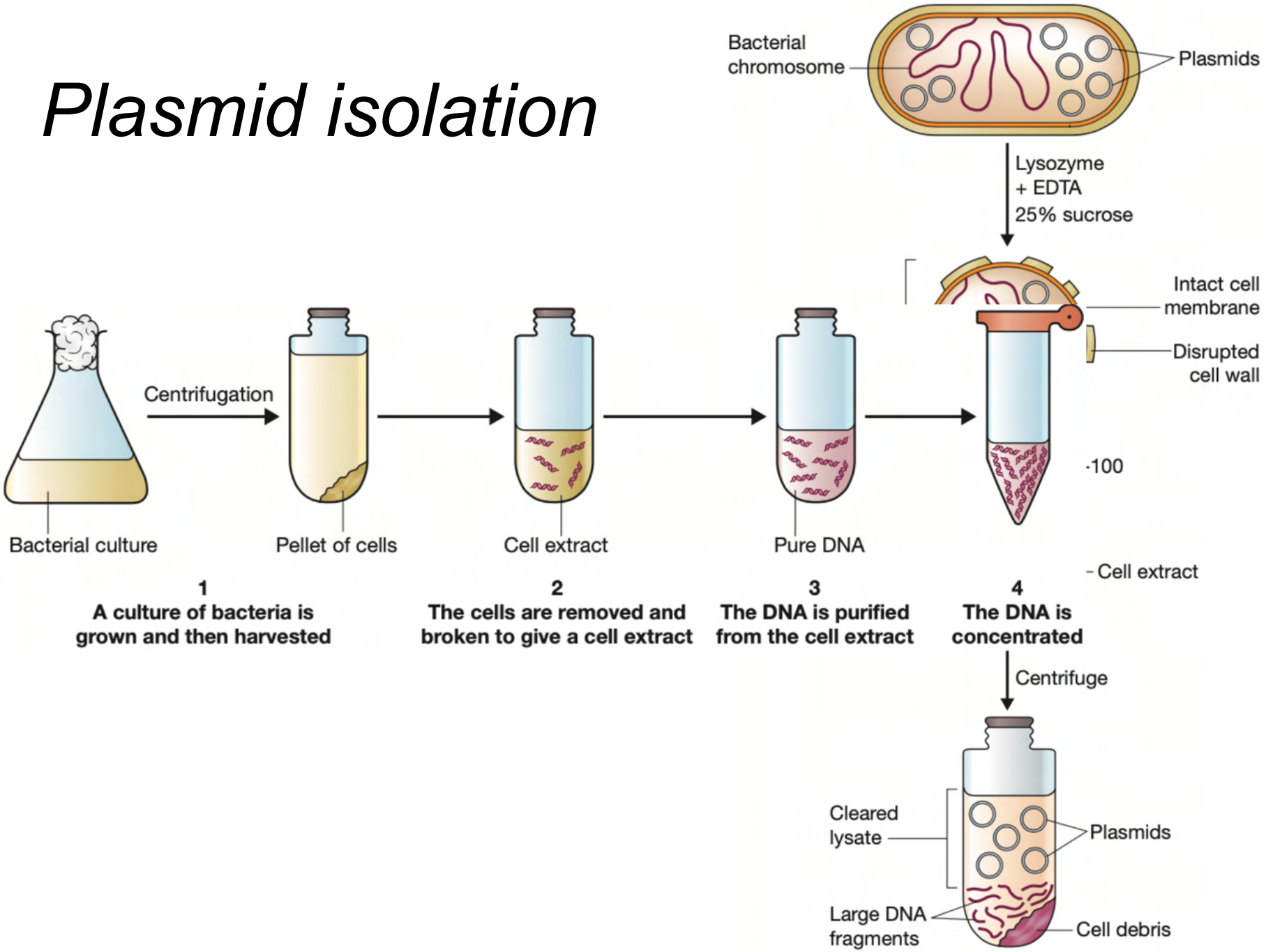
Purification of DNA from Living Cells







Plasmid isolation



GENE CLONING

Part II: Manipulating the DNA

Manipulation of the DNA

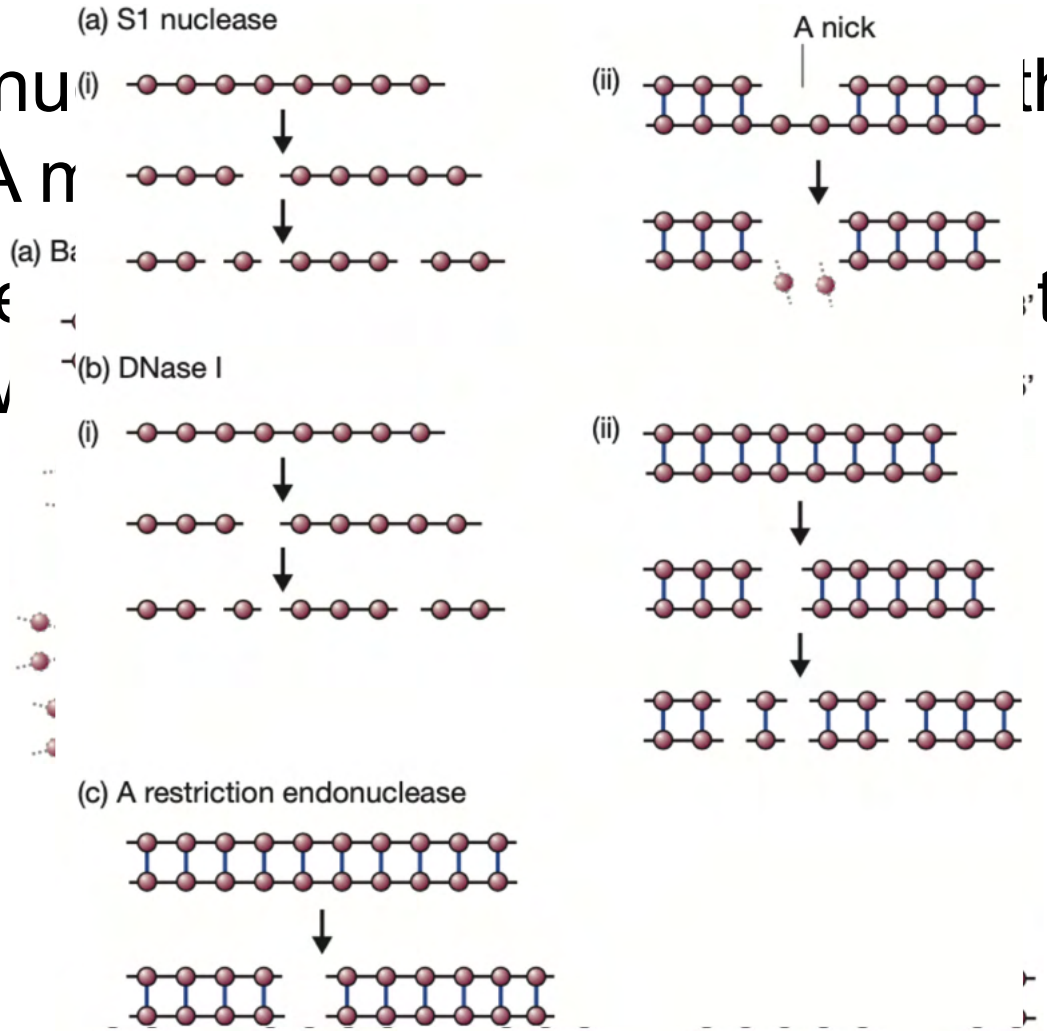
1. The range of DNA manipulative enzymes.
2. Enzymes for cutting DNA—restriction endonucleases.
3. Ligation—joining DNA molecules together.

DNA manipulative enzymes

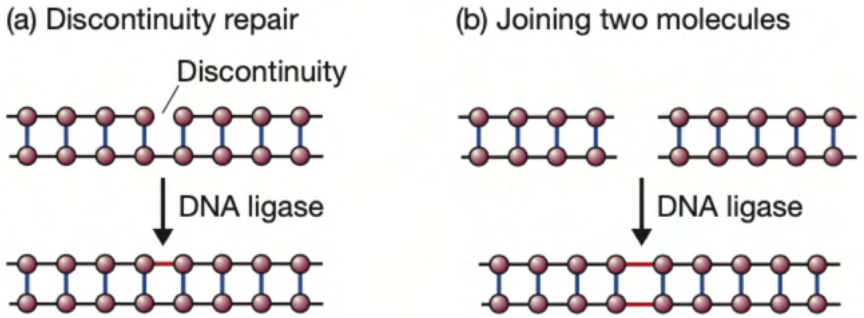
- Nucleases, are enzymes that cut, shorten, or degrade NA molecules.
- Ligases, join NA molecules together.
- Polymerases, makes copies of NA molecules.
- Modifying enzymes, remove/add chemical groups.

Nucleases

- Exonucleases, remove nu
  of a DNA m
- Endonucleases, are able
  bonds v



Ligases

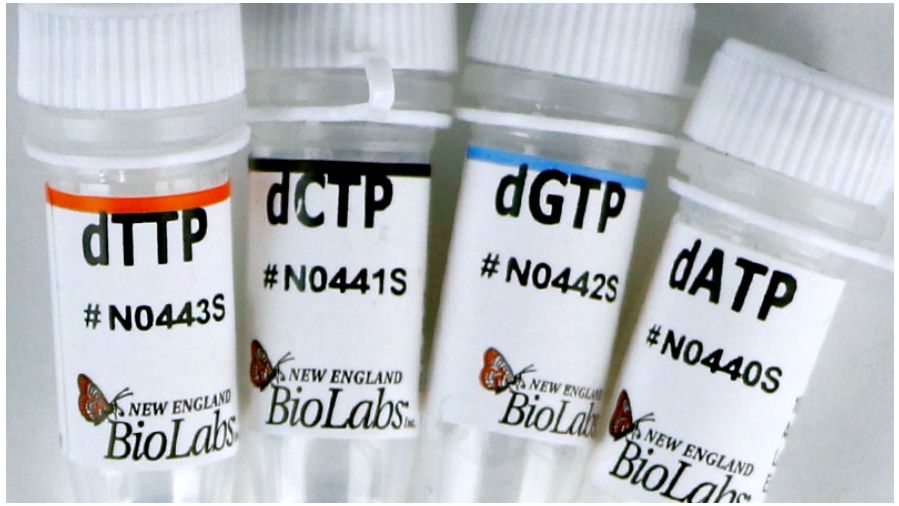

(a) Discontinuity repair
Discontinuity
DNA ligase
(b) Joining two molecules
DNA ligase


invitrogen
15224-025
Store at: -30 °C
T4 DNA Ligase
Qty: 500 U (1 U/µL)
1296528
Mar2015
For Research Use Only
5791 Van Allen Way Carlsbad, CA 92008
Read SDS
Made in USA
For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.
24-hour Medical or Spill Emergency Response (Chemtrec)
North America +1 866 536 0631 / Other +1 301 431 8585
5X T4 DNA
Lot no. 1253588
T4 DNA Ligase

DNA polymerase

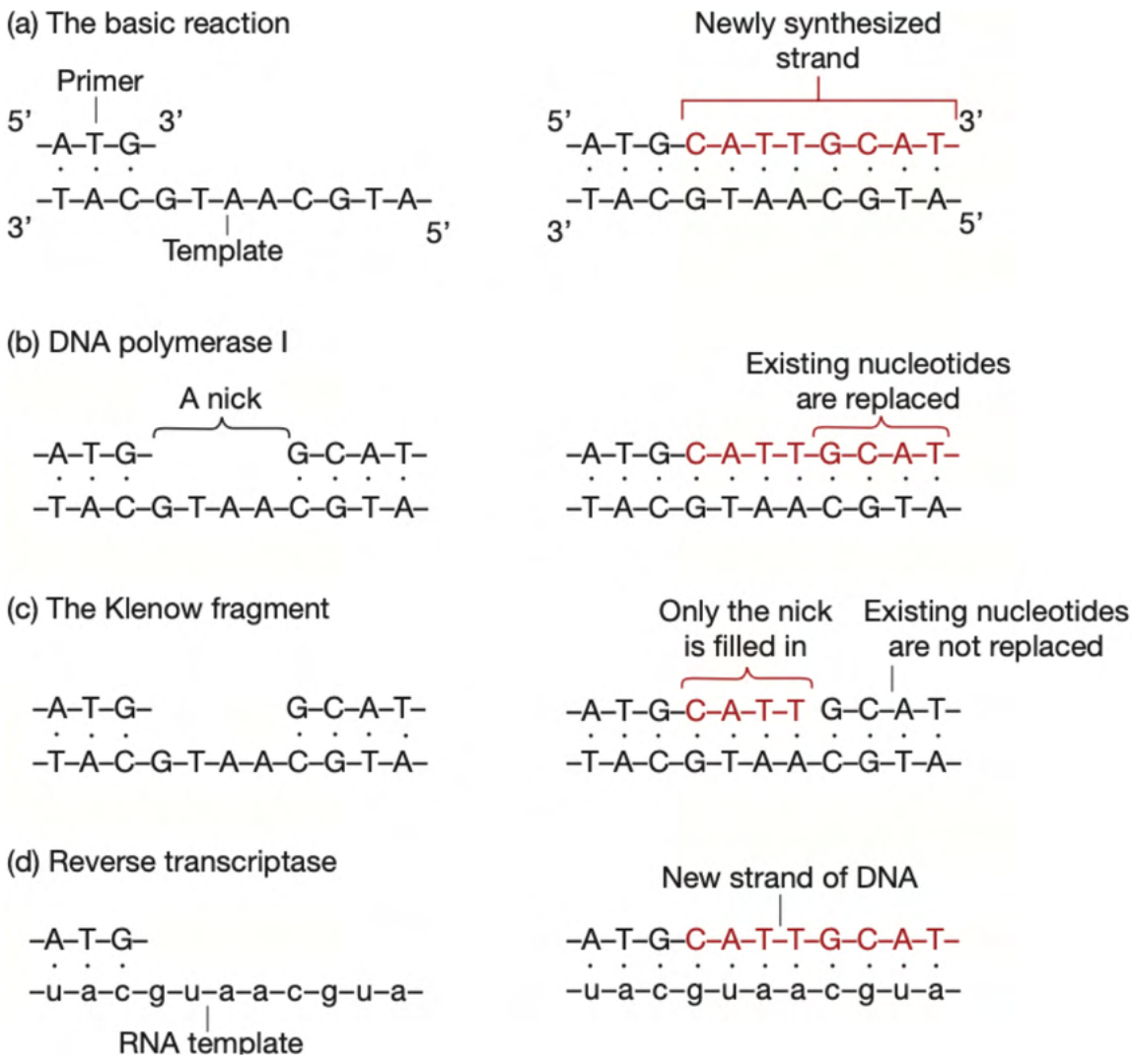
DNA polymerase

dNTPs



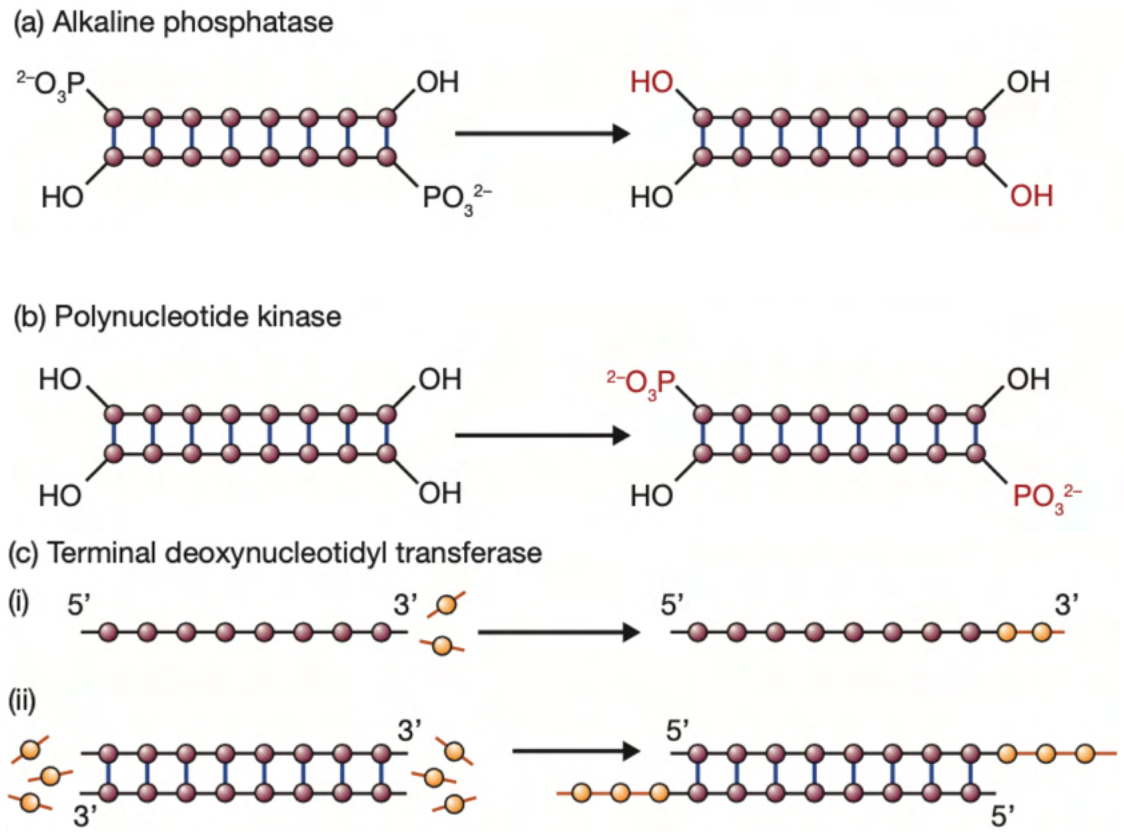
Primers

catalyzed by DNA polymerase

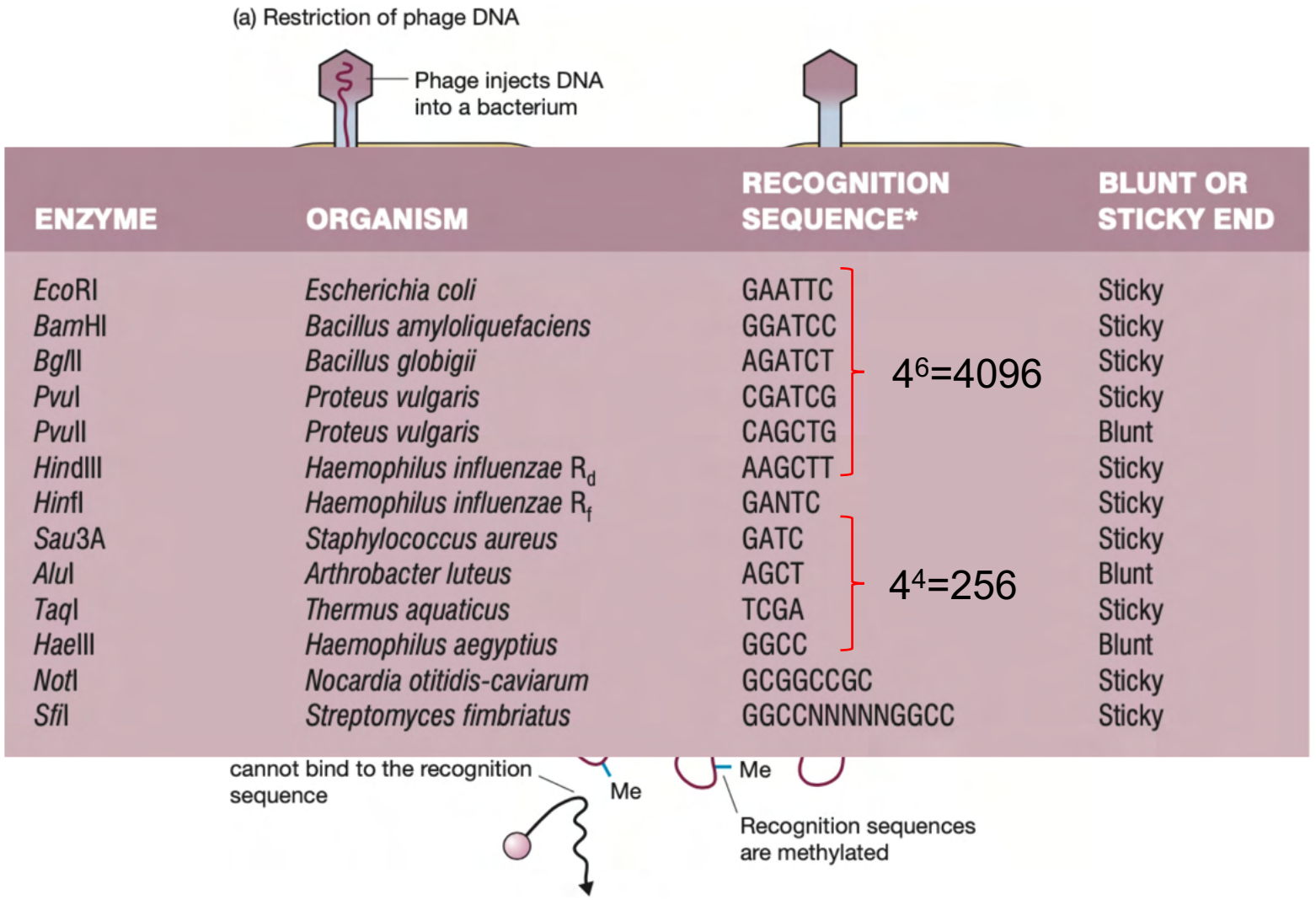


DNA modifying enzymes

- Alkaline phosphatase, removes the phosphate group present at the 5′ terminus of a DNA molecule.
- Polynucleotide kinase adds phosphate groups onto free 5′ termini
- Terminal deoxynucleotidyl transferase adds one or more deoxyribonucleotides onto the 3′ terminus of a DNA molecule.

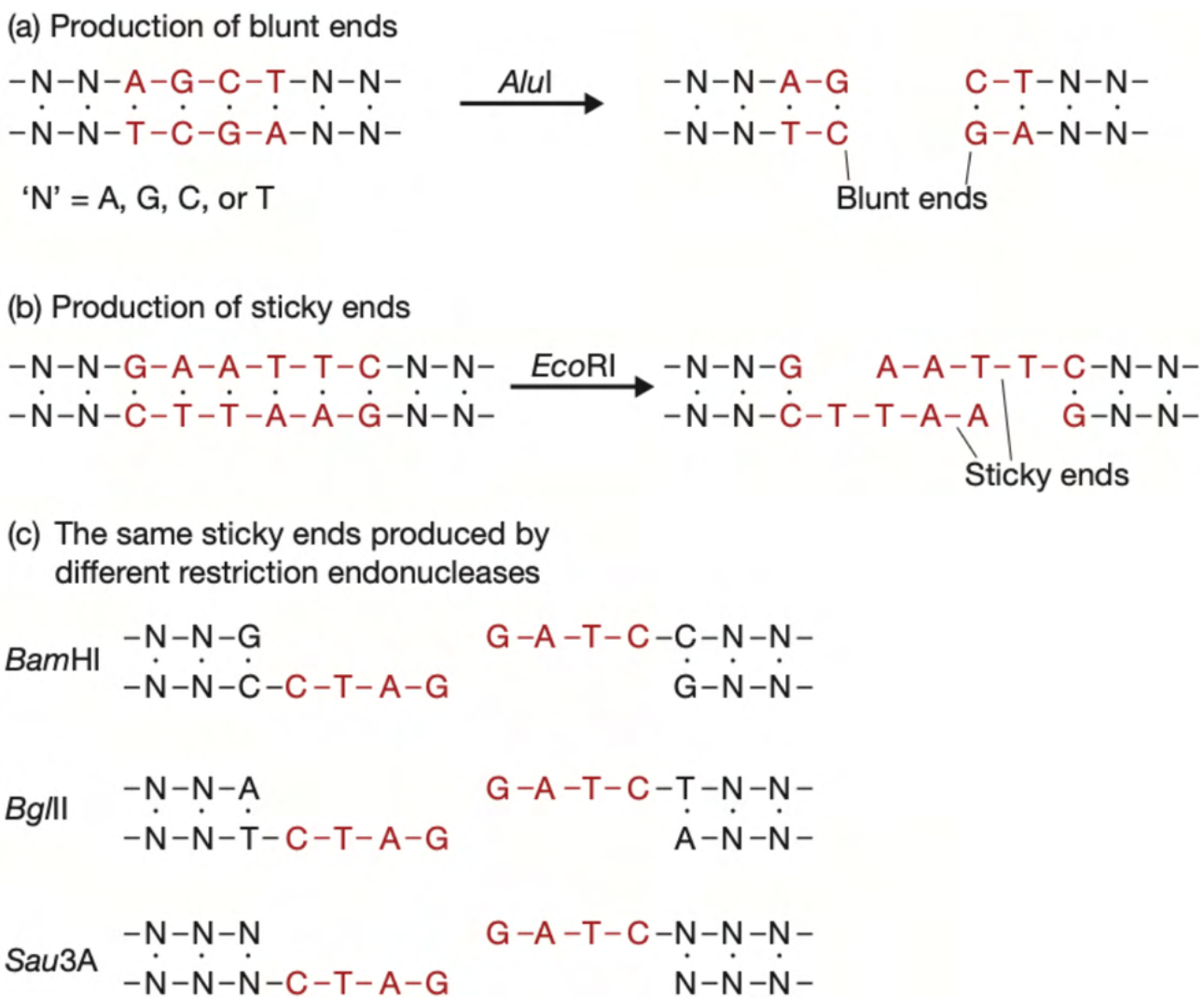


Restriction endonucleases

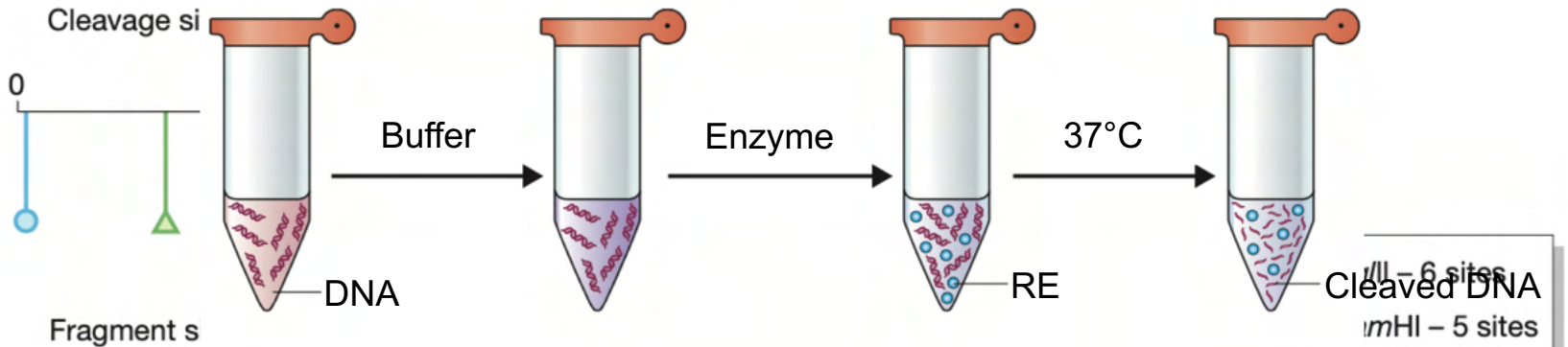


ENZYME	ORGANISM	RECOGNITION SEQUENCE*		BLUNT OR STICKY END
*Eco*RI	*Escherichia coli*	GAATTC		Sticky
*Bam*HI	*Bacillus amyloliquefaciens*	GGATCC		Sticky
*Bgl*II	*Bacillus globigii*	AGATCT	4^6=4096	Sticky
*Pvu*I	*Proteus vulgaris*	CGATCG		Sticky
*Pvu*II	*Proteus vulgaris*	CAGCTG		Blunt
*Hind*III	*Haemophilus influenzae* R_d	AAGCTT		Sticky
*Hin*fI	*Haemophilus influenzae* R_f	GANTC		Sticky
*Sau*3A	*Staphylococcus aureus*	GATC		Sticky
*Alu*I	*Arthrobacter luteus*	AGCT	4^4=256	Blunt
*Taq*I	*Thermus aquaticus*	TCGA		Sticky
*Hae*III	*Haemophilus aegyptius*	GGCC		Blunt
*Not*I	*Nocardia otitidis-caviarum*	GCGGCCGC		Sticky
*Sfi*I	*Streptomyces fimbriatus*	GGCCNNNNNGGCC		Sticky

blunt vs sticky ends



Restriction digestion @lab



*Bgl*II

22 010
13 286
9688
2392
651
415
60

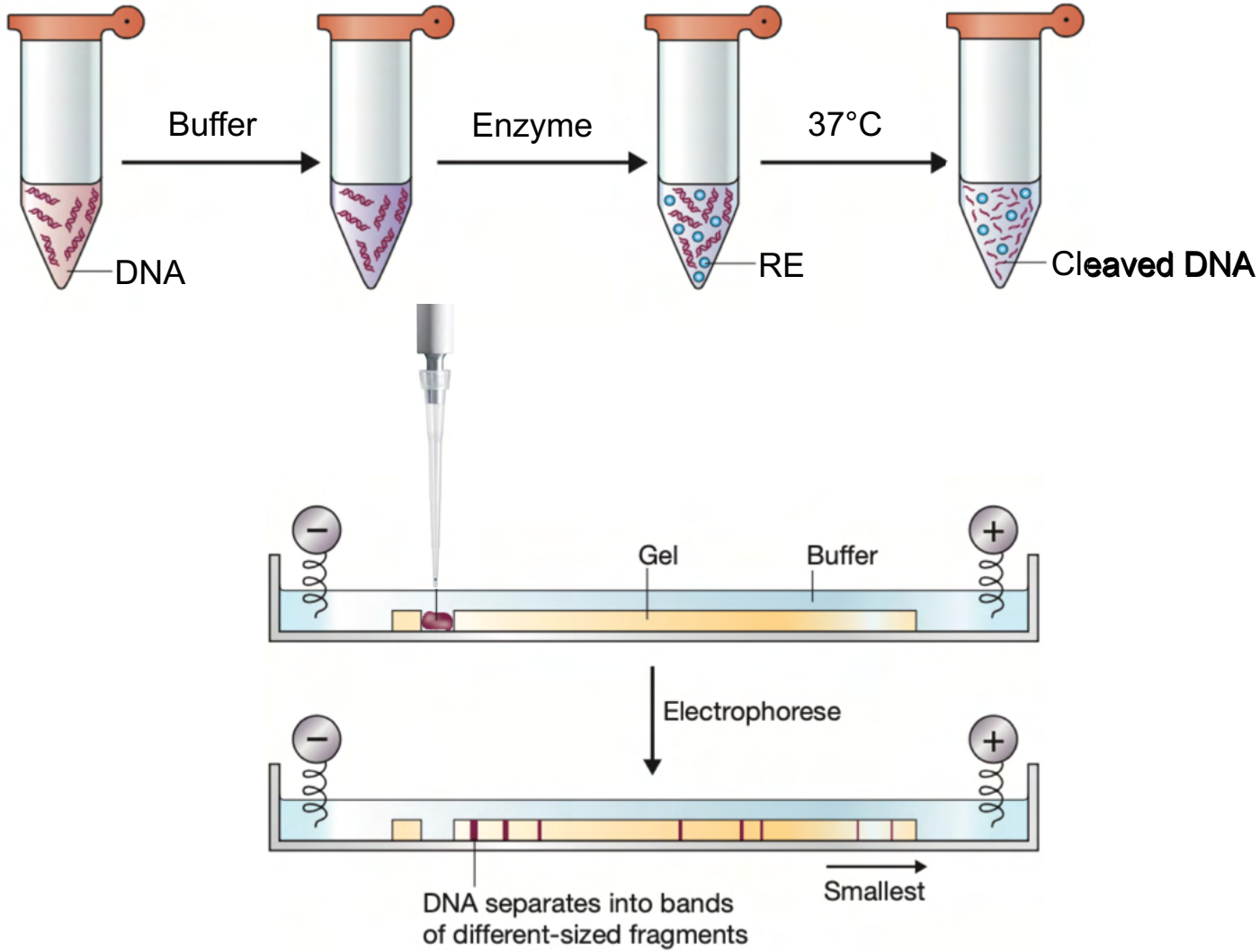
*Bam*HI

16 841
7233
6770
6527
5626
5505

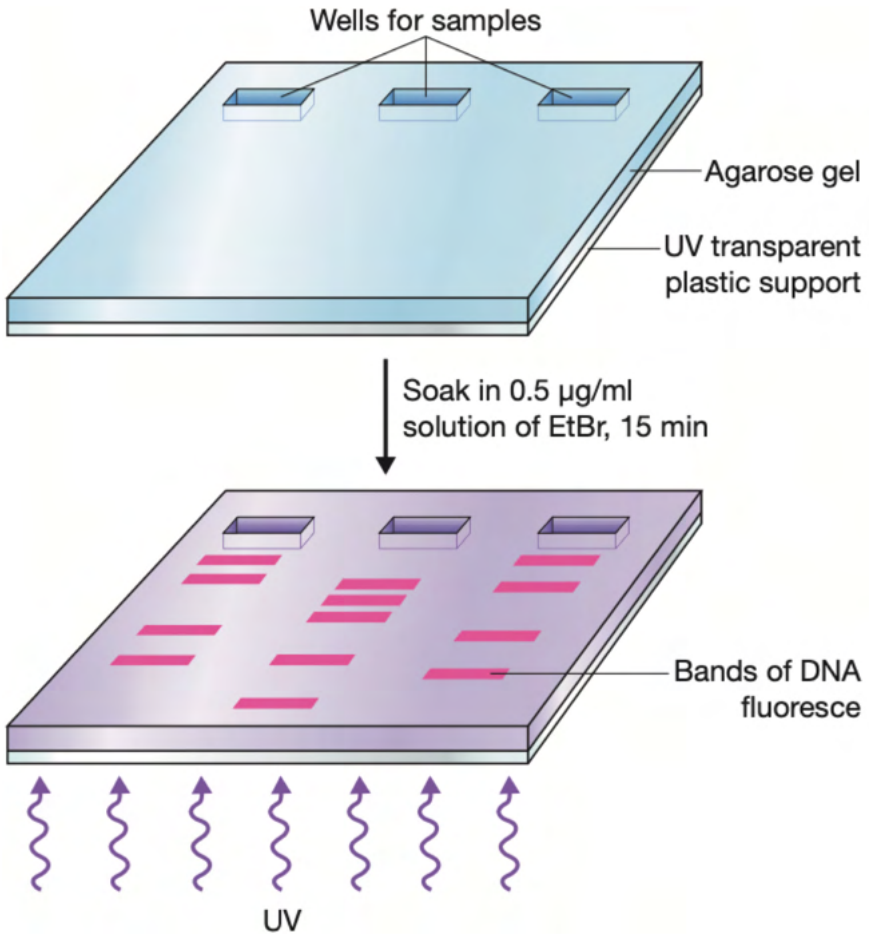
*Sal*I

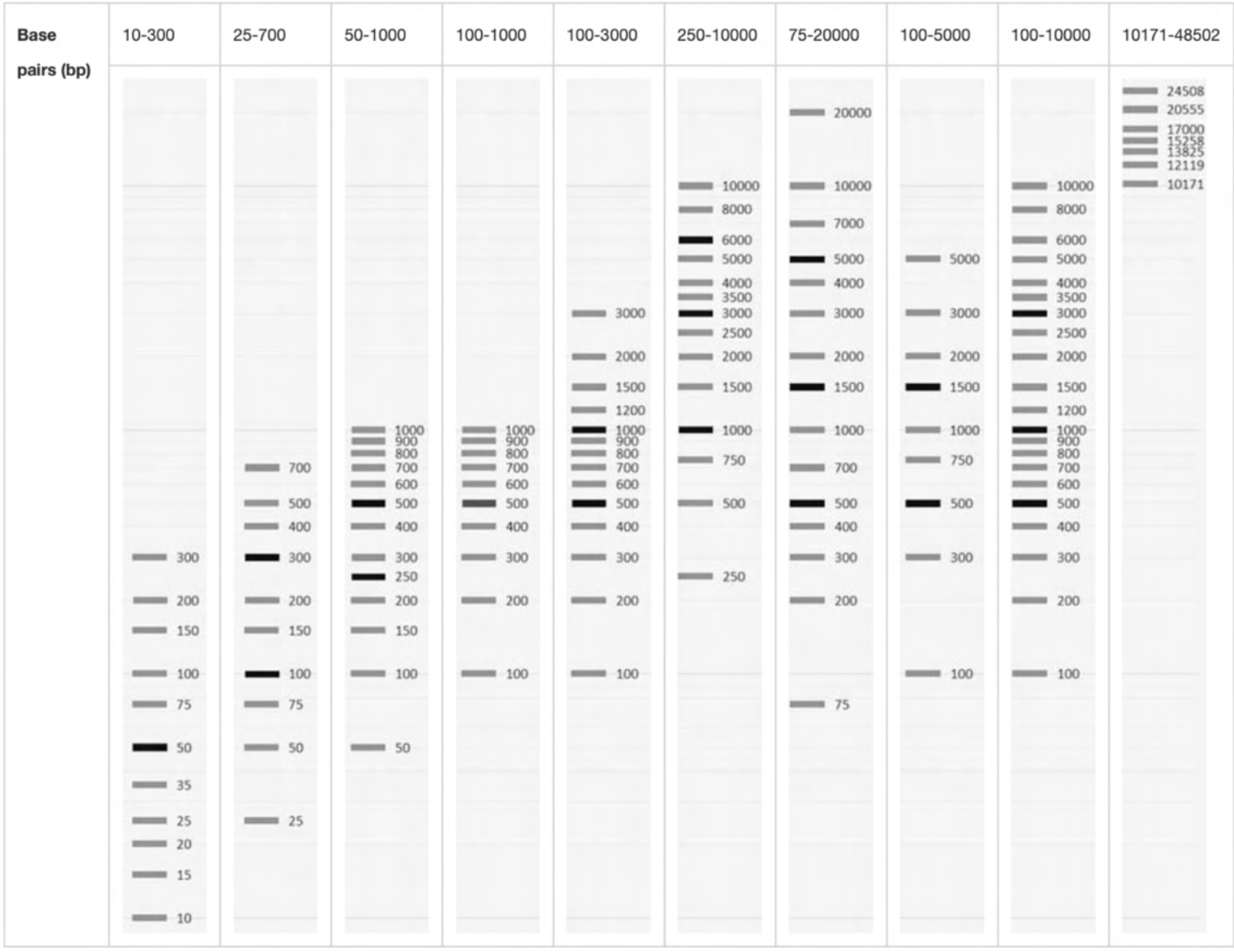
32 745
15 258
499

Restriction digestion @lab

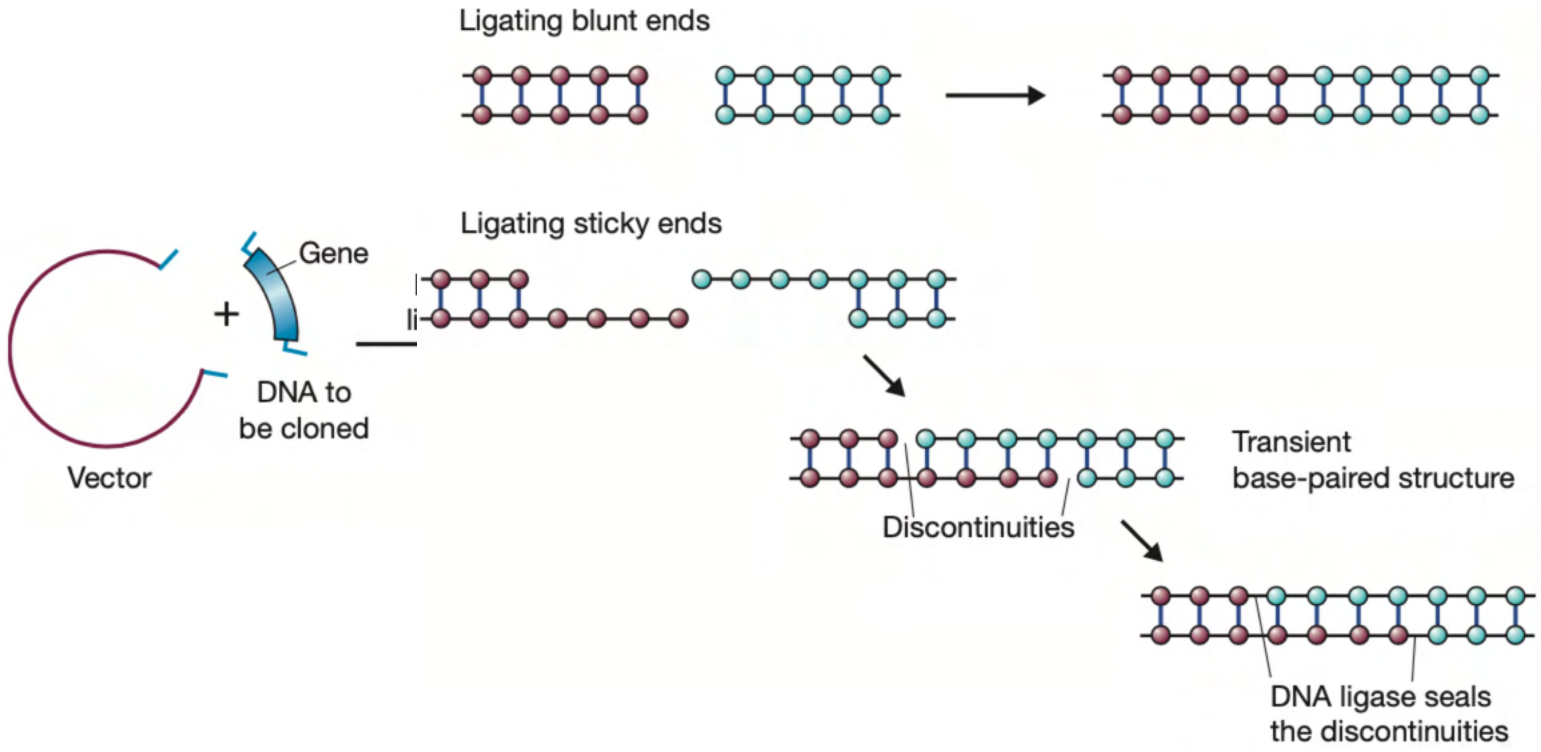


seeing the DNA

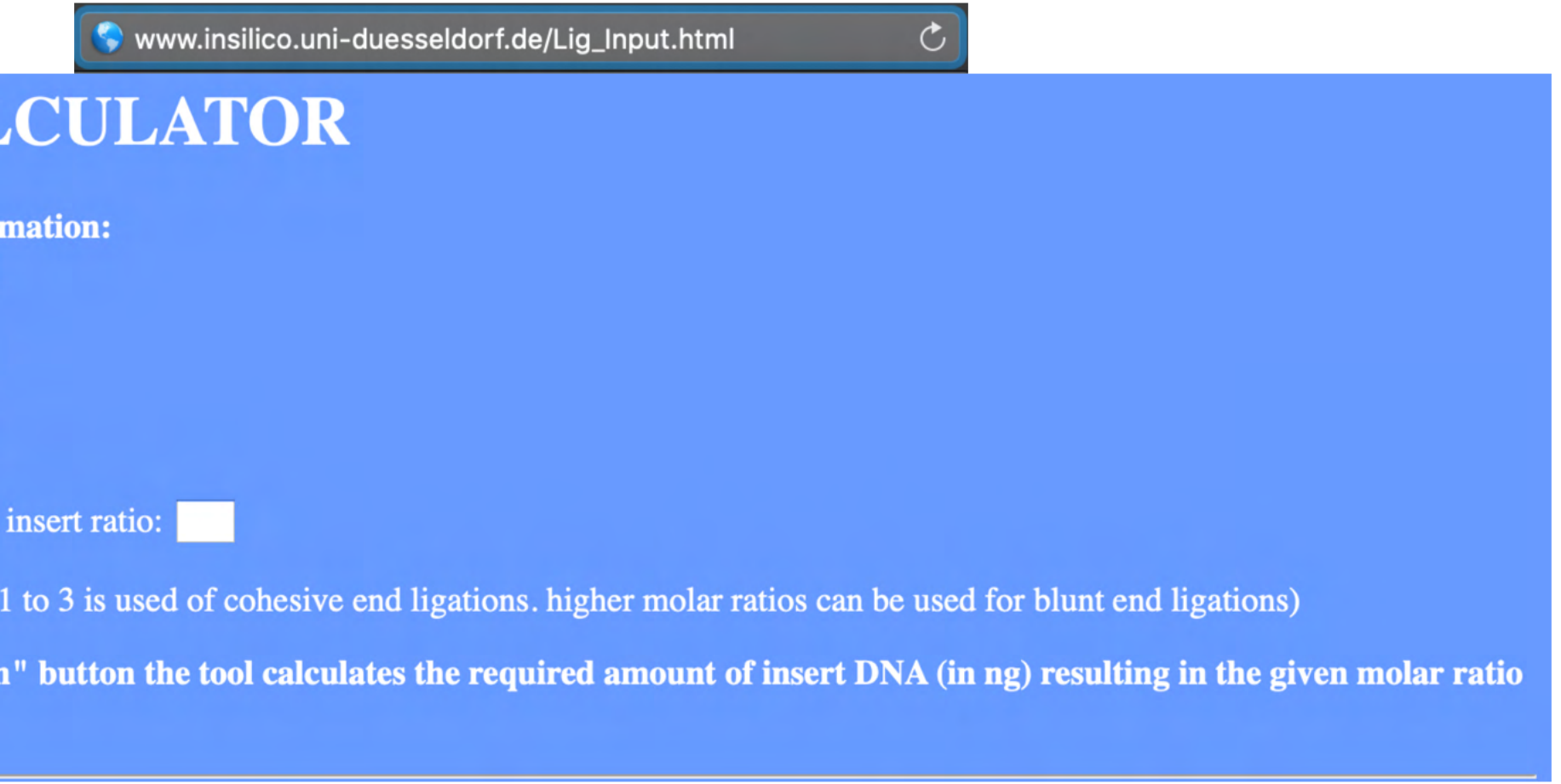




ligating the DNA

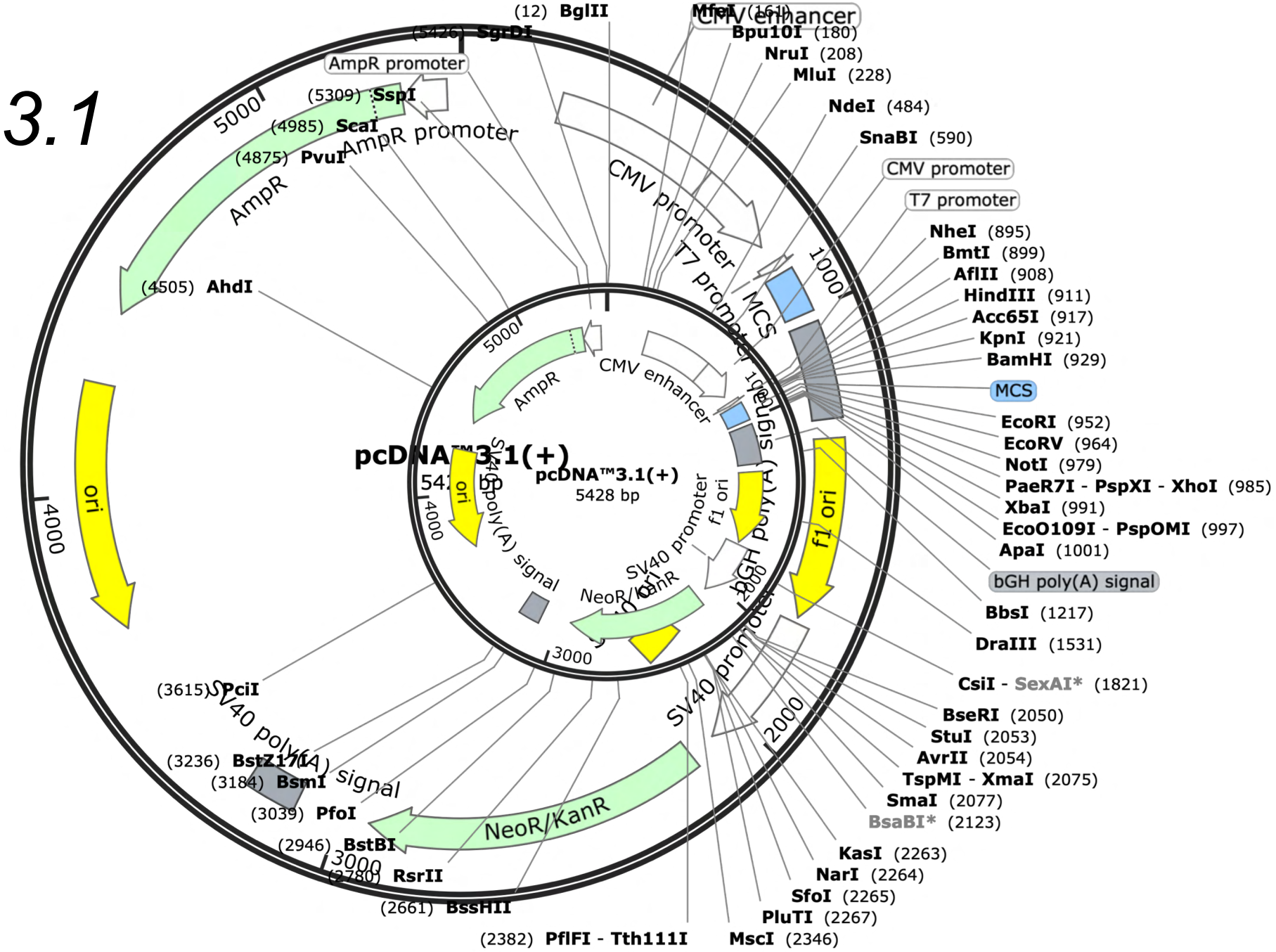


calculating the DNA to insert ratio



INTRODUCTION OF DNA INTO LIVING CELLS

pcDNA3.1


pcDNA™3.1(+)
5428 bp
CMV enhancer
CMV promoter
T7 promoter
MCS
AmpR promoter
AmpR
ori
f1 ori
SV40 promoter
SV40 poly(A) signal
bGH poly(A) signal
NeoR/KanR
1000
2000
3000
4000
5000
(12) BglII
(5426) SgrDI
(5309) SspI
(4985) ScaI
(4875) PvuI
(4505) AhdI
(3615) PciI
(3236) BstZ17I
(3184) BsmI
(3039) PfoI
(2946) BstBI
(2780) RsrII
(2661) BssHII
(2382) PflFI - Tth111I
MscI (2346)
PluTI (2267)
SfoI (2265)
NarI (2264)
KasI (2263)
BsaBI* (2123)
SmaI (2077)
TspMI - XmaI (2075)
AvrII (2054)
StuI (2053)
BseRI (2050)
CsiI - SexAI* (1821)
DraIII (1531)
BbsI (1217)
ApaI (1001)
EcoO109I - PspOMI (997)
XbaI (991)
PaeR7I - PspXI - XhoI (985)
NotI (979)
EcoRV (964)
EcoRI (952)
MCS
BamHI (929)
KpnI (921)
Acc65I (917)
HindIII (911)
AflII (908)
BmtI (899)
NheI (895)
T7 promoter
CMV promoter
SnaBI (590)
NdeI (484)
MluI (228)
NruI (208)
Bpu10I (180)
MfeI (161)