

# Lecture 8 - 9

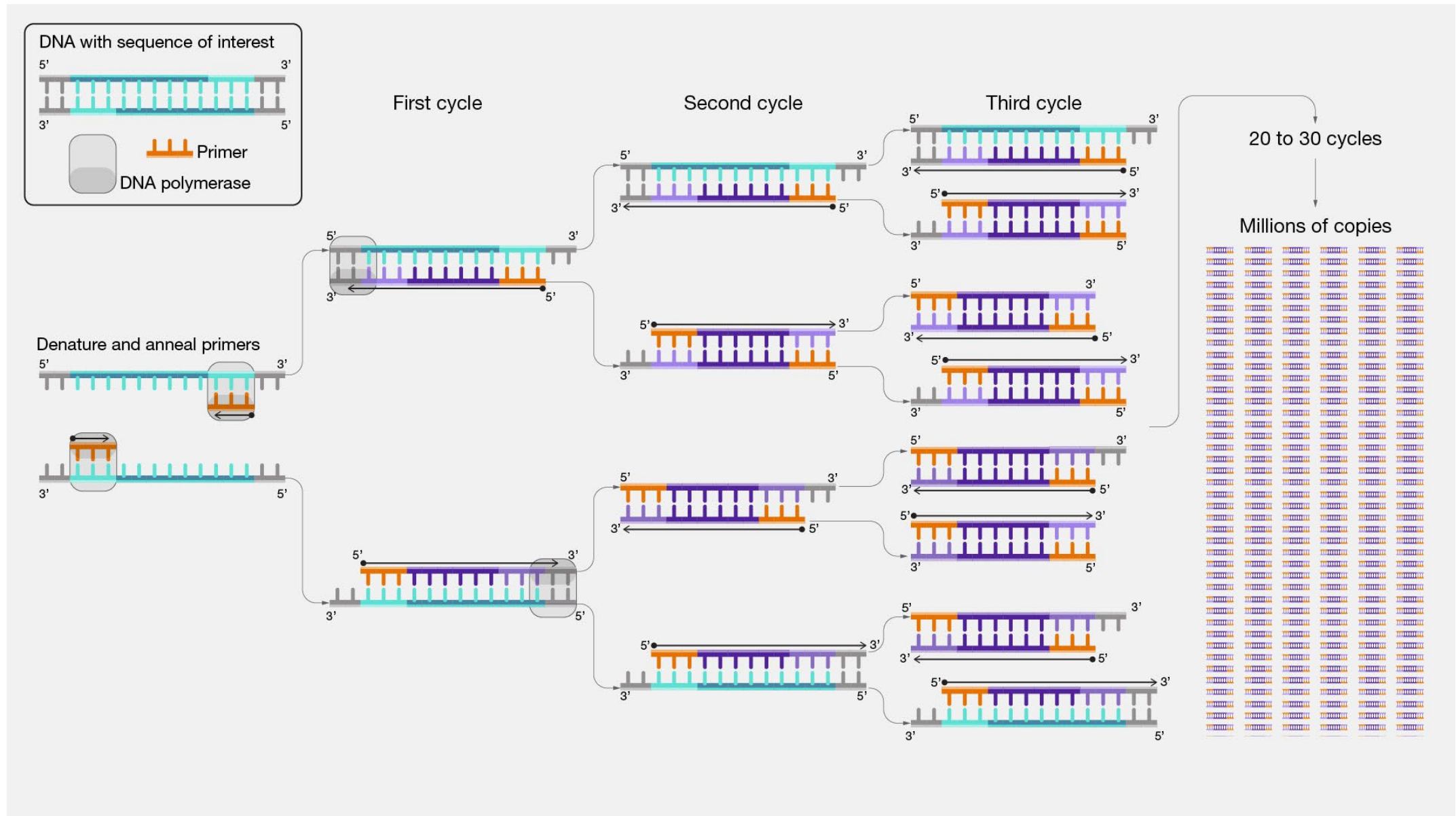
## Techniques & Methods



Prof. Sebastian Maerkl

PCR

# Polymerase Chain Reaction (PCR)



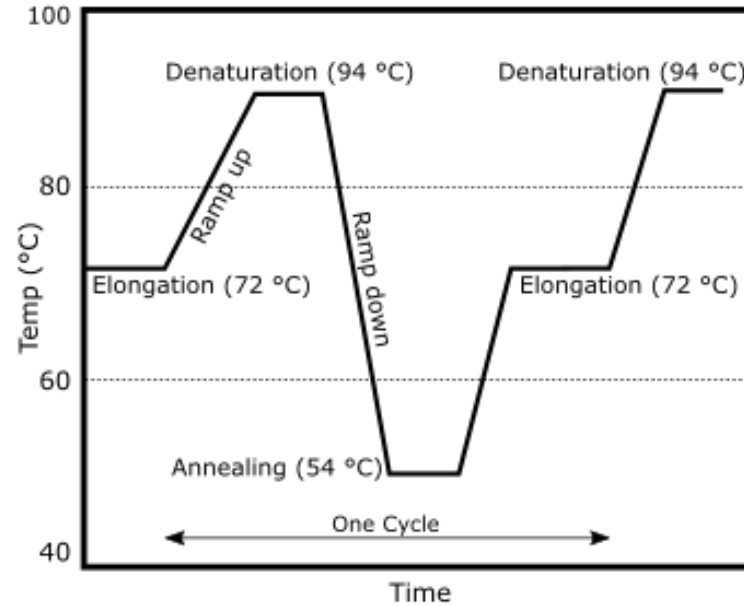
# Polymerase Chain Reaction (PCR)

## Materials:

- Sequence to be amplified
- DNA polymerase
- Buffer solution
- Primers
- dNTP
- Thermal cycler
- PCR Tubes

## Thermal Cycles:

- Denaturation
  - ~94-98°C
  - ~10s
- Annealing
  - ~50-55°C depends on primer  $T_m$ )
  - ~10s
- Elongation
  - 72°C or optimal temperature for polymerase)
  - 15-30 s/kb
- Generally 25-35 cycles



# Agarose Gels

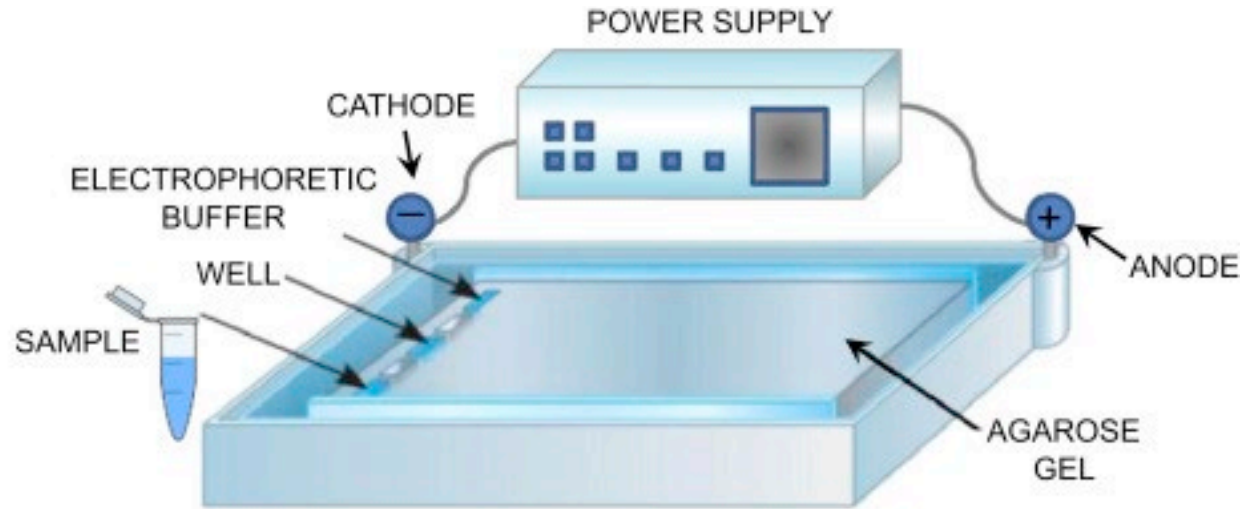
# Agarose gel electrophoresis: casting and loading

## Gel parameters:

- Number of wells = number of samples
- Gel density = 0.5 – 2%
  - Lower gel density for high MW
  - Higher gel density for lower MW
- Length
  - Shorter = lower resolution
  - Longer = higher resolution
- Running Buffer
  - TAE (tris base, acetic acid, EDTA)
  - TBE (tris base, boric acid, EDTA)
- Loading Buffer
  - Xylene cyanol / bromophenol blue

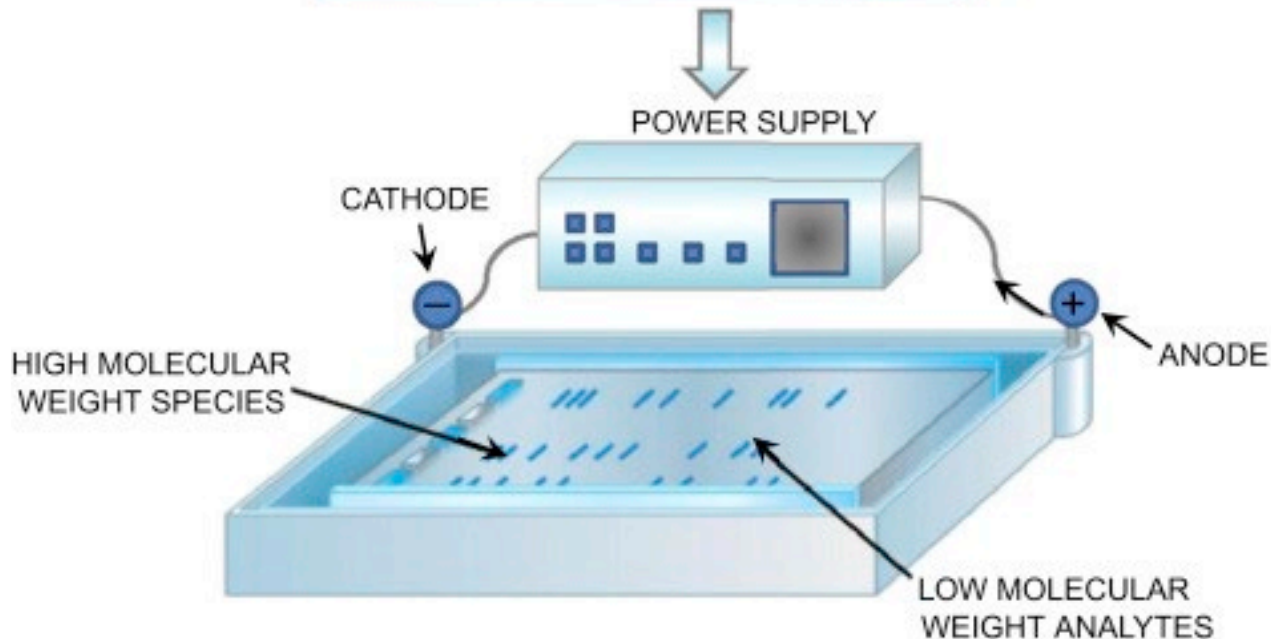


# Agarose gel electrophoresis: running gels



## Running parameters:

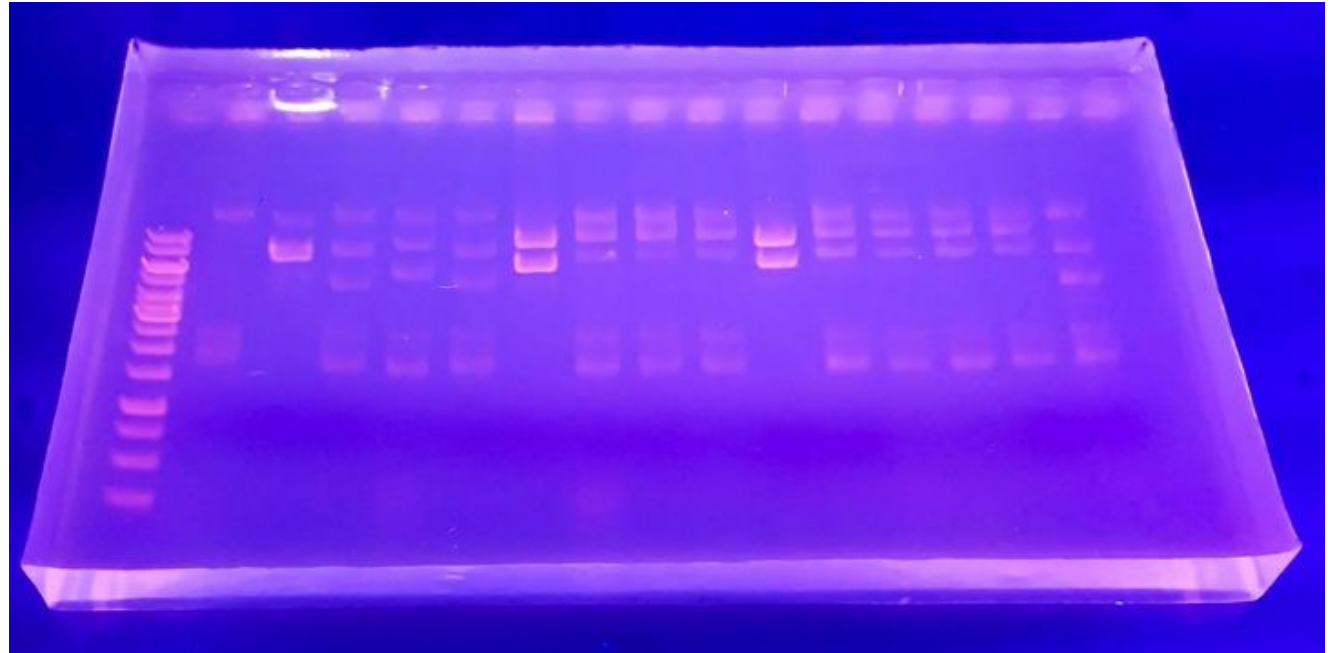
- Voltage:
  - ~100 Volts (10V/cm of gel length)
- Time:
  - Until loading dye reaches bottom of gel



# Agarose gel electrophoresis: imaging

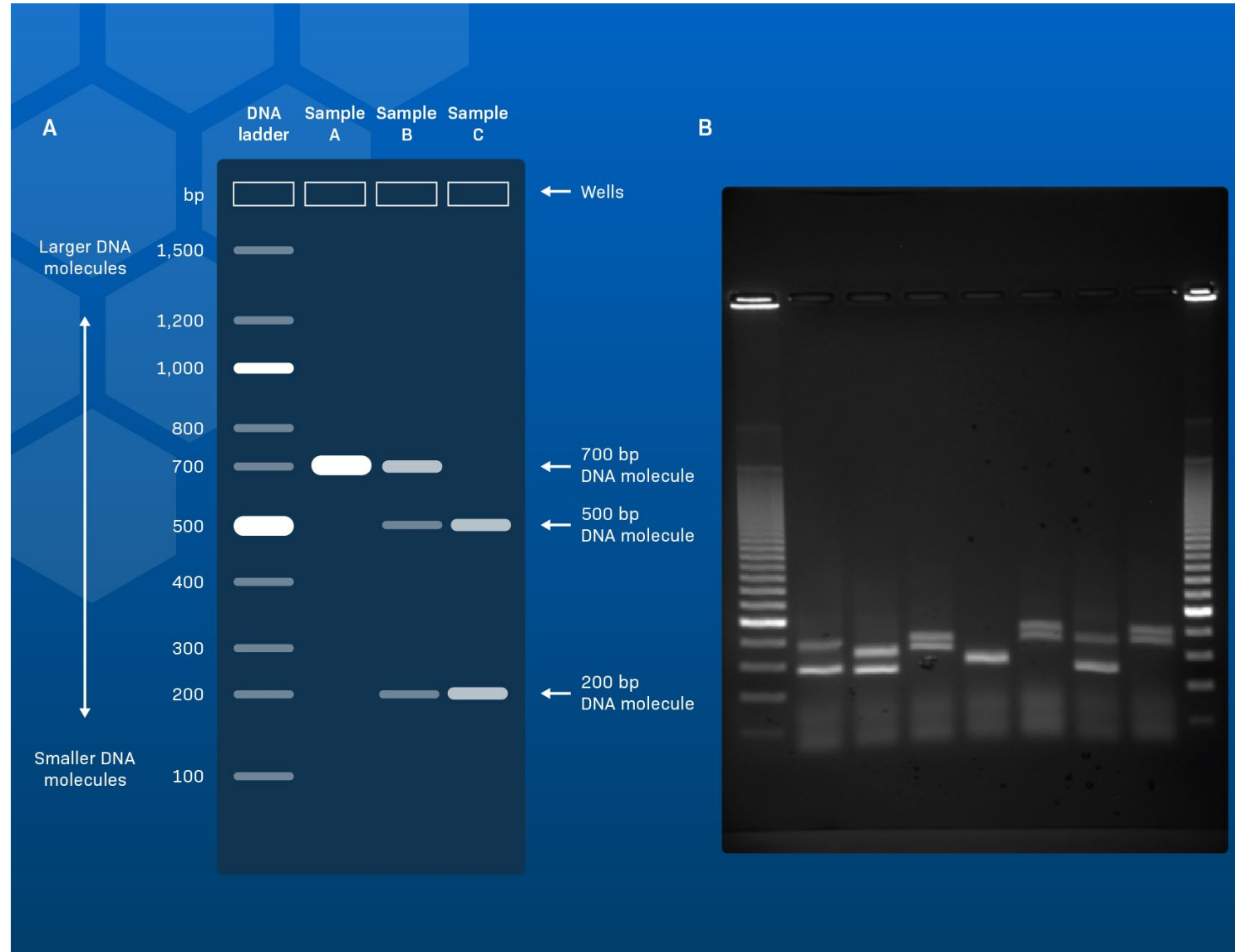
## Imaging:

- DNA stain:
  - Ethidium bromide (mutagenic)
  - SYBR green (mutagenic?)



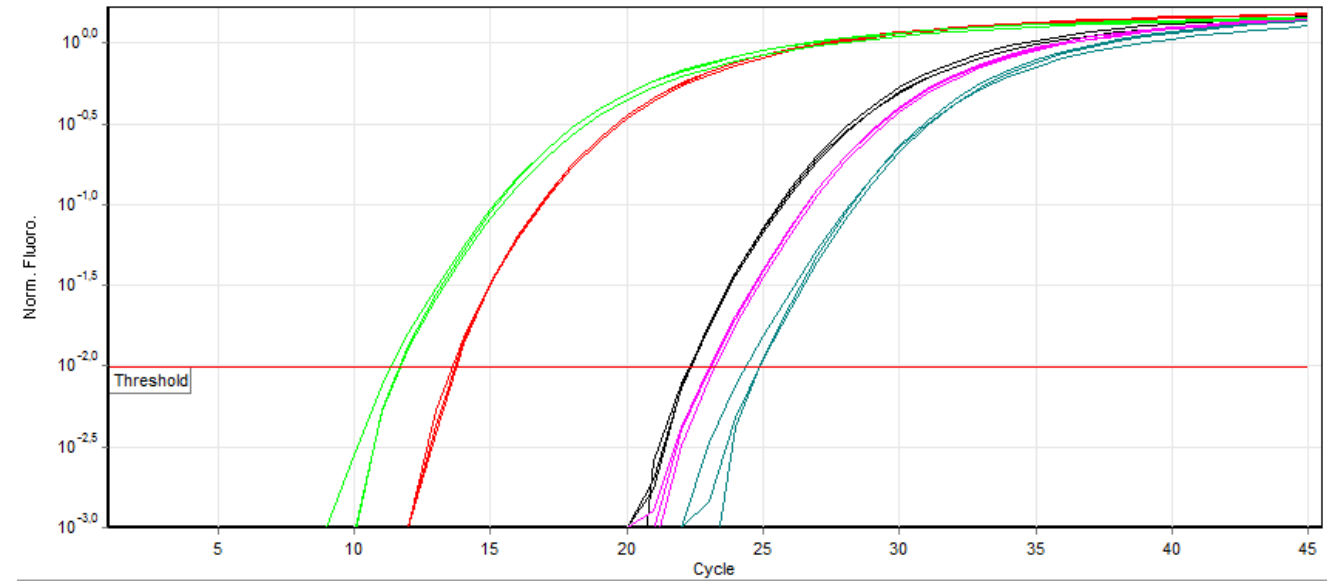
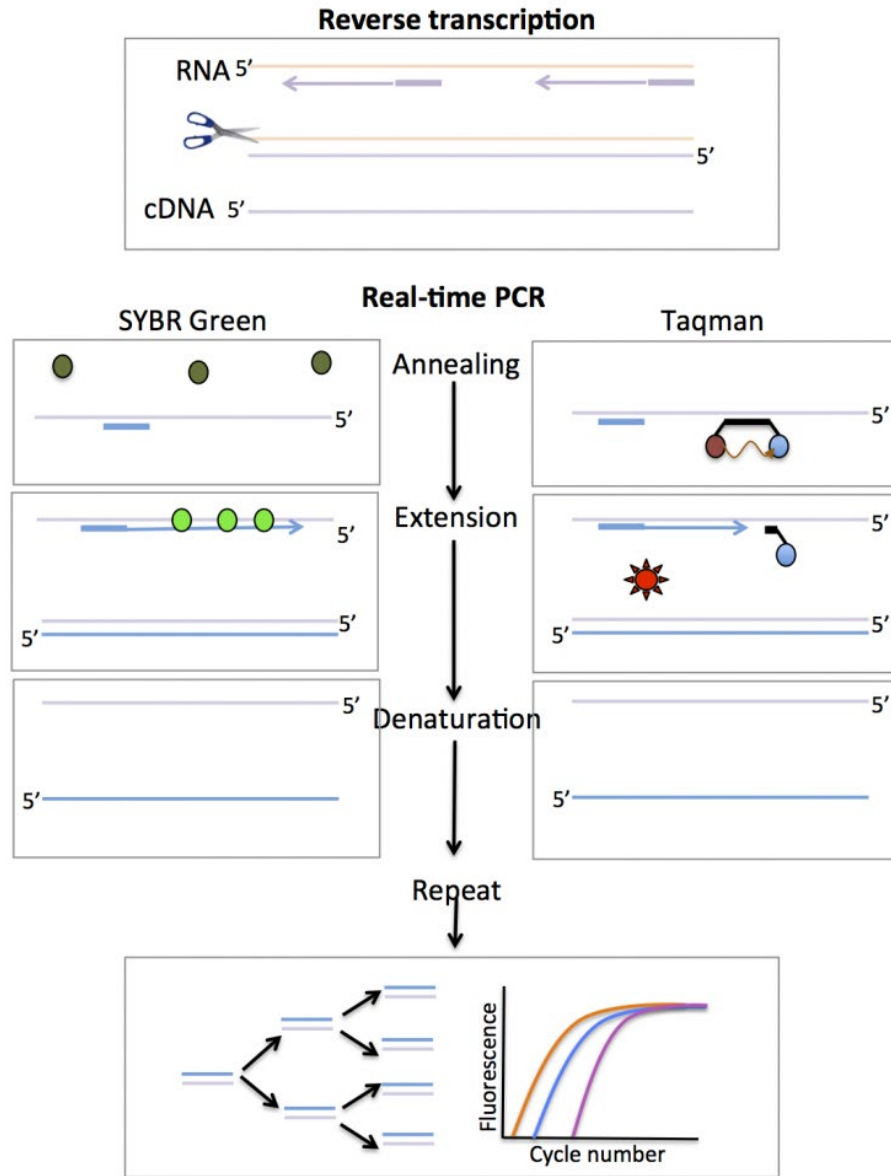


# Agarose gel electrophoresis: interpreting results



PCR variants

# Real-time RT-PCR



# Real-time RT-PCR

## How does COVID-19 real-time RT-PCR testing work?

### 1 Sample

A person's blood, saliva or mucus is sampled.

Chemical solutions are added to remove substances such as proteins and fats.

Mix of a person's genetic material. + The virus's RNA (if present).

### 2 From RNA to DNA

In order for PCR to work, the RNA needs to be converted into cDNA.

RT - Reverse transcription

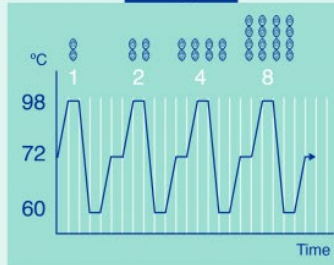
RNA → cDNA

### 3 Copying and dyeing the DNA

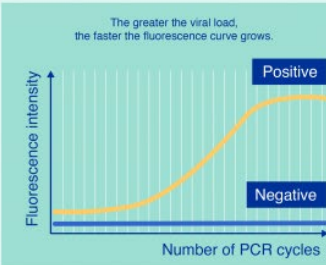
Chemical reagents, including probes with fluorescent dyes that will mark any viral cDNA found, are added in order to build copies of the genetic material.

The samples are then placed in a PCR thermocycler machine.

#### PCR cycles



Different temperature cycles trigger chemical reactions that replicate the original genetic material.



If the virus is present, the copies will generate fluorescence. The more fluorescence, the more viral material.

In about one hour, billions of copies of the original genetic material are made.

1 hour



# Digital PCR

## Droplet Digital PCR (ddPCR)

Emulsify with oil to generate droplets



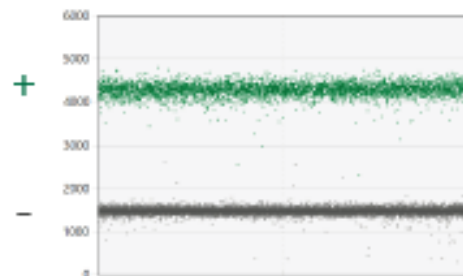
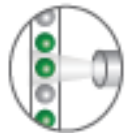
Size and number of droplets can be variable



Tube-based PCR amplification



Droplets counted with laser



Sample  
(DNA, primers, probes,  
reaction components)



## Physical partitioning for dPCR

Partition sample on nanowell plate



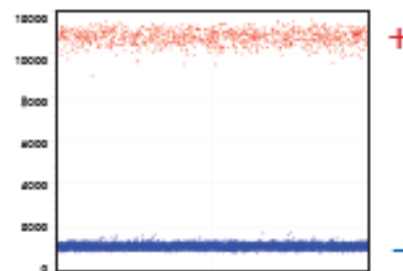
Uniform size and  
consistent number  
of partitions



Amplification on  
flat thermocycler



Plate scanned and  
image collected

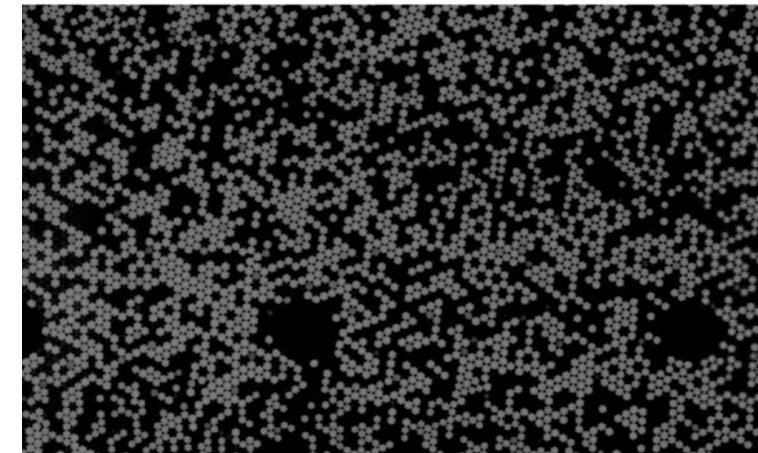


Partition sample  
into microreactions

Amplify  
target regions

Collect  
data

Visualize results  
(1D charts)



# Isothermal PCRs

## RCA

## Rolling Circle Amplification

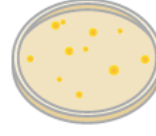
### Input materials



Purified plasmid



Liquid culture



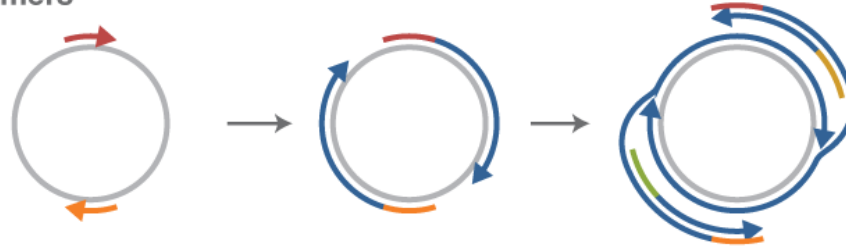
Cultured colony



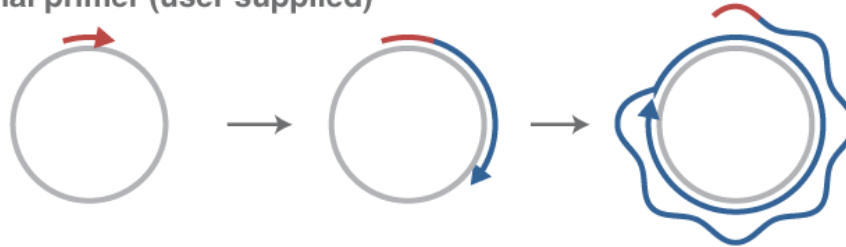
Glycerol stock

### Rolling Circle Amplification (RCA)

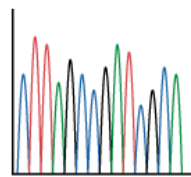
#### Random primers



#### Unidirectional primer (user supplied)



### Applications



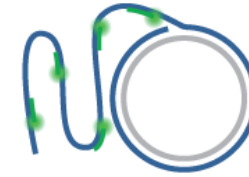
Sequencing



Cell-free DNA enrichment



Cell-free protein expression

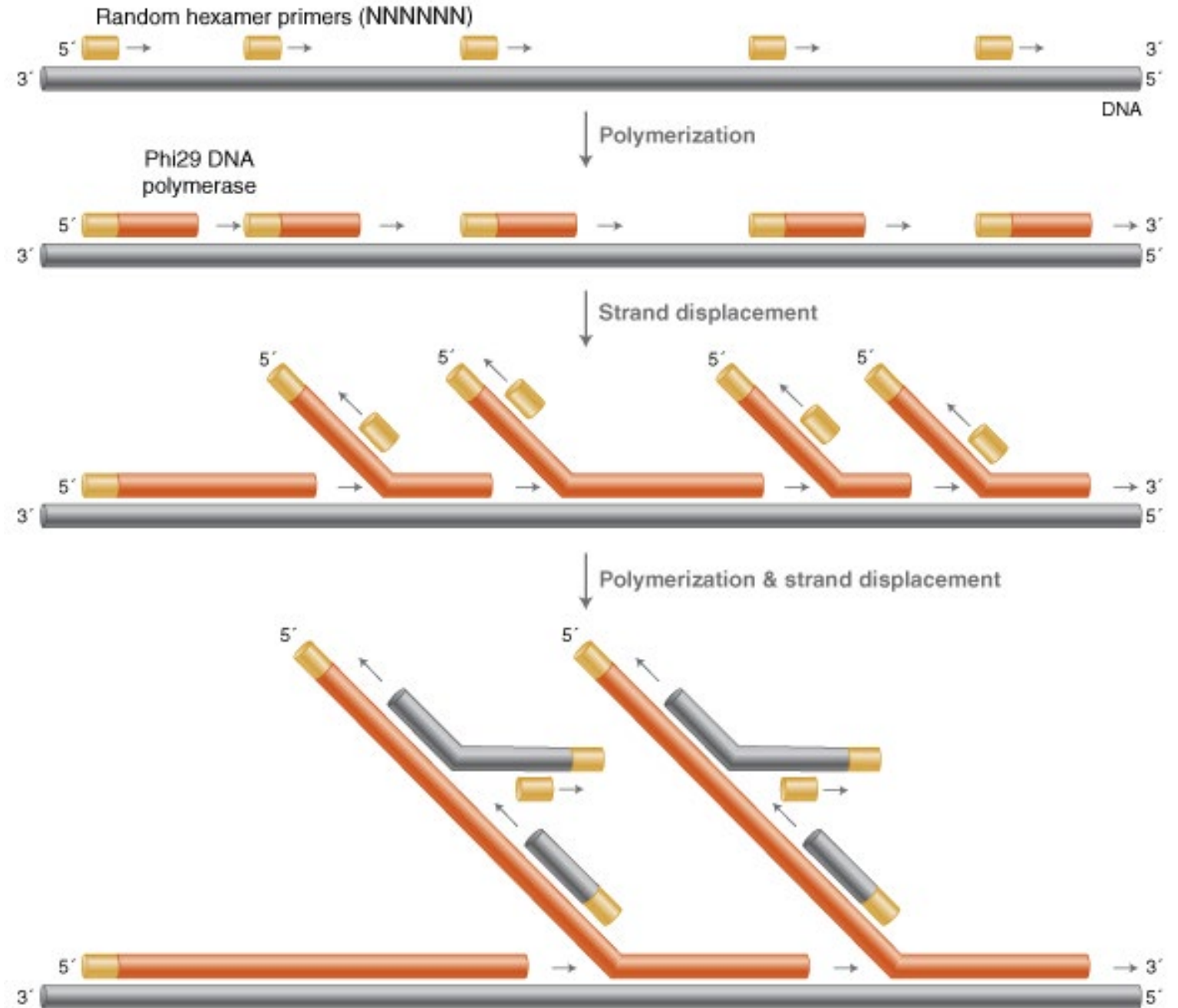


Biosensor

# Isothermal PCRs

## WGA

## Whole Genome Amplification

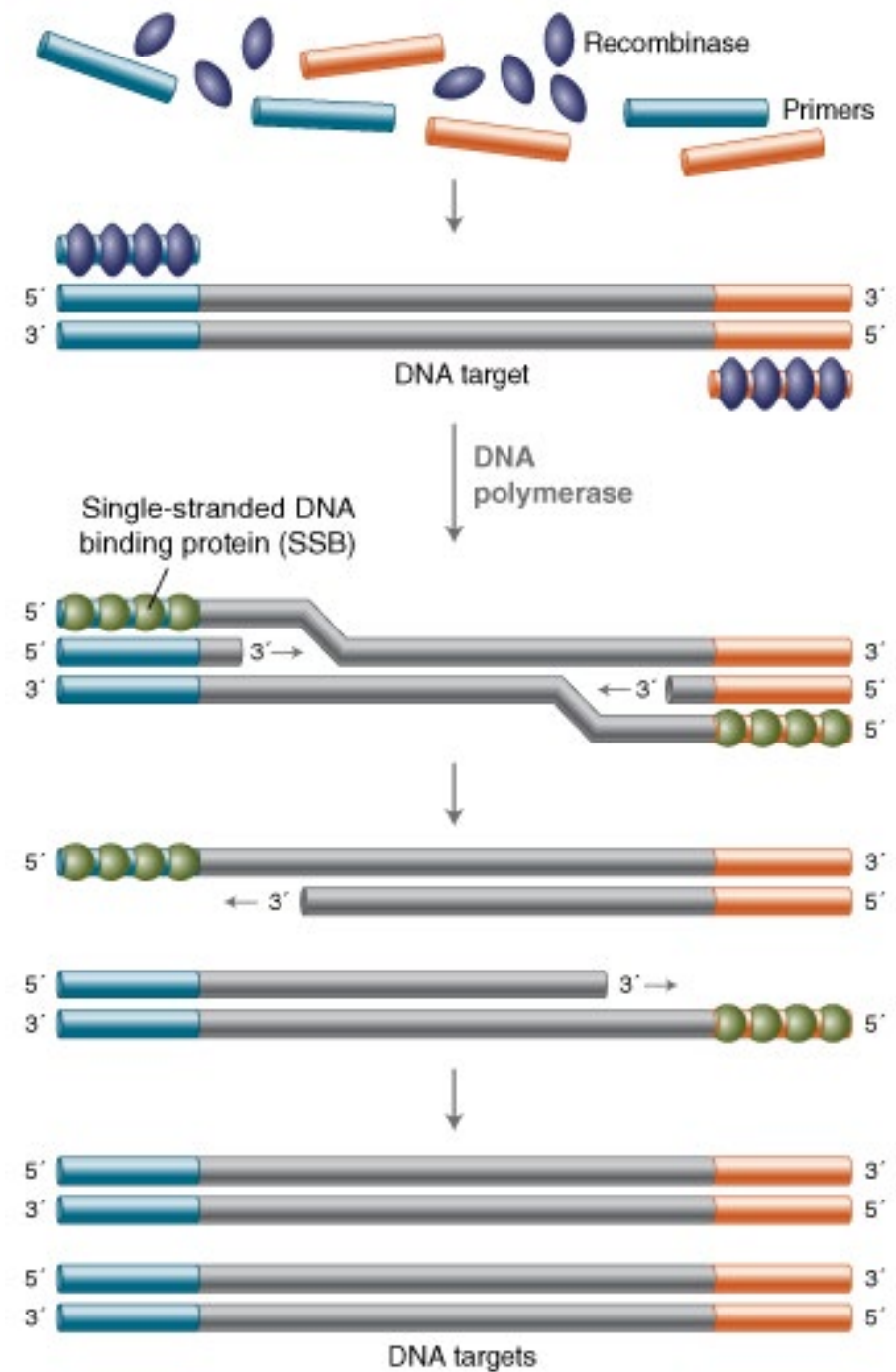




# Isothermal PCRs

## RPA

## Recombinase Polymerase Amplification

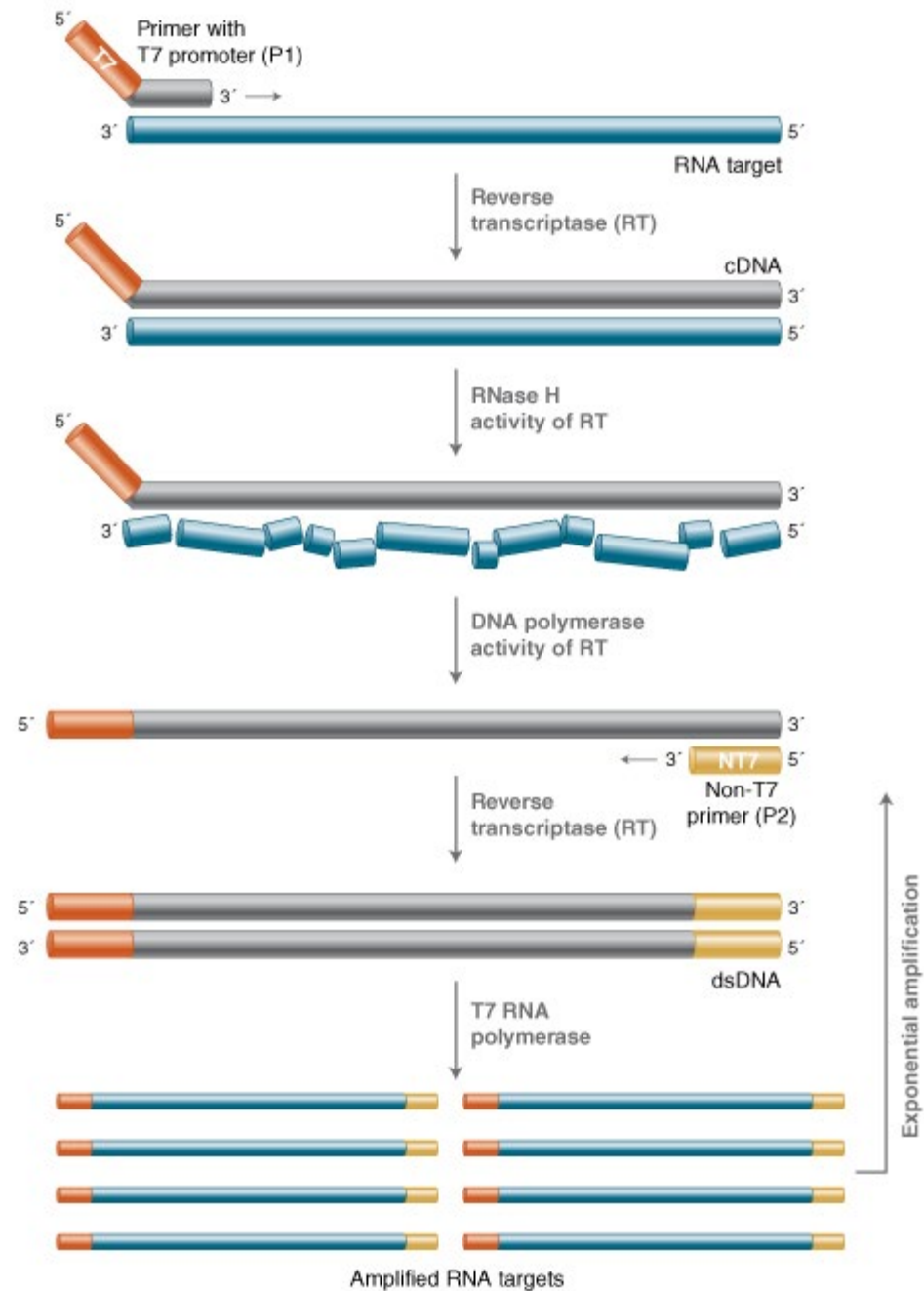




# Isothermal PCRs

## NASBA

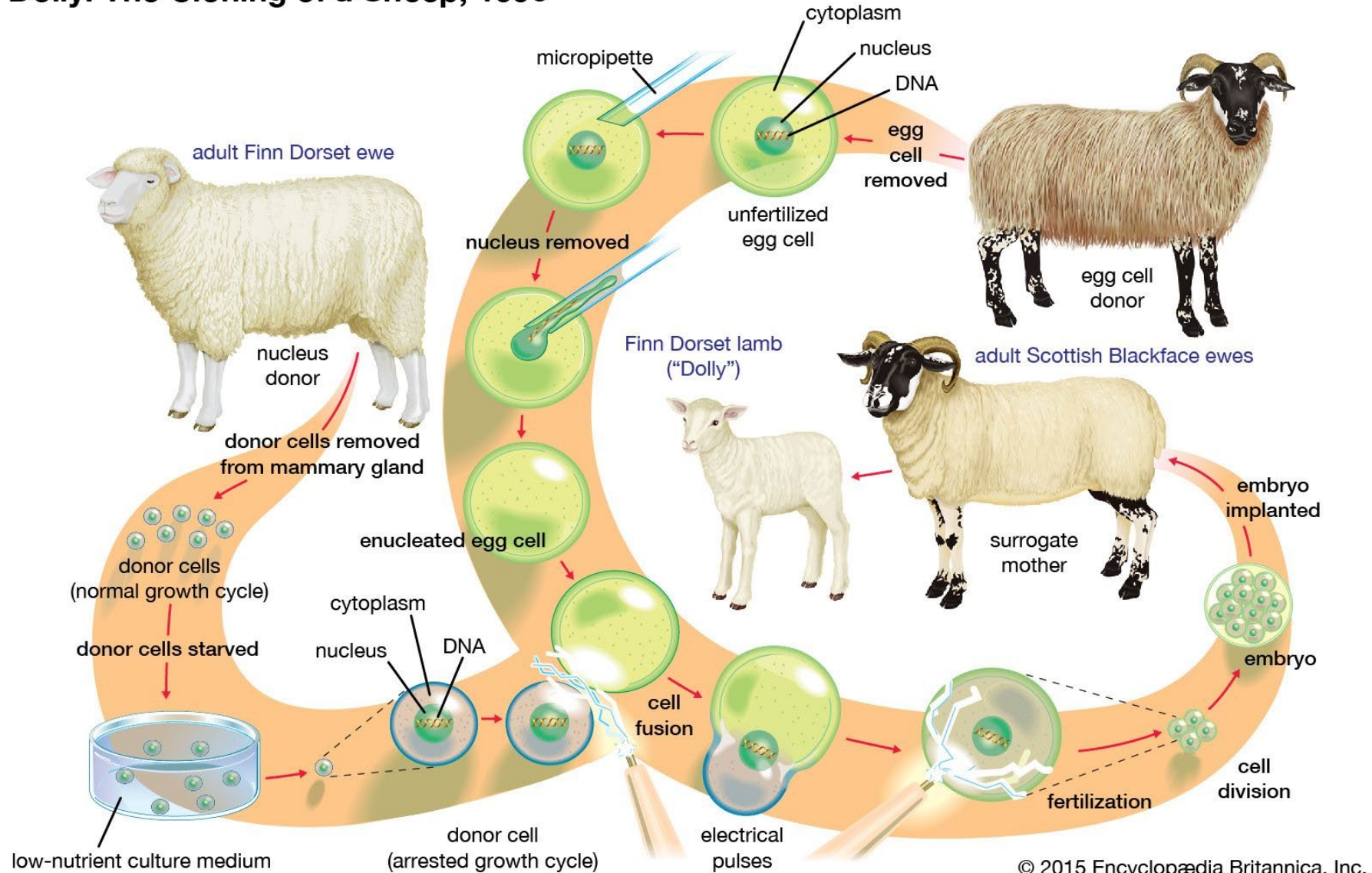
### Nucleic Acid Sequenced Based Amplification and Transcription Mediated Amplification



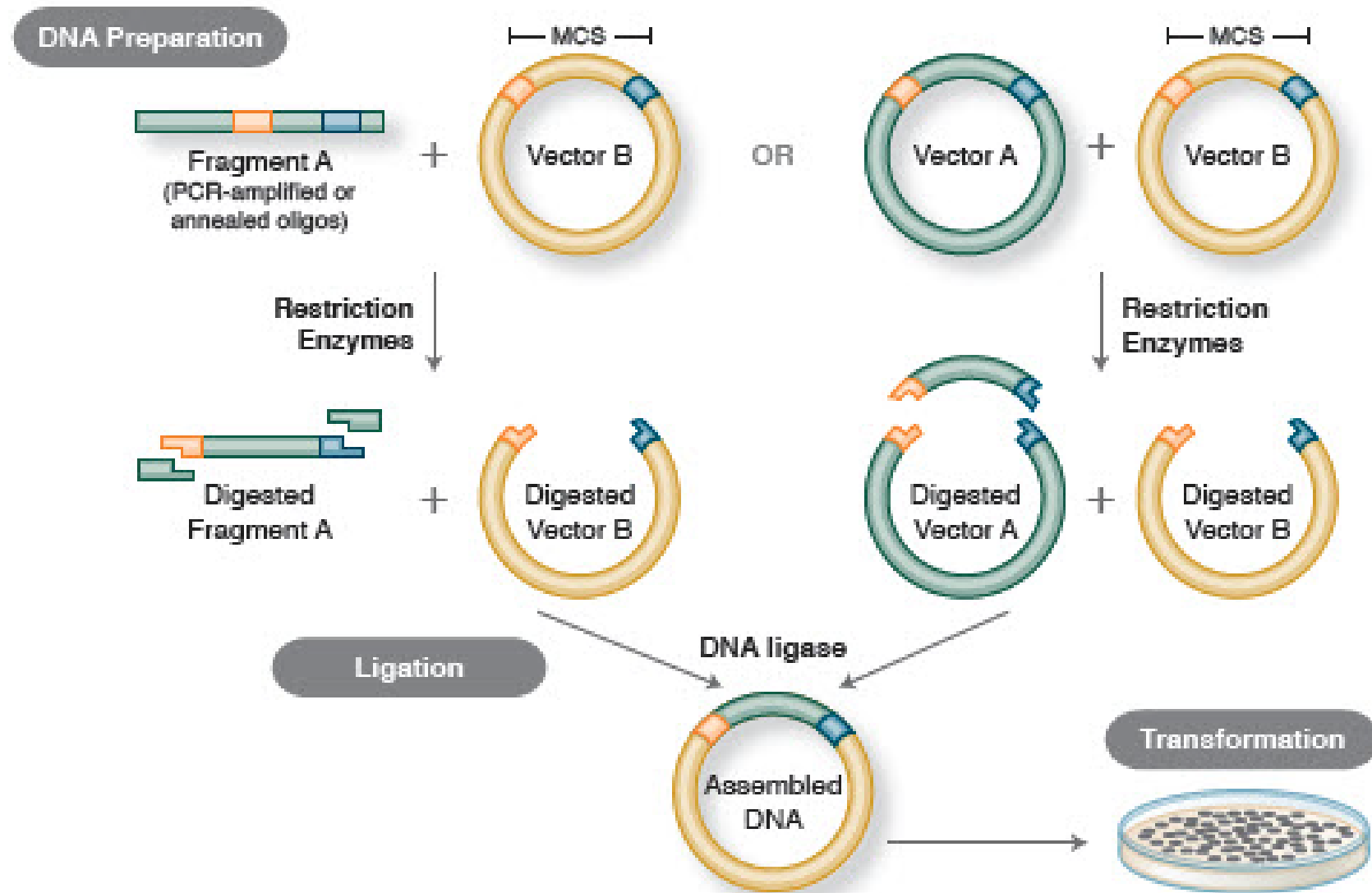
# Molecular Cloning

# Not this cloning!!!

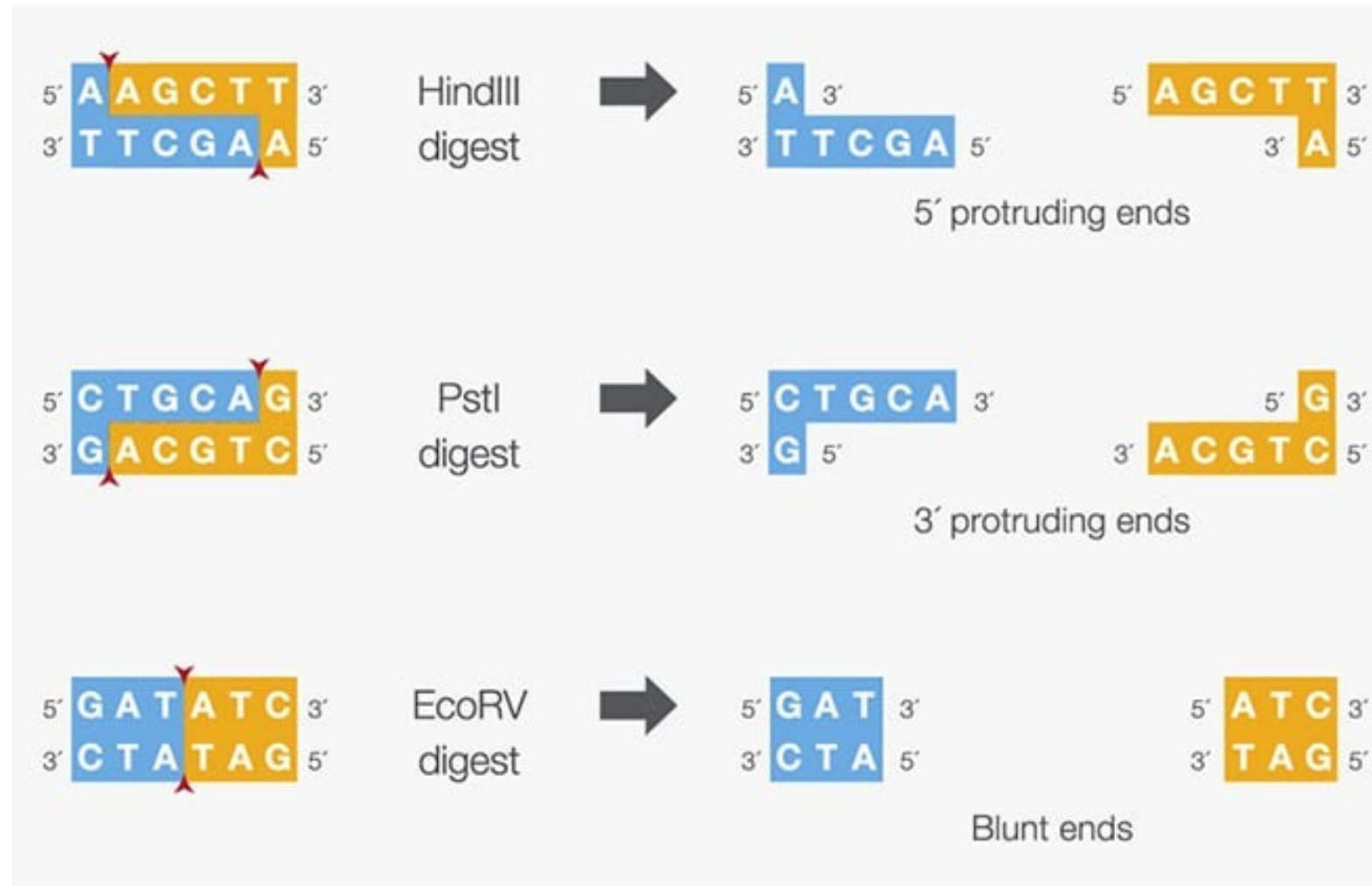
## Dolly: The Cloning of a Sheep, 1996



# Molecular Cloning

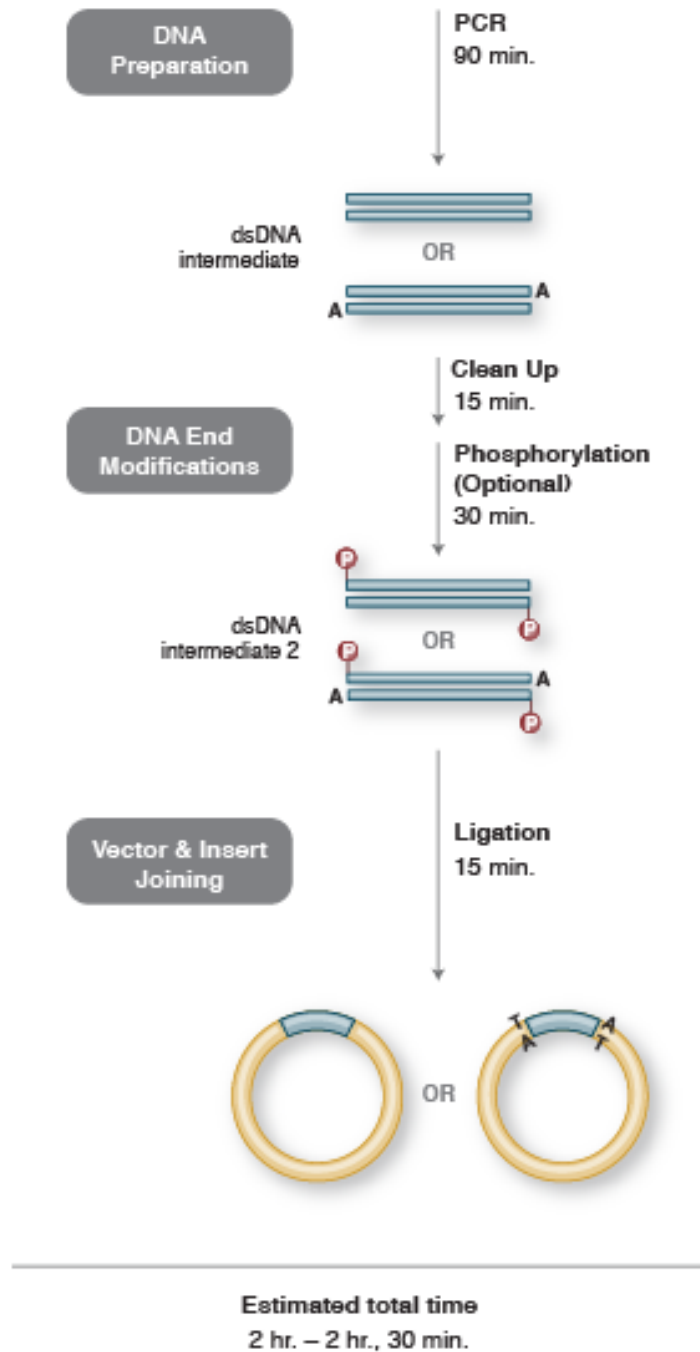


# Restriction Enzymes

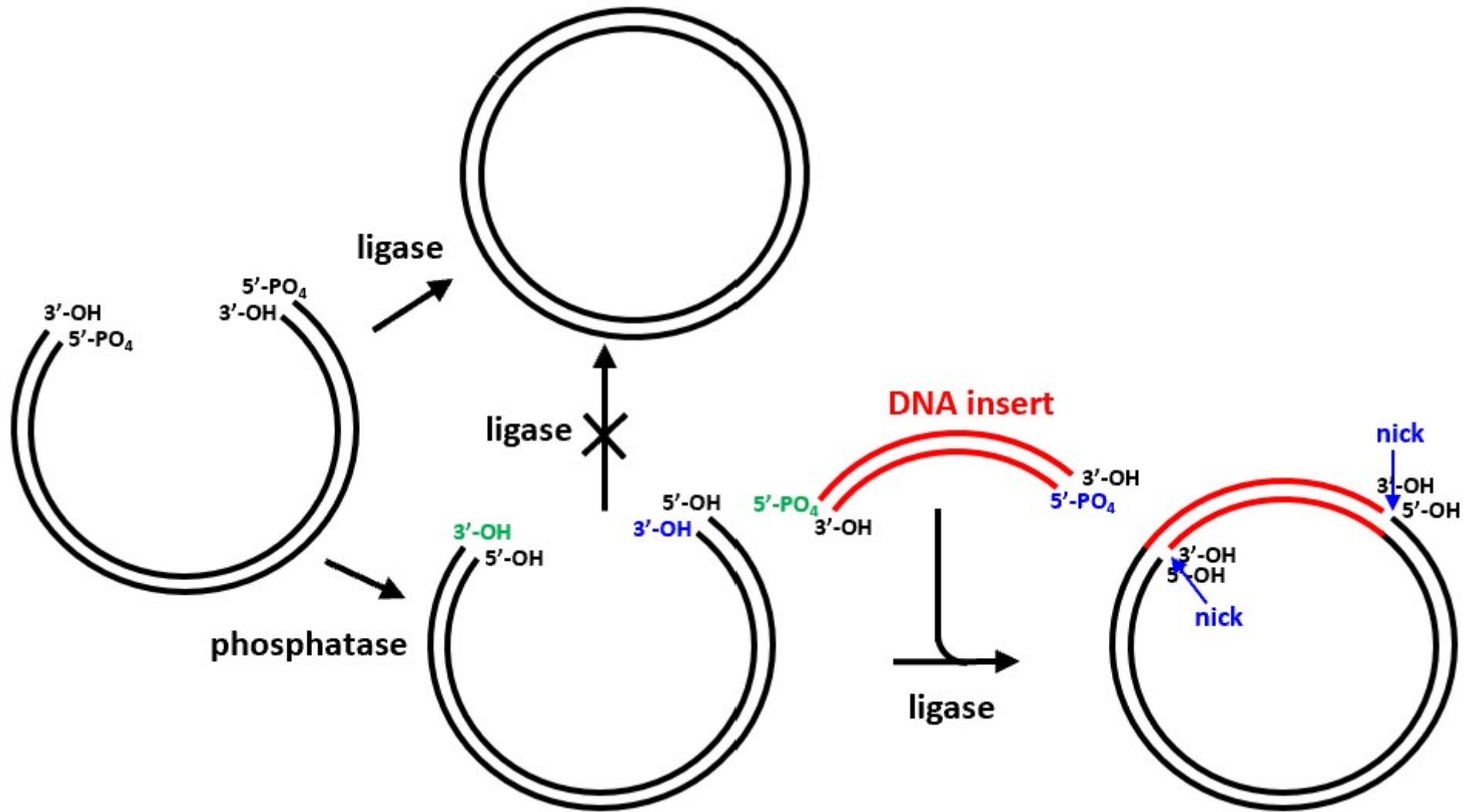


# Phosphorylation

(needed for blunt end cloning of PCR products)



# De-phosphorylation



# Cloning Steps Summary:

## Design and Preparation of Insert and Vector:

- Select the **DNA fragment (insert)** and an appropriate **plasmid vector**.
- Add matching **restriction sites** via PCR if needed.

## Restriction Digest:

- Digest both **insert** and **vector** with the same or compatible **restriction enzymes** to create compatible ends.

## Phosphorylation and Dephosphorylation (Vector Only):

- Phosphorylate the PCR product if necessary using **T4 polynucleotide kinase**
- Treat the **digested vector** with **alkaline phosphatase** (e.g., CIP or SAP) to remove 5' phosphate groups.
- This prevents **vector self-ligation** (re-circularization without insert).

## Purification:

- Purify both digested vector and insert DNA using gel extraction or column purification to remove enzymes and unwanted fragments.

## Ligation:

- Mix purified **insert** and **dephosphorylated vector** with **T4 DNA ligase**.
- The insert must have 5' phosphates for ligation to occur.

## Transformation:

- Introduce the ligation product into **competent E. coli** cells via heat shock or electroporation.

## Selection:

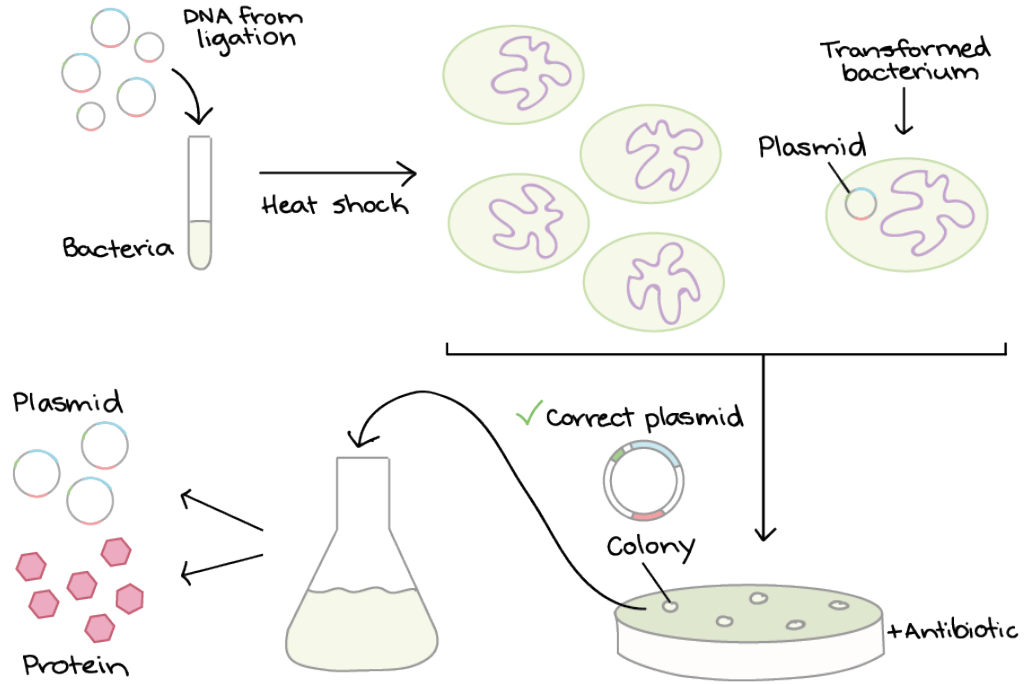
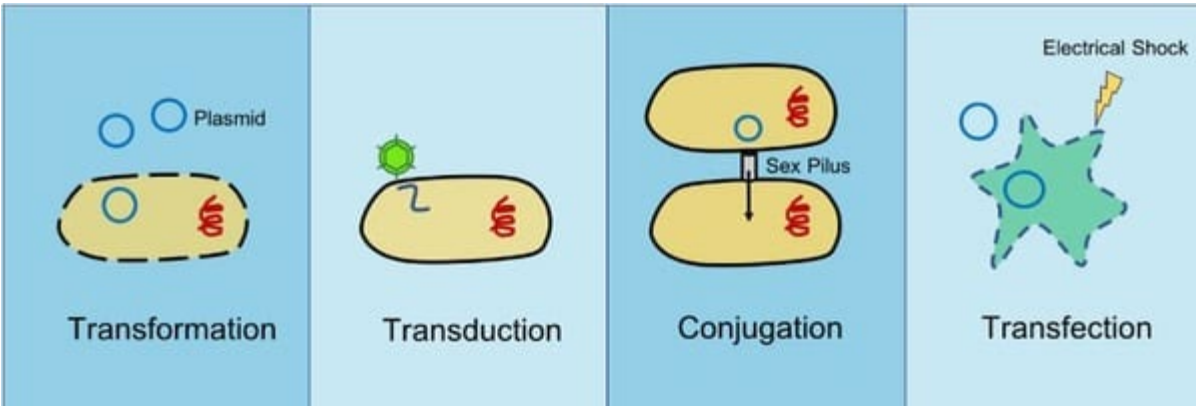
- Plate transformed cells on **antibiotic-containing agar** to select for colonies carrying the plasmid.

## Screening and Confirmation:

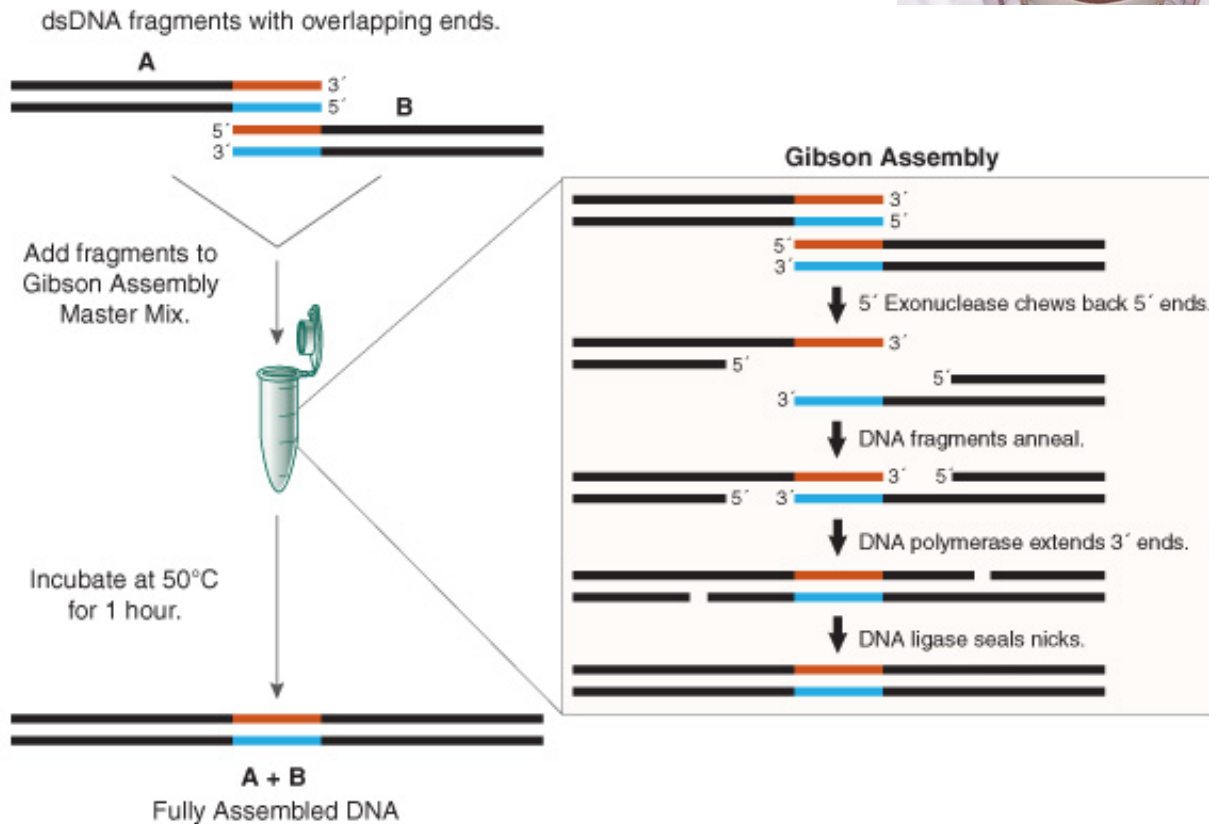
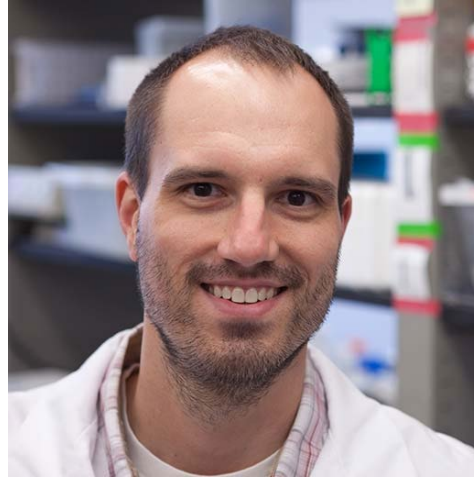
- Pick colonies and verify insert presence by **colony PCR**, **restriction digestion**, or **sequencing**.



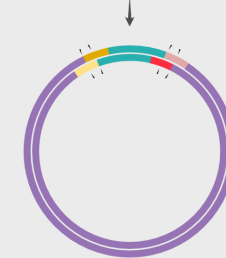
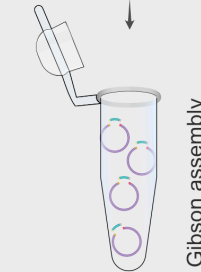
# Transformation / Transfection



# Gibson Assembly

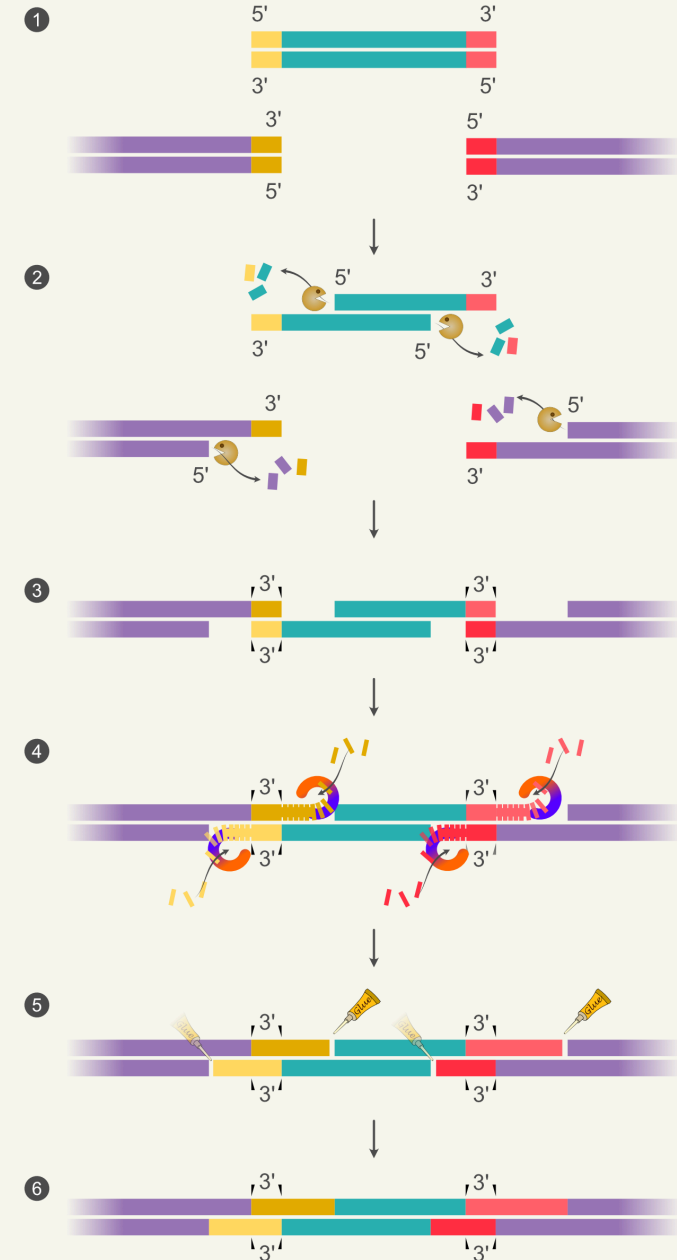


## Gibson assembly - Single insert



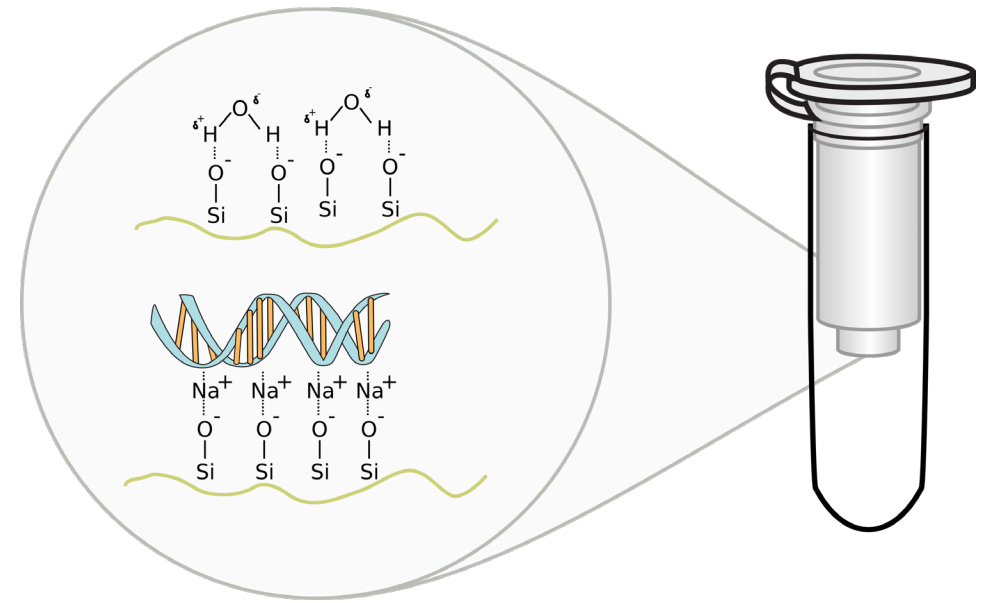
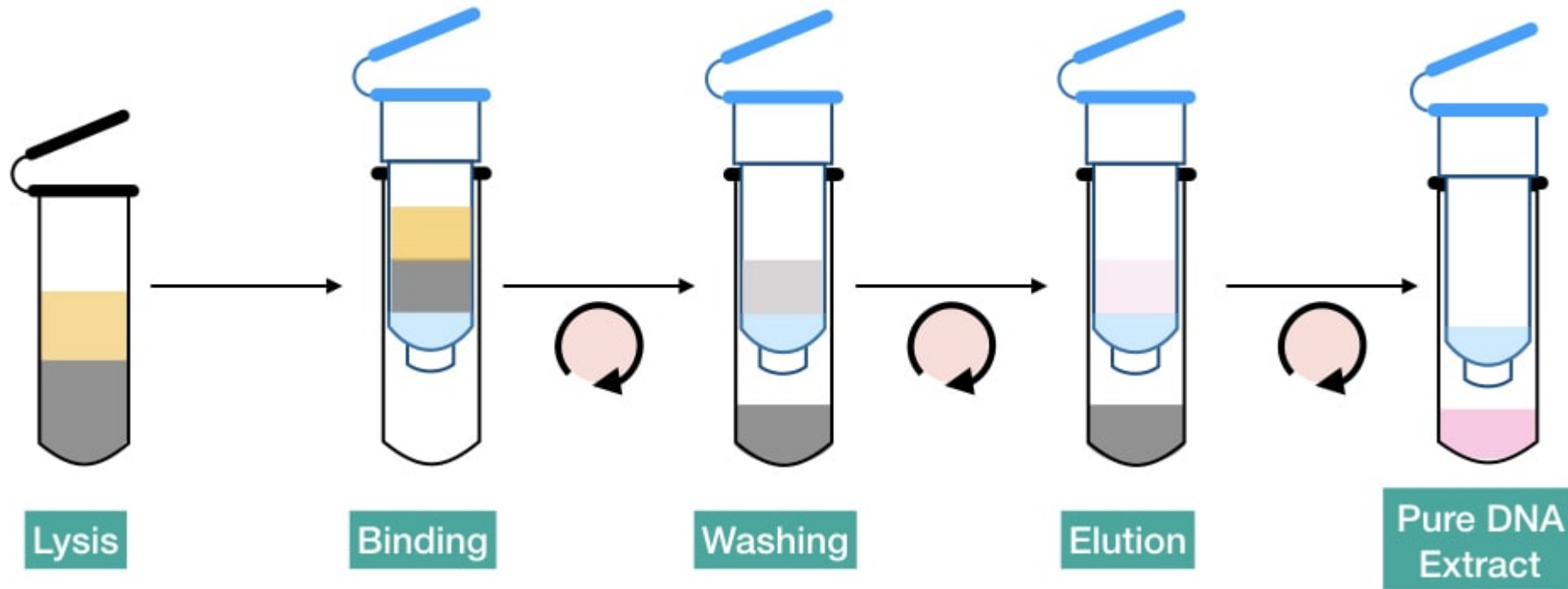
- T5 Exonuclease
- DNA polymerase
- Ligase
- Newly replicated DNA
- Homology sequence used in the primer design
- Homology regions of adjuscent fragments

sharebiology.com



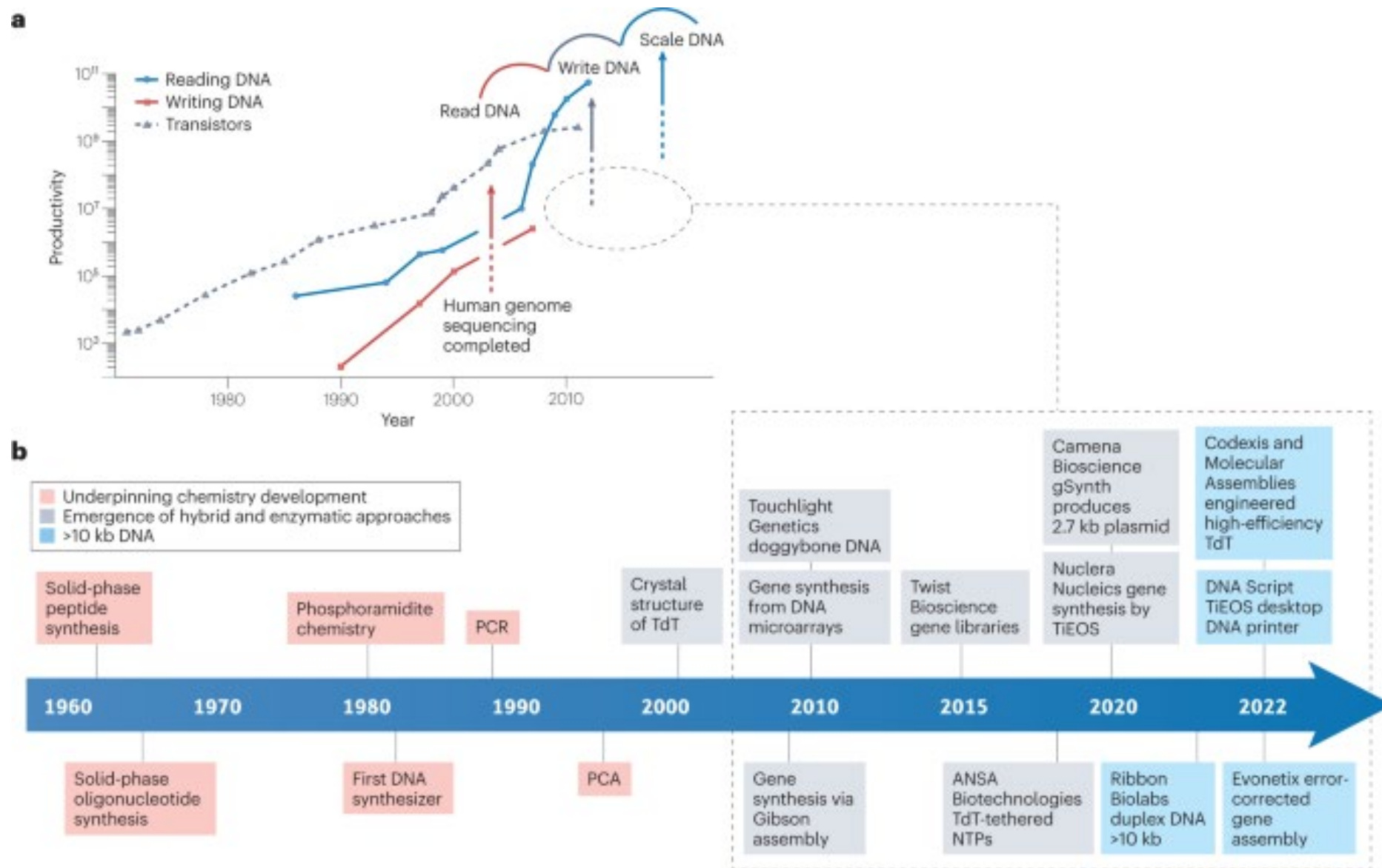
# DNA Purification

# Spin column DNA purification

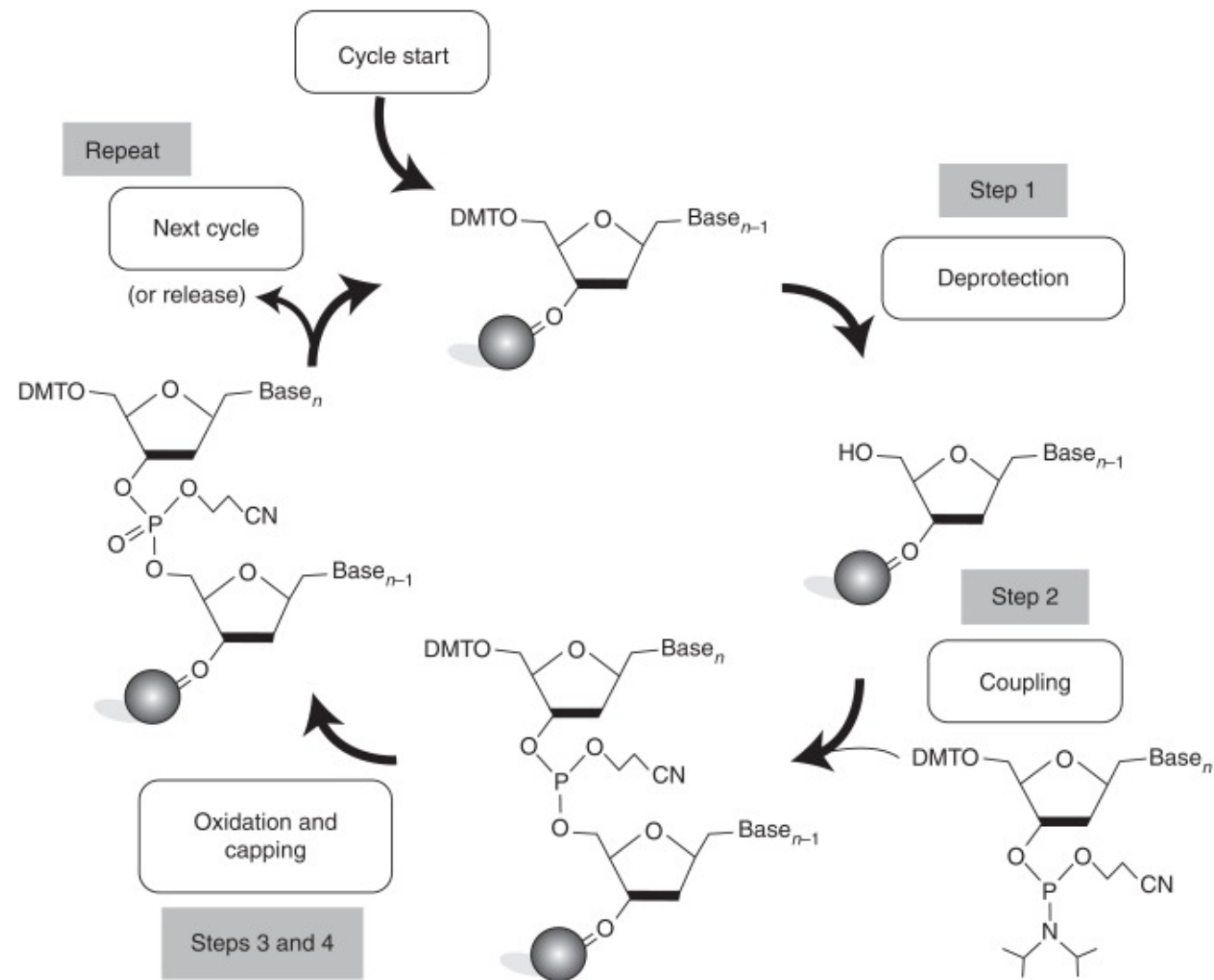


# DNA Synthesis

# DNA Synthesis History

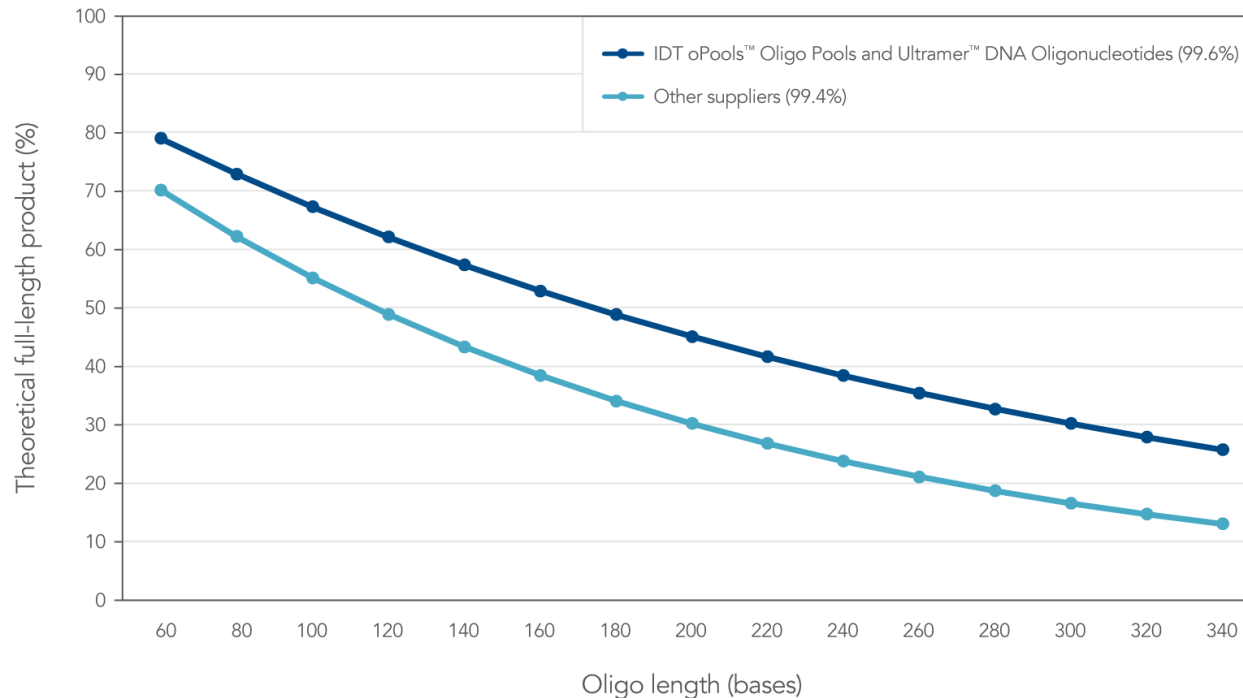


# Solid Phase Synthesis



# Limits of Solid Phase Synthesis

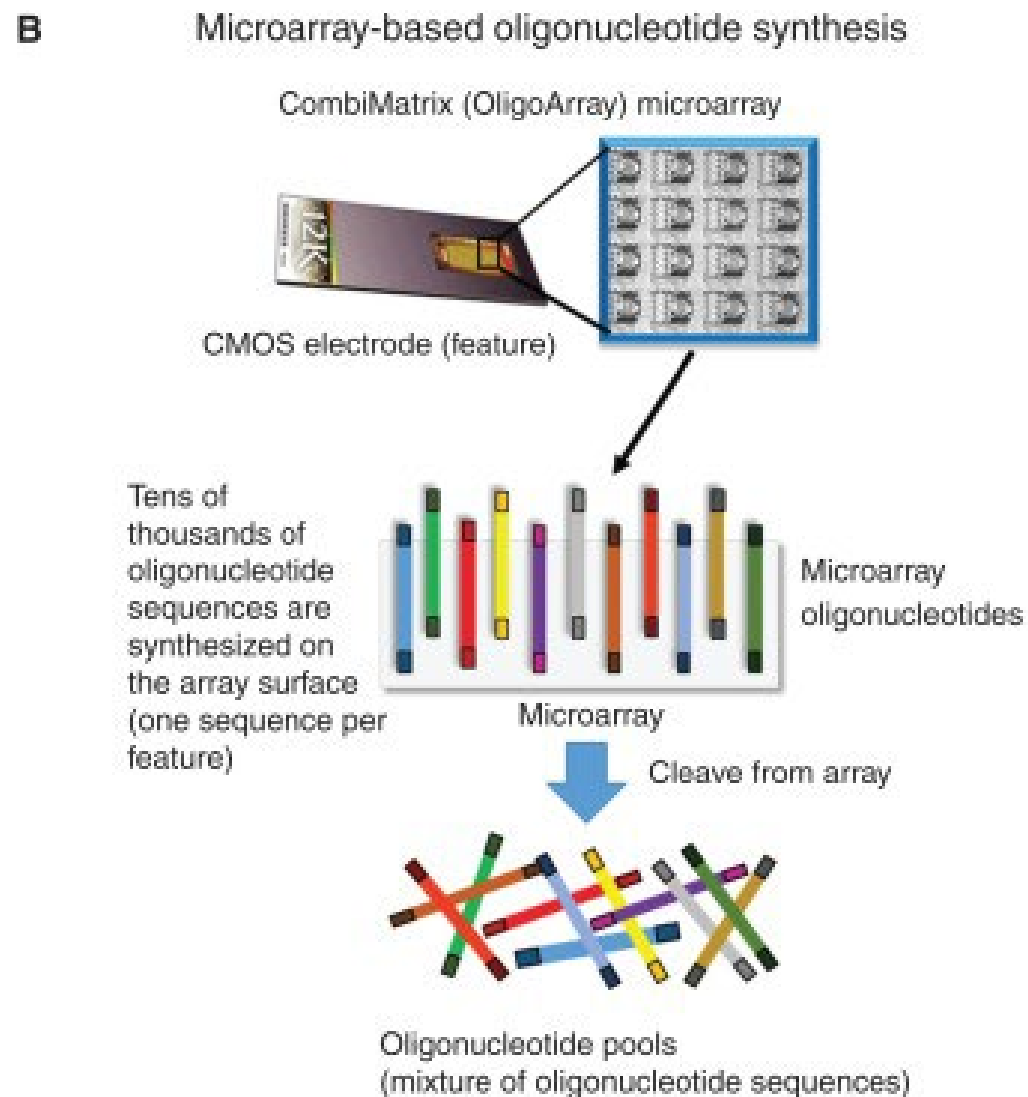
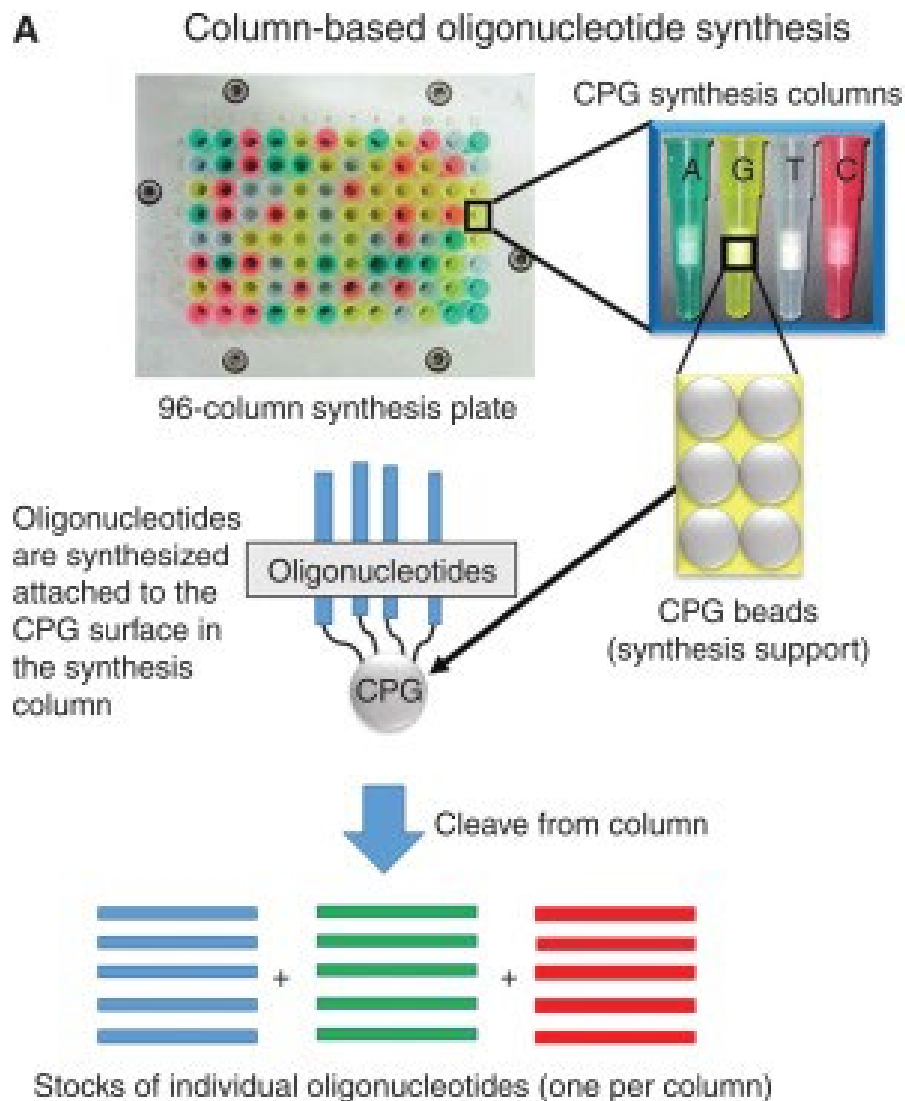
- Throughput is limited (~48-96 oligos at a time)
- Oligo Length is limited to 200 – 300 nt (coupling efficiency is ~99.5%)



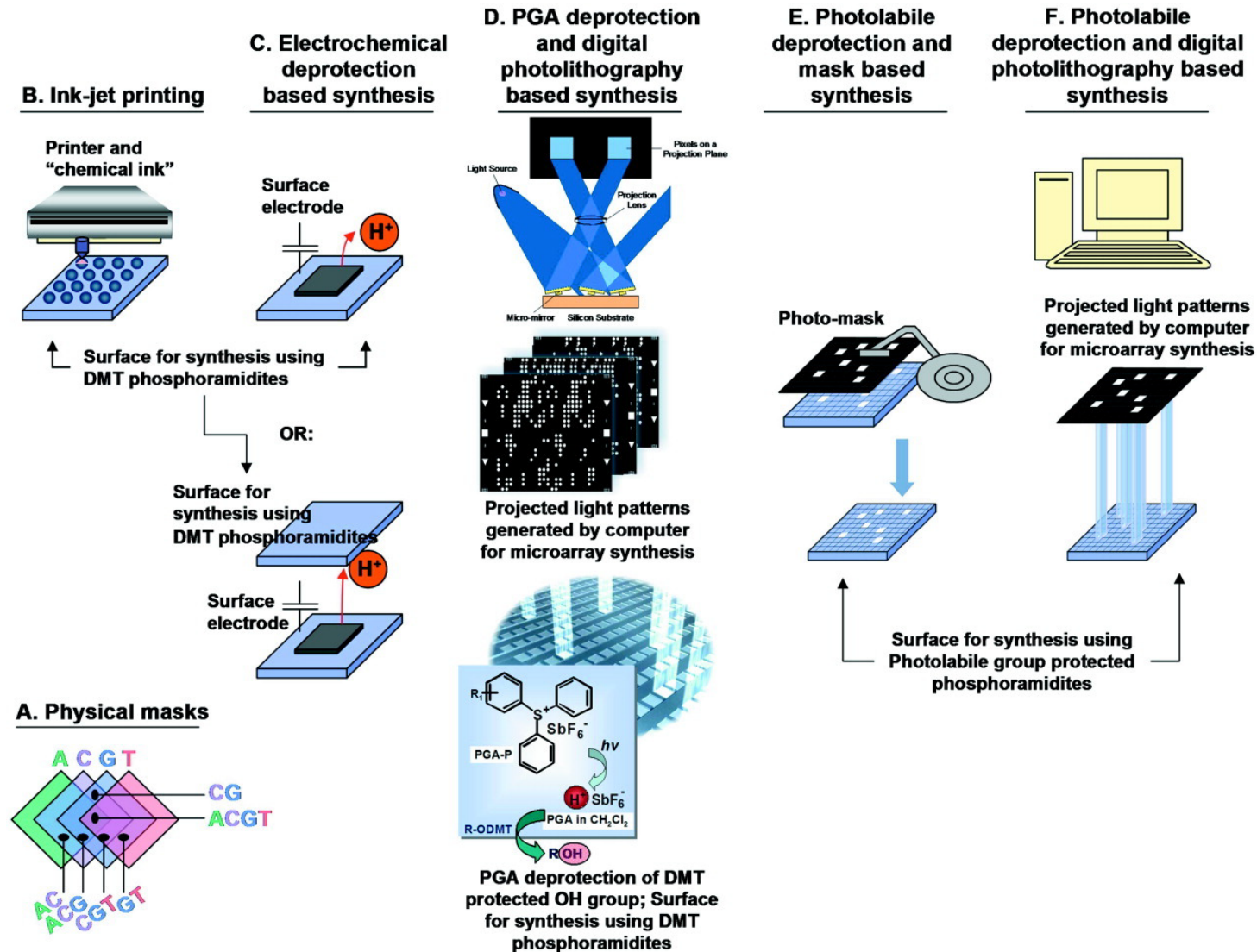


Microarray-based oligo synthesis  
(increased throughput)

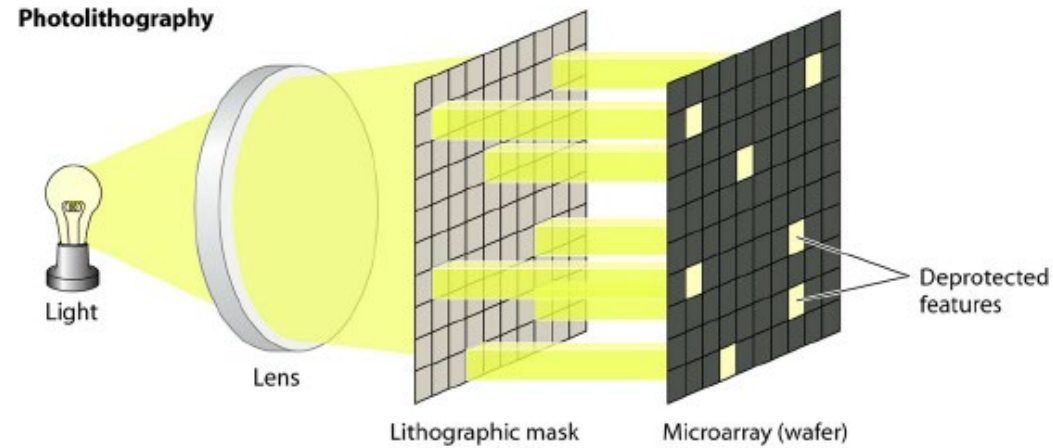
# Microarray-based oligo synthesis



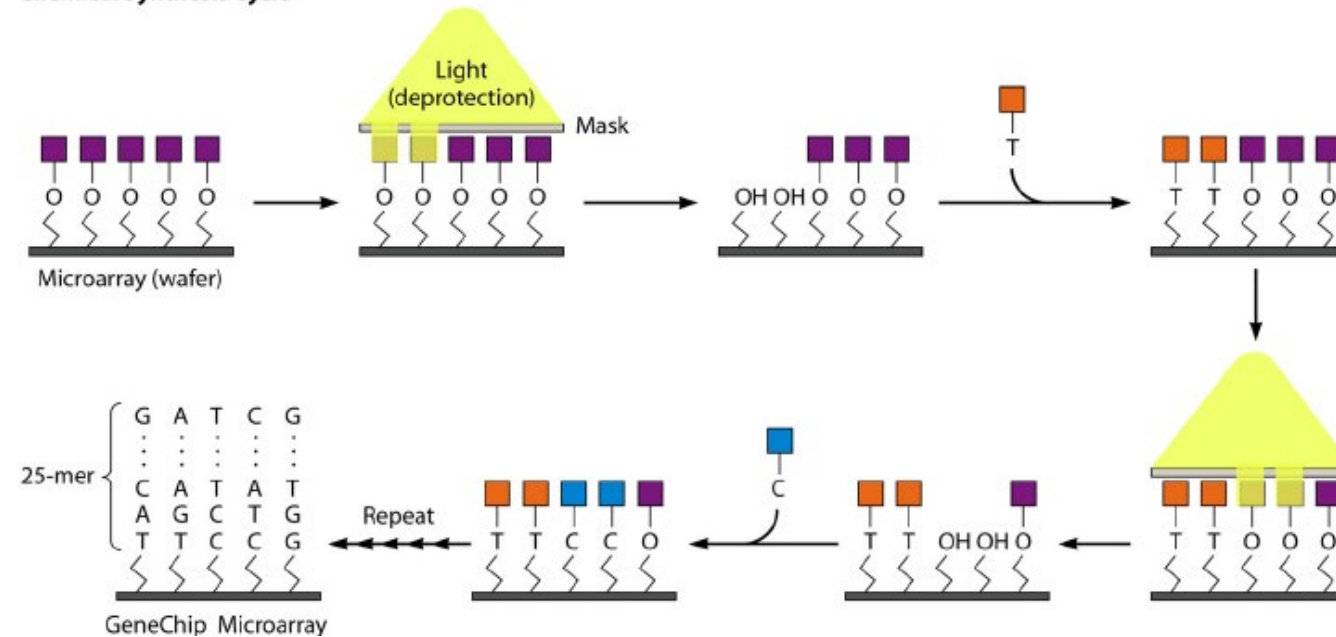
# Microarray-based oligo synthesis: inkjet and digital photolithography



# Digital Photolithography



**Chemical Synthesis Cycle**

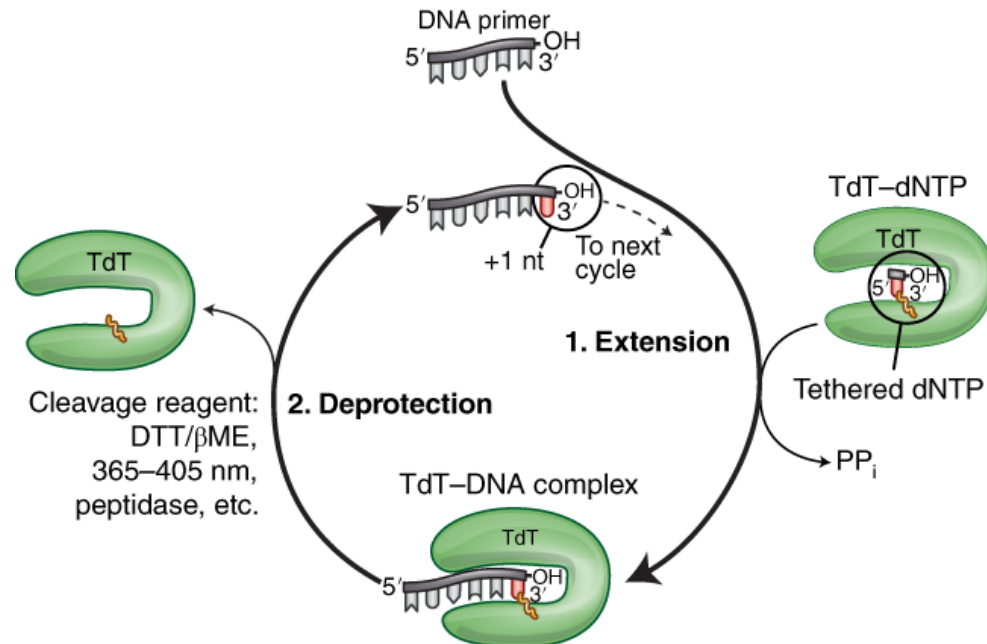


# Enzyme Based Oligo Synthesis (increased cycling efficiency)

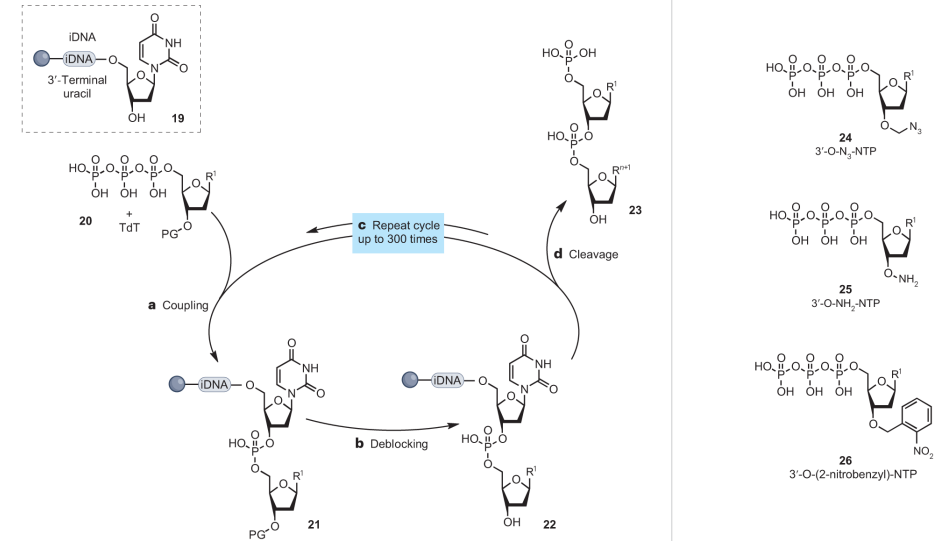
# TiEOS: template-independent enzymatic oligonucleotide synthesis

Enzyme:

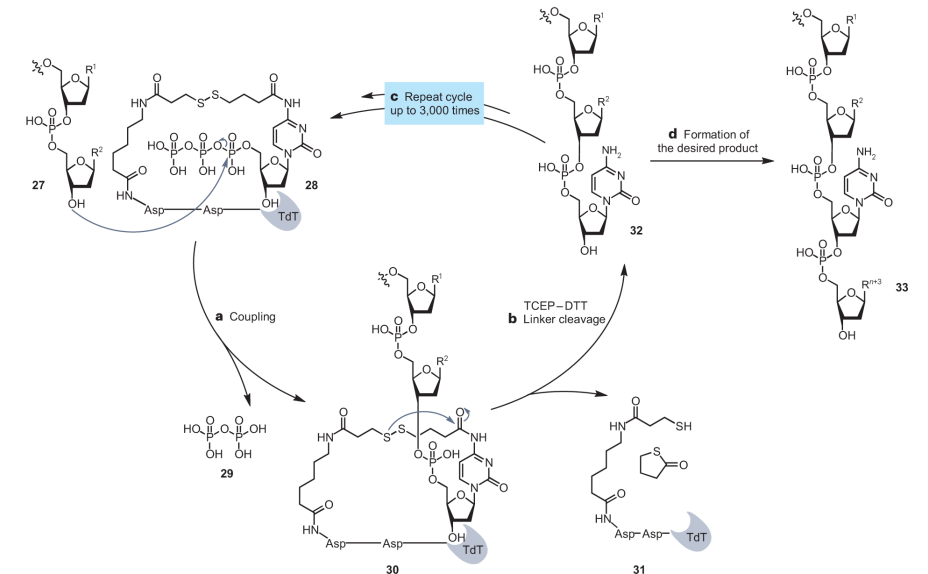
- terminal deoxynucleotidyl transferase (TdT)
- Elongation cycle efficiency of 99.7% or potentially higher



**A** TiEOS: the 3'-protected NTP approach

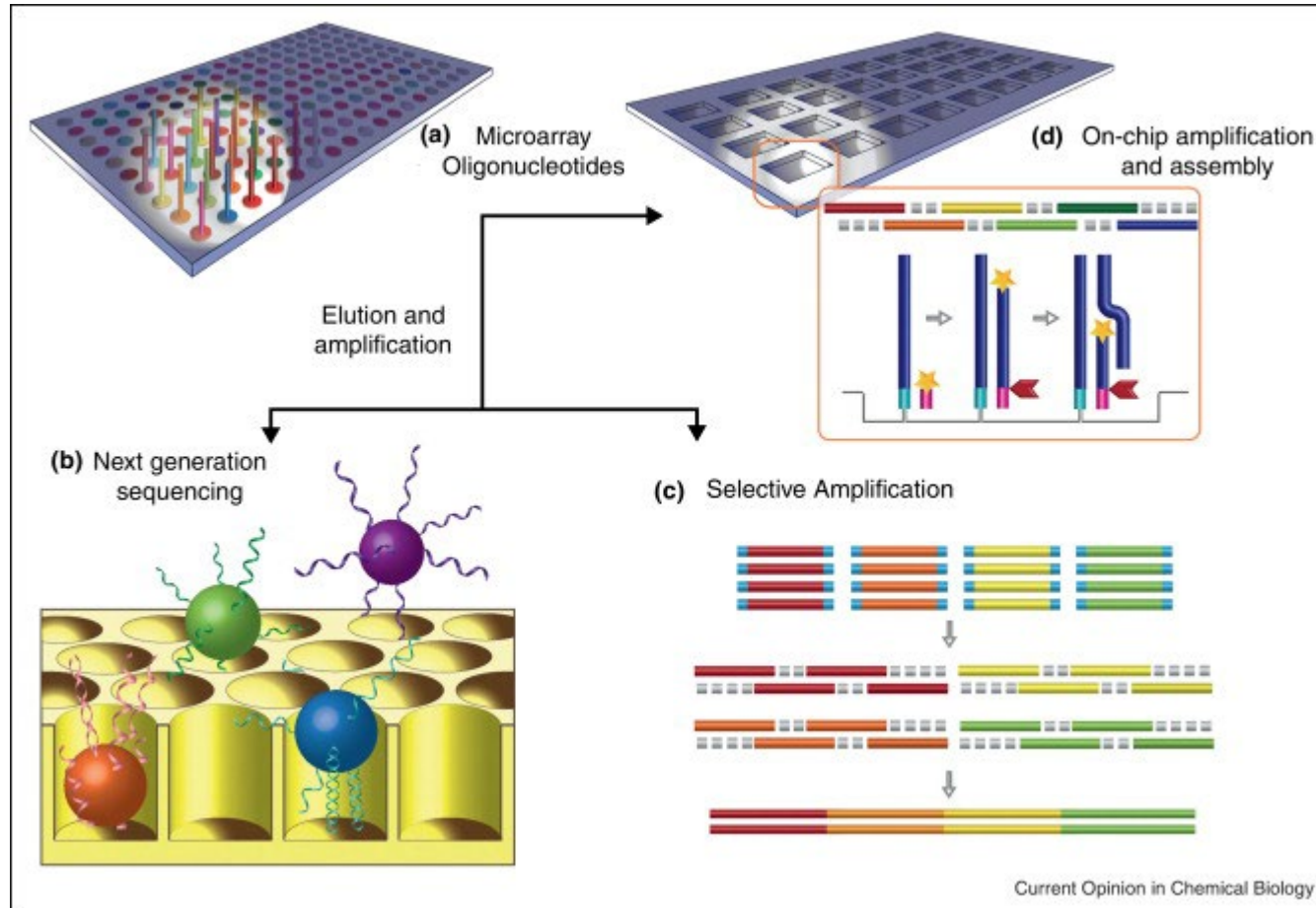


**C** TiEOS: the tethered NTP approach

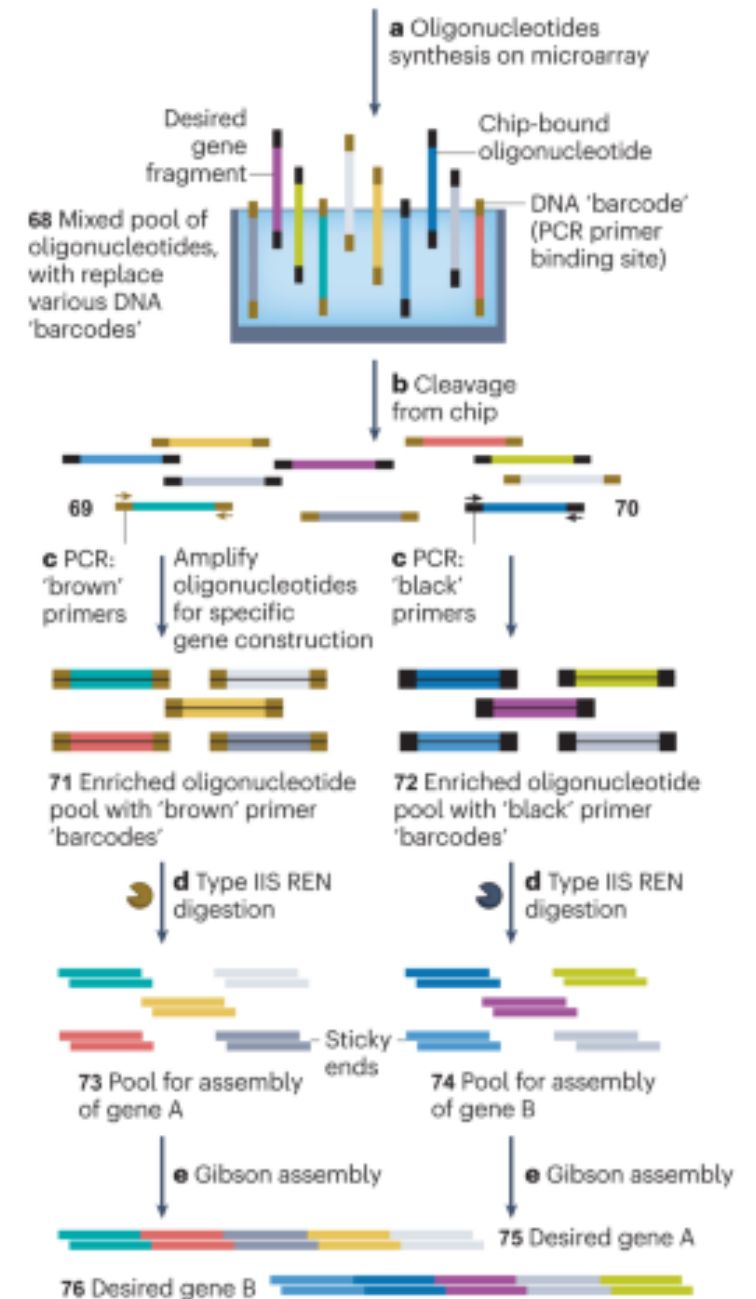


# Gene and Genome Synthesis

# Gene Synthesis



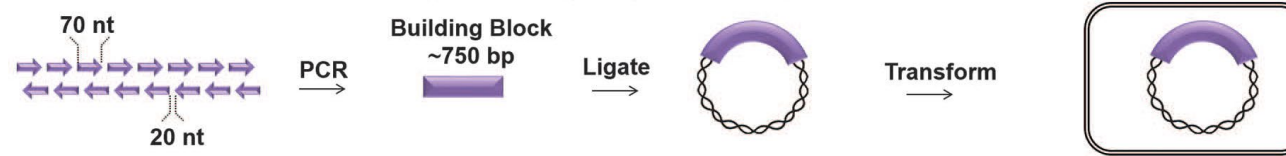
## B Gene synthesis from DNA microarrays



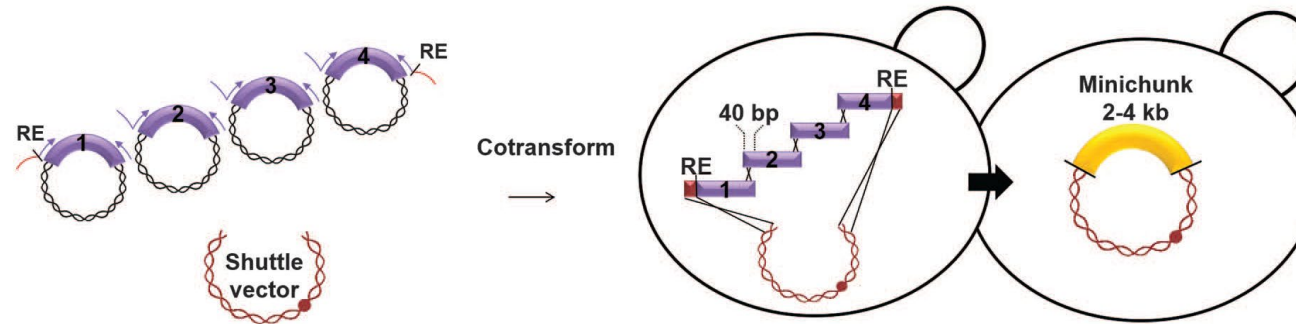


# Genome Synthesis

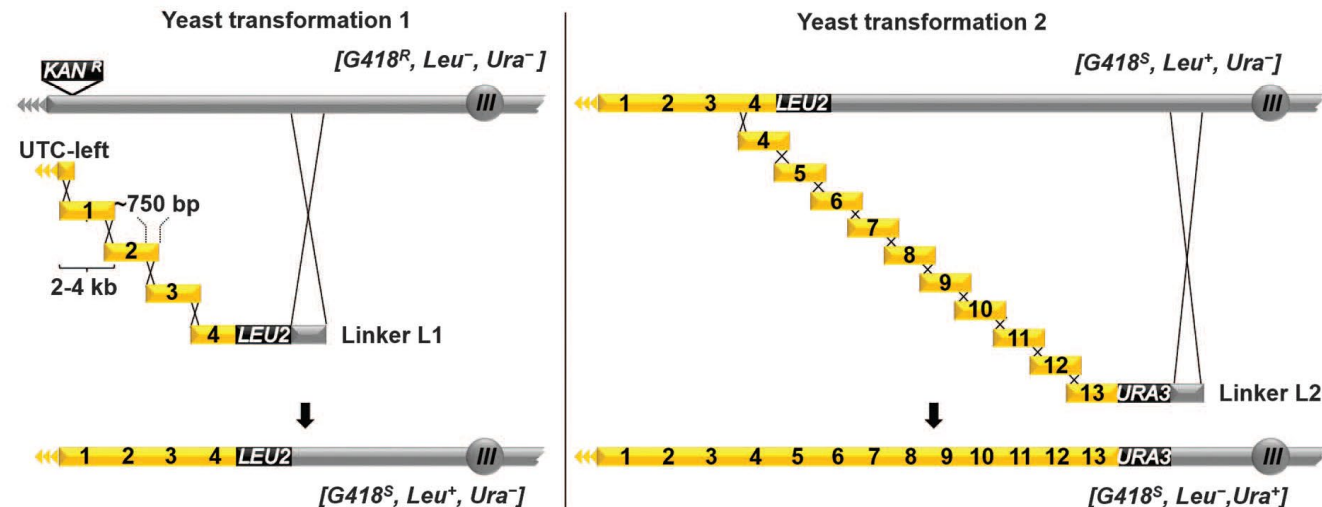
## A Step 1: Synthesize Building Blocks (BBs) from oligonucleotides



## B Step 2: Assemble 2-4 kb minichunks

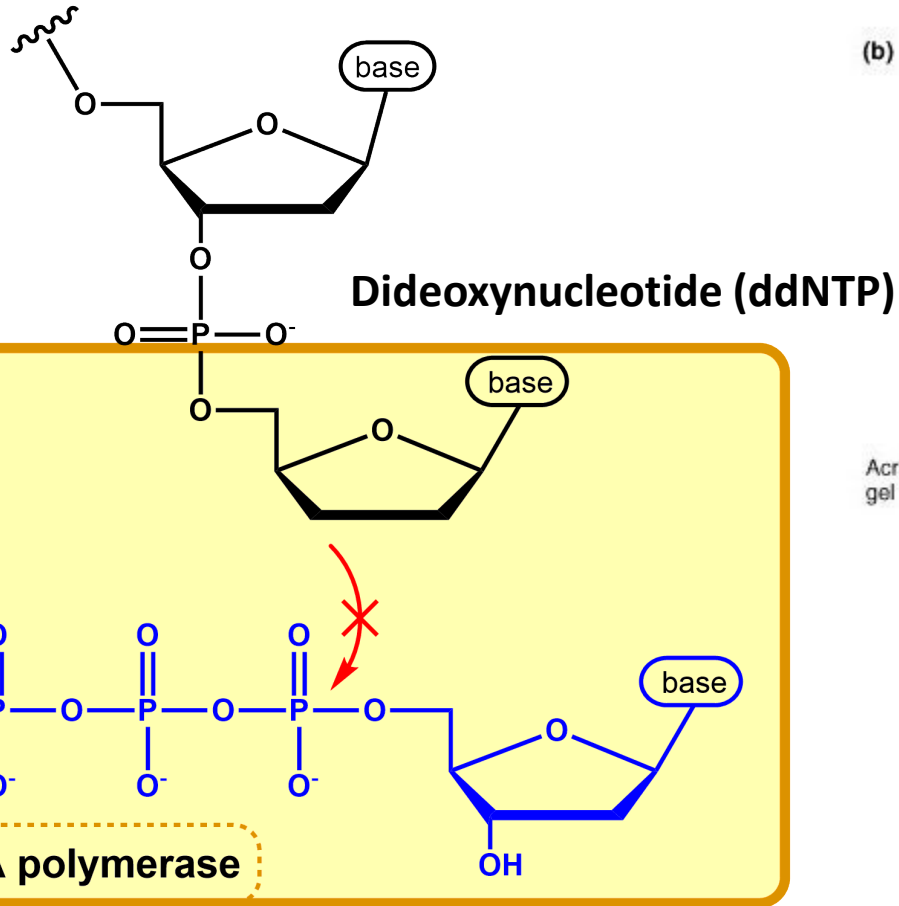


## C Step 3: Replace native III with minichunks

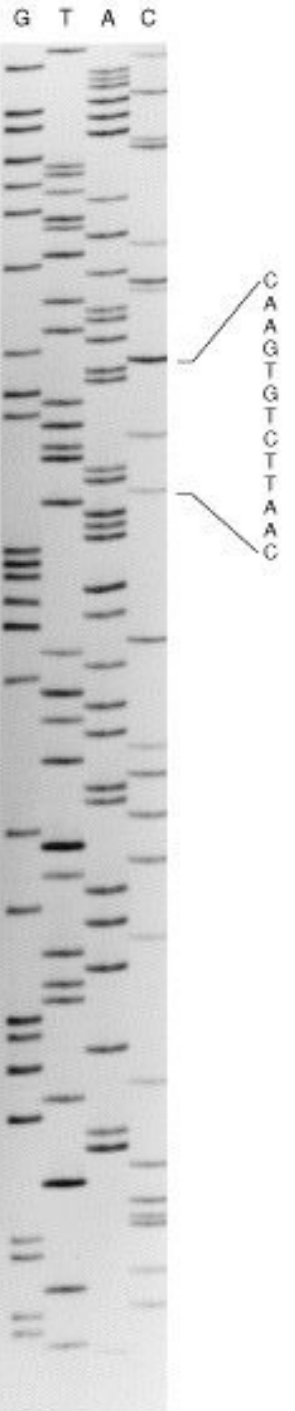
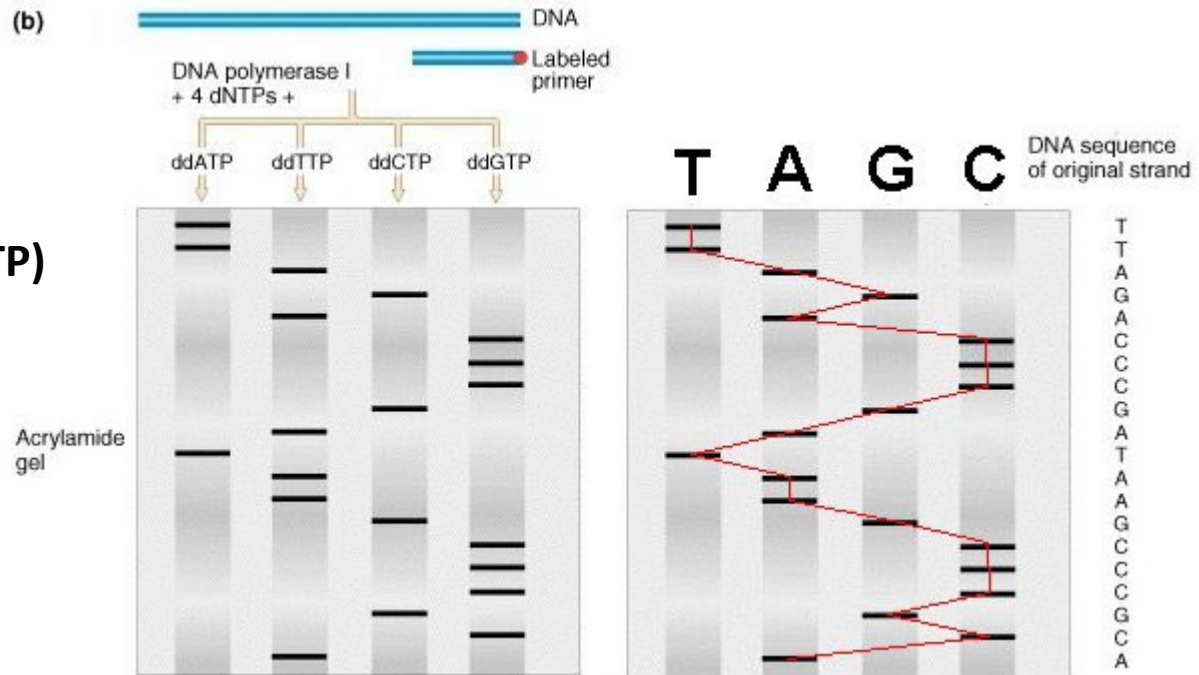


# Sequencing

# Radioactive DNA Sequencing



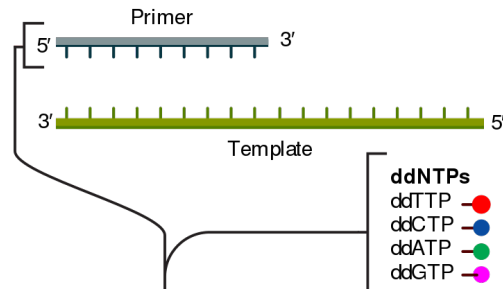
(b)



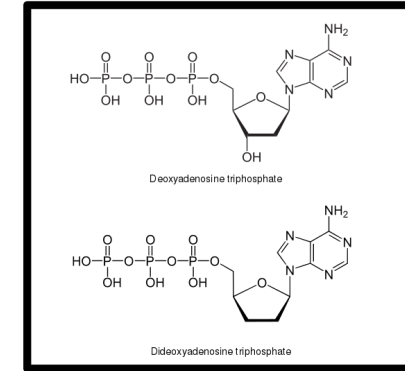
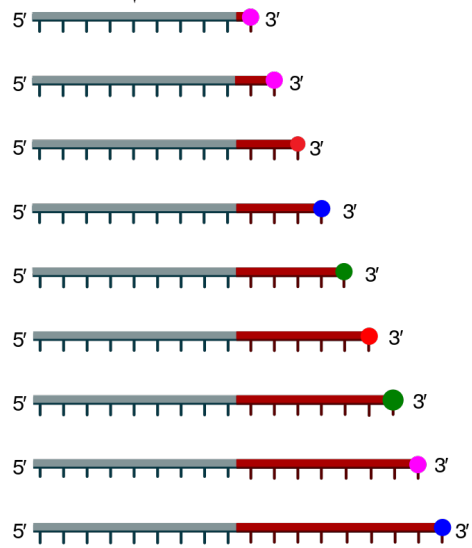
# Sanger Sequencing

## ① Reaction mixture

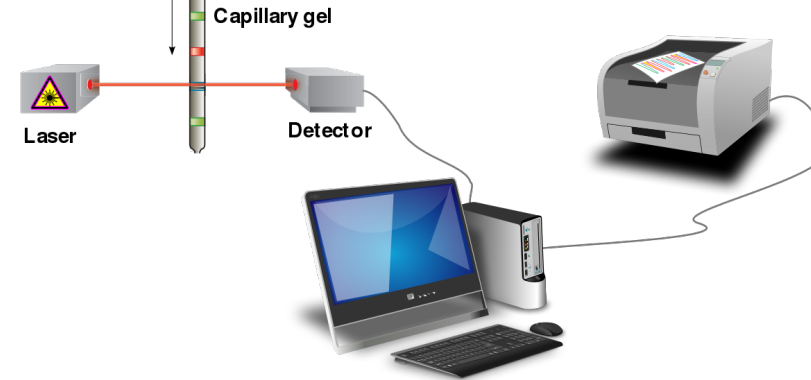
- ▶ Primer and DNA template
- ▶ DNA polymerase
- ▶ ddNTPs with flouorochromes
- ▶ dNTPs (dATP, dCTP, dGTP, and dTTP)



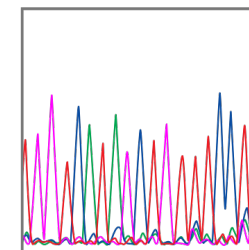
## ② Primer elongation and chain termination



## ③ Capillary gel electrophoresis separation of DNA fragments

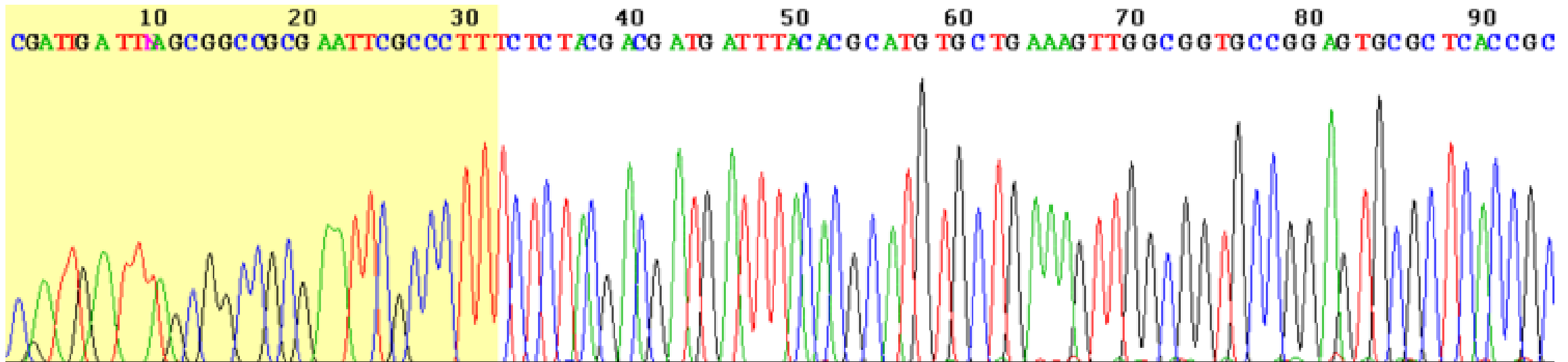


## ④ Laser detection of flouorochromes and computational sequence analysis

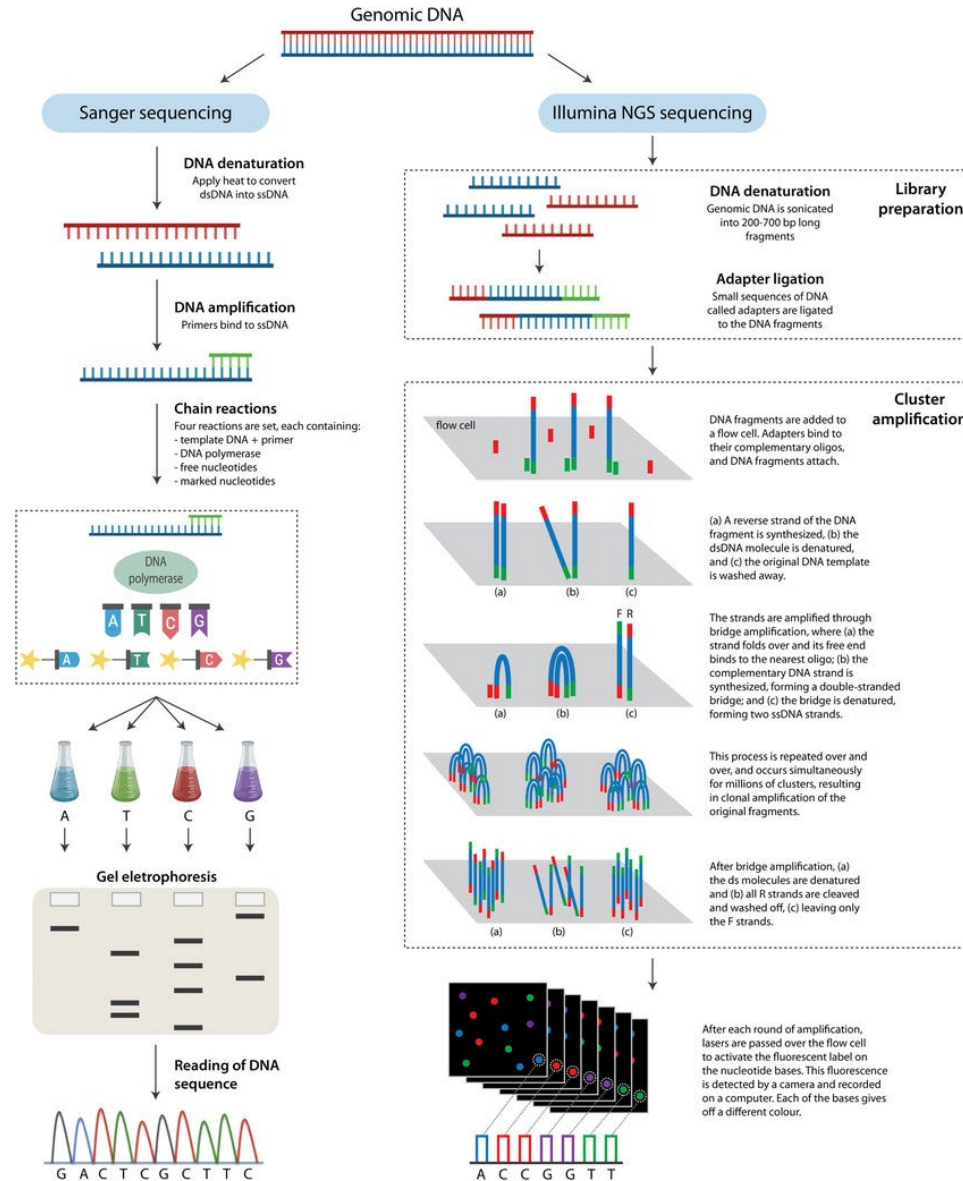


Chromatograph

# Sanger Sequencing Trace



# Next Generation Sequencing



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News Feature | Published: 12 January 2023

## Method of the year: long-read sequencing

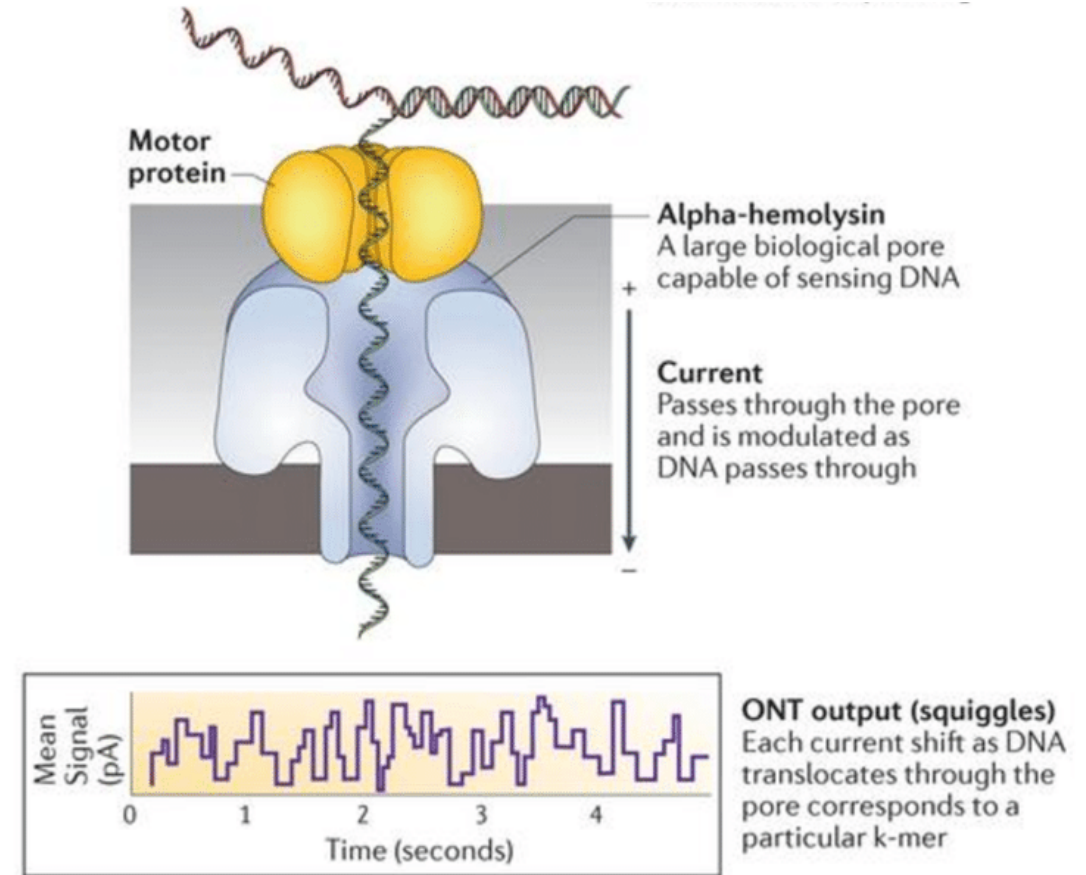
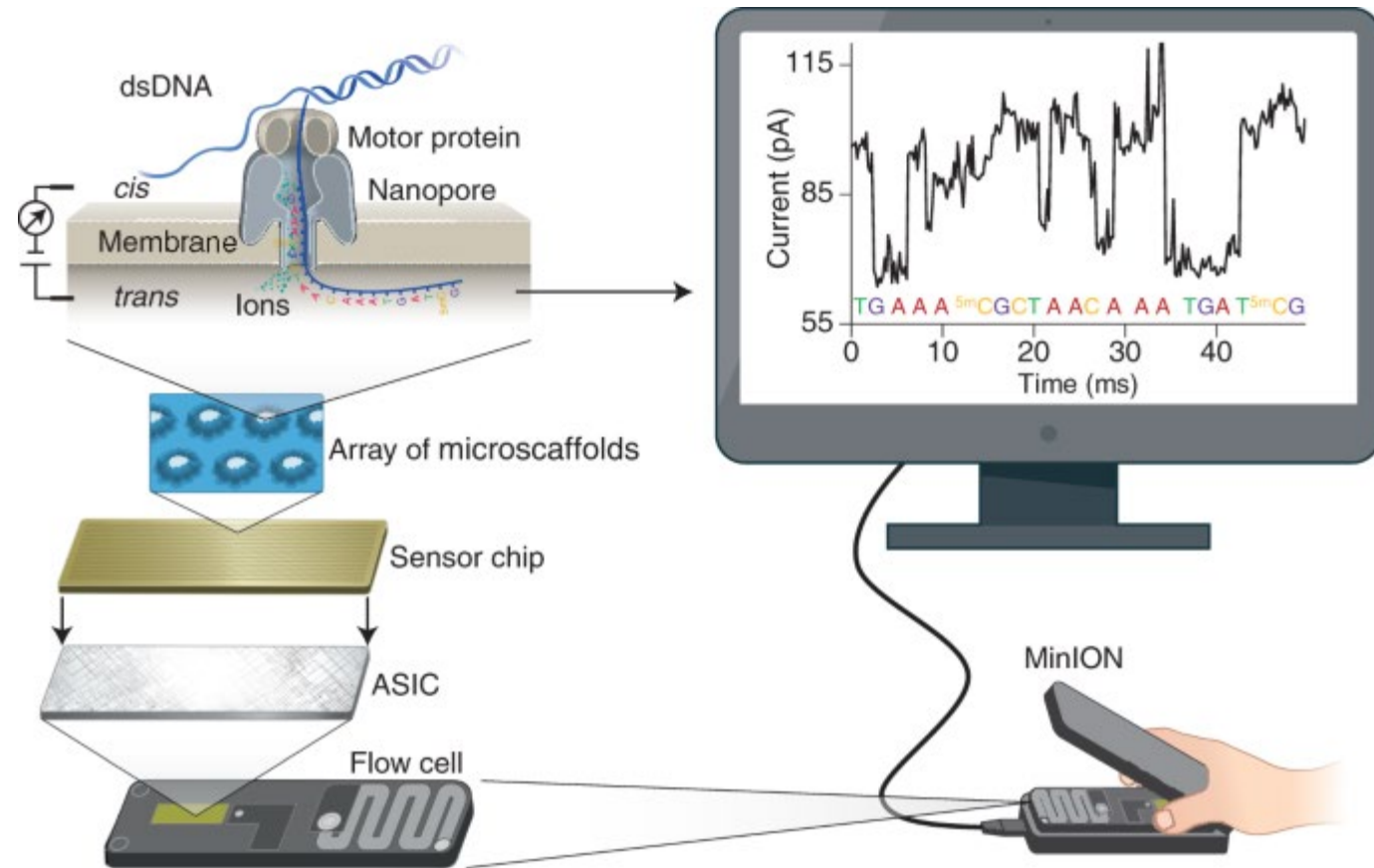
[Vivien Marx](#) 

[Nature Methods](#) **20**, 6–11 (2023) | [Cite this article](#)

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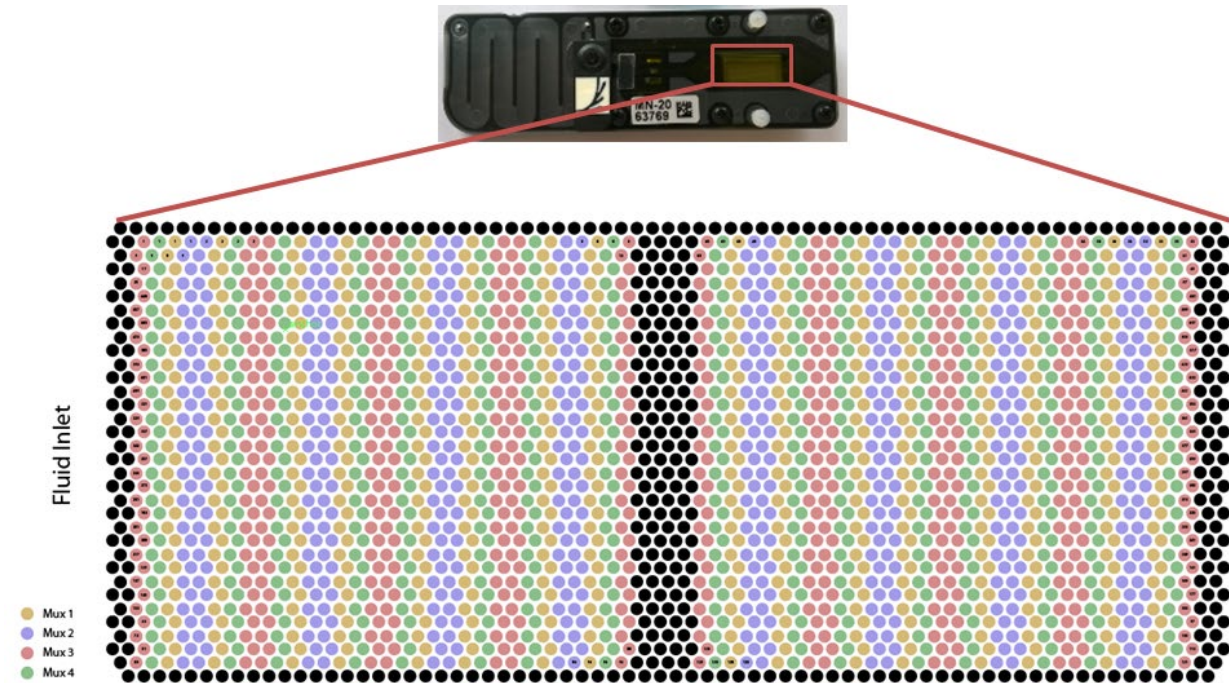
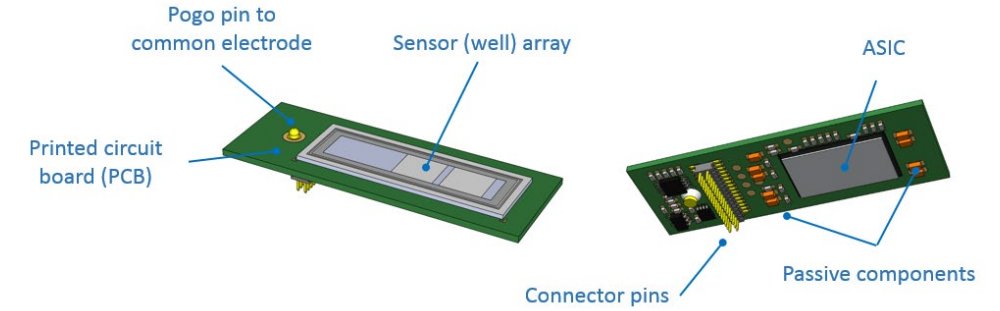
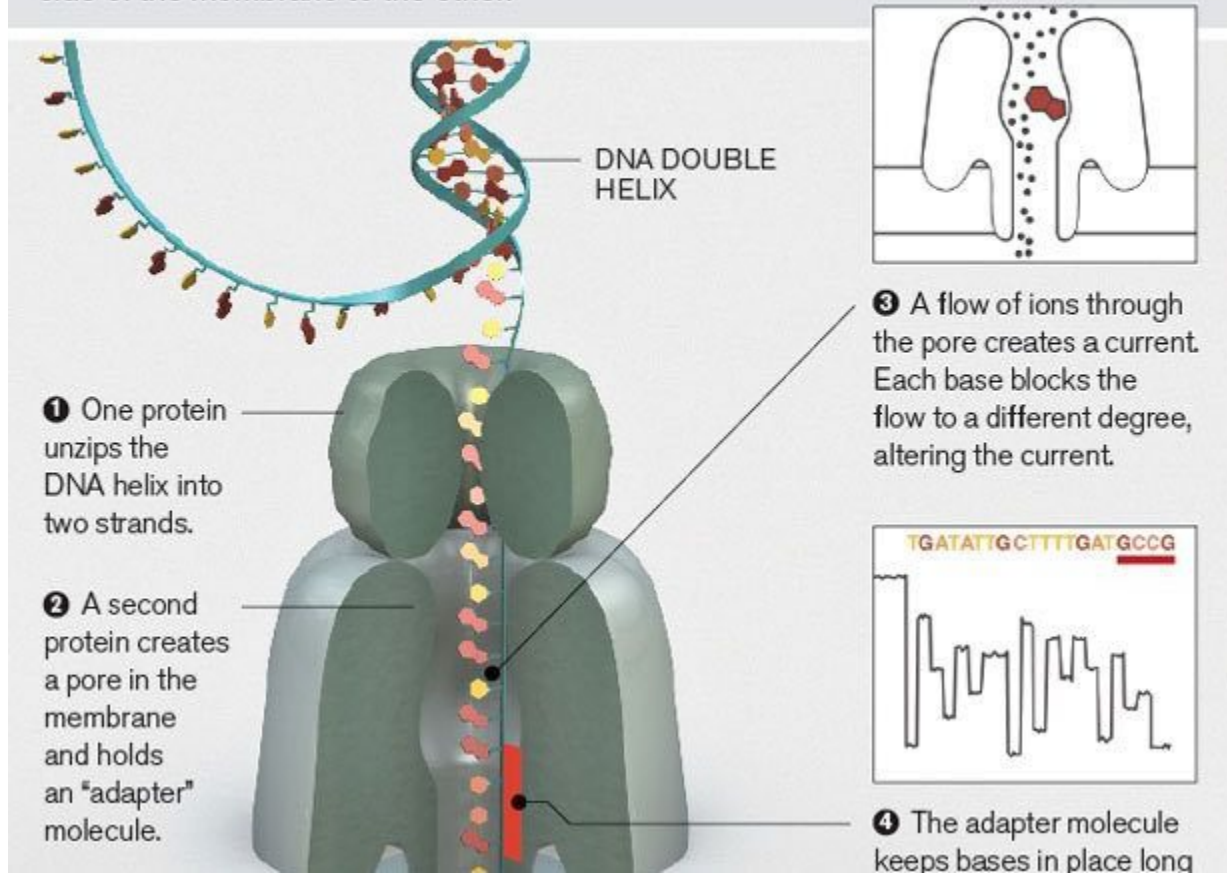
# Nanopore Sequencing



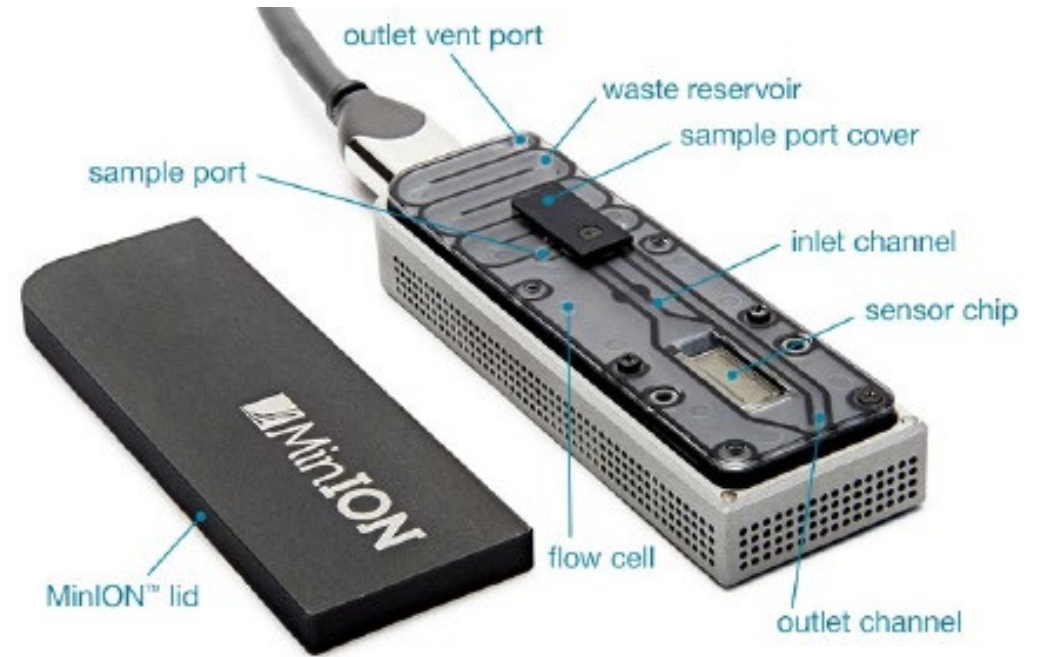


# Nanopore Sequencing

DNA can be sequenced by threading it through a microscopic pore in a membrane. Bases are identified by the way they affect ions flowing through the pore from one side of the membrane to the other.



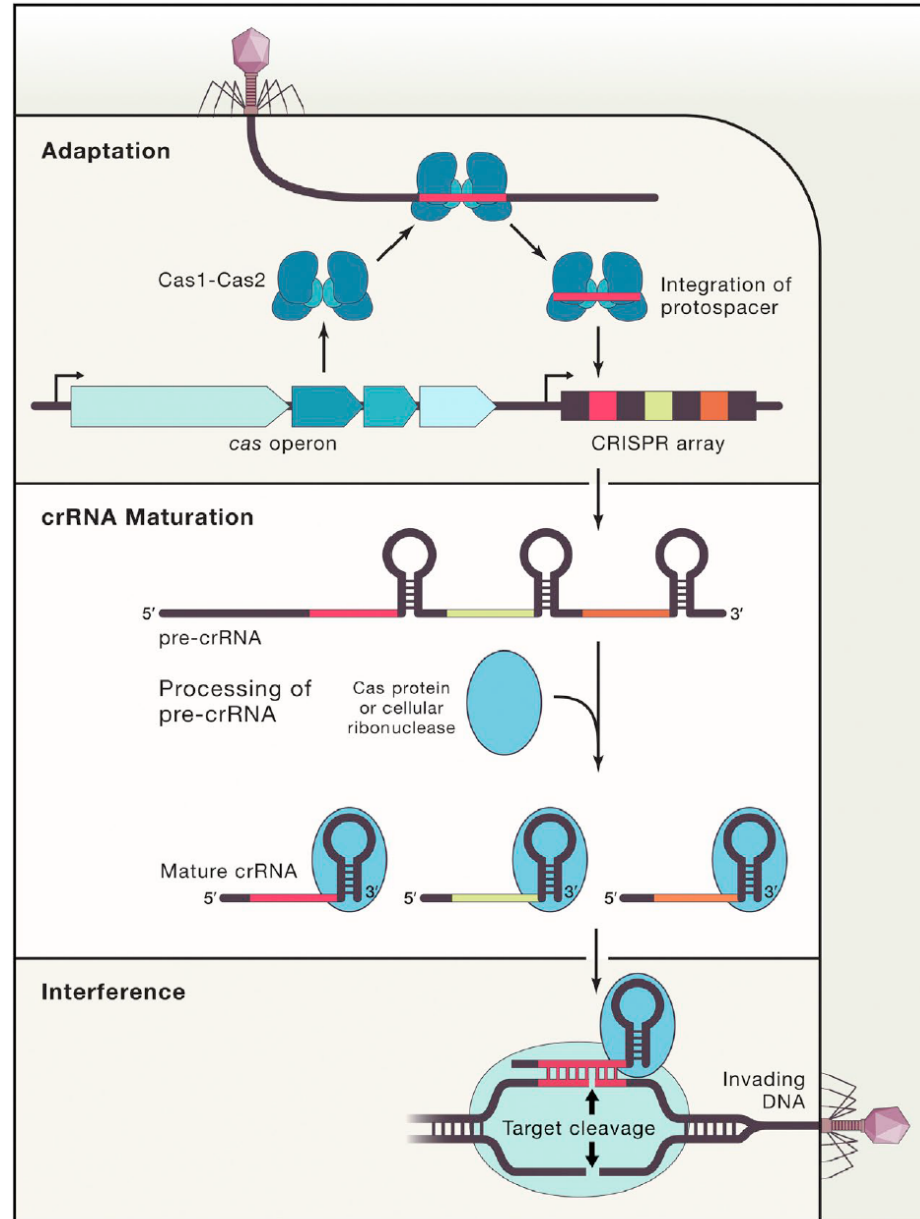
# Nanopore Sequencing



Future: direct single protein sequencing!

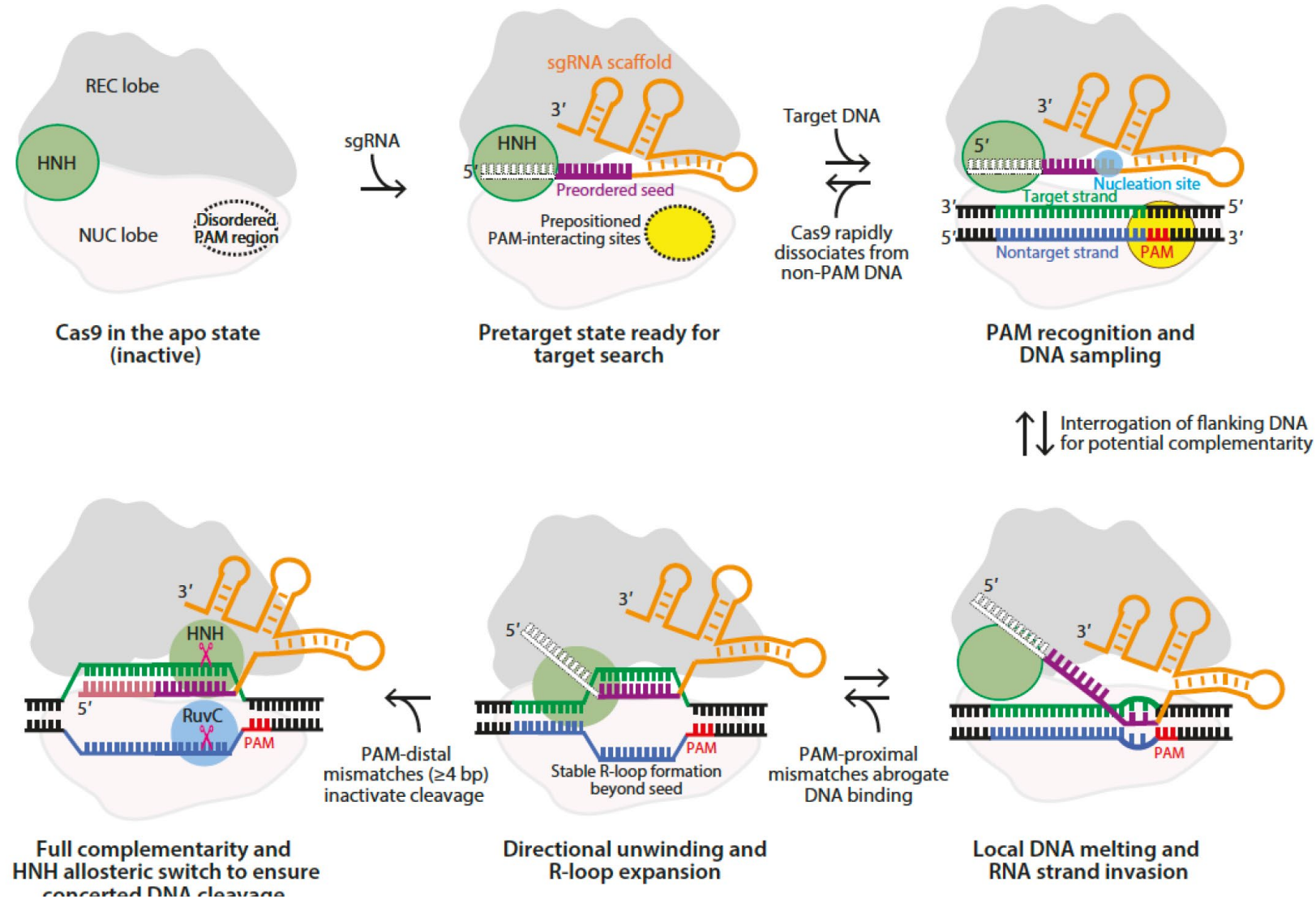
CRISPR  
Nobel Chemistry 2020:  
Emmanuelle Charpentier  
and  
Jennifer Doudna

# CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

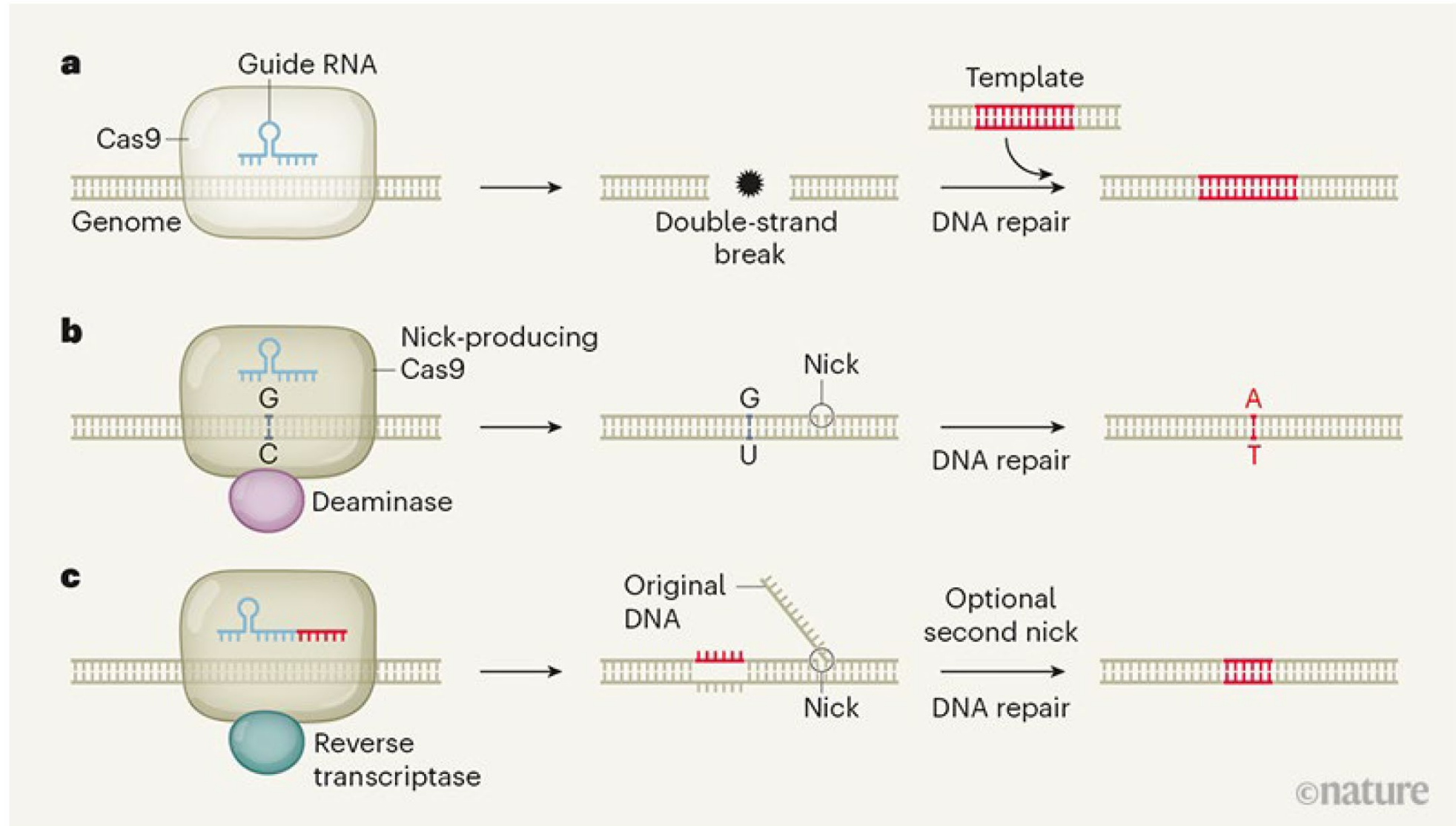




# How CRISPR-Cas9 cuts target DNA



# Genome Editing!



# Did CRISPR help—or harm—the first-ever gene-edited babies?

He Jiankui's attempt to knock out the CCR5 gene was messy—and so are debates about potential consequences

1 AUG 2019 • BY [JON COHEN](#)

People inherit two copies of *CCR5*, one from each parent. He chose the gene as a target because he knew that about 1% of Northern European populations are born with both copies missing 32 base pairs, resulting in a truncated protein that doesn't reach the cell surface. These people, known as *CCR5*Δ32 homozygotes, appear healthy and are highly resistant to HIV infection.

In the embryos He's team edited, the researchers did not attempt to delete these exact 32 base pairs; rather, the group designed CRISPR to cut *CCR5* at the base pair at one end of the natural deletion. The error-prone cell-repair mechanism, which CRISPR depends on to finish knocking out genes, then deleted 15 base pairs in one of Lulu's copies of the gene, but none in the other. With one normal *CCR5*, she is expected to have no protection from HIV. Nana, according to [the data He presented in a slide at an international genome-editing summit held in November 2018 in Hong Kong, China](#), had bases added to one *CCR5* copy and deleted from the other, which likely would cripple both genes and provide HIV resistance.

He added the genes for the CRISPR machinery almost immediately after each embryo was created through in vitro fertilization, but several researchers who closely studied the slide caution that it may have done its editing after Nana's embryo was already past the one-cell stage. That means she [could be a genetic "mosaic"](#) who has some unaffected cells with normal *CCR5*—and ultimately might have no protection from HIV.

