

Lecture 8 - 9

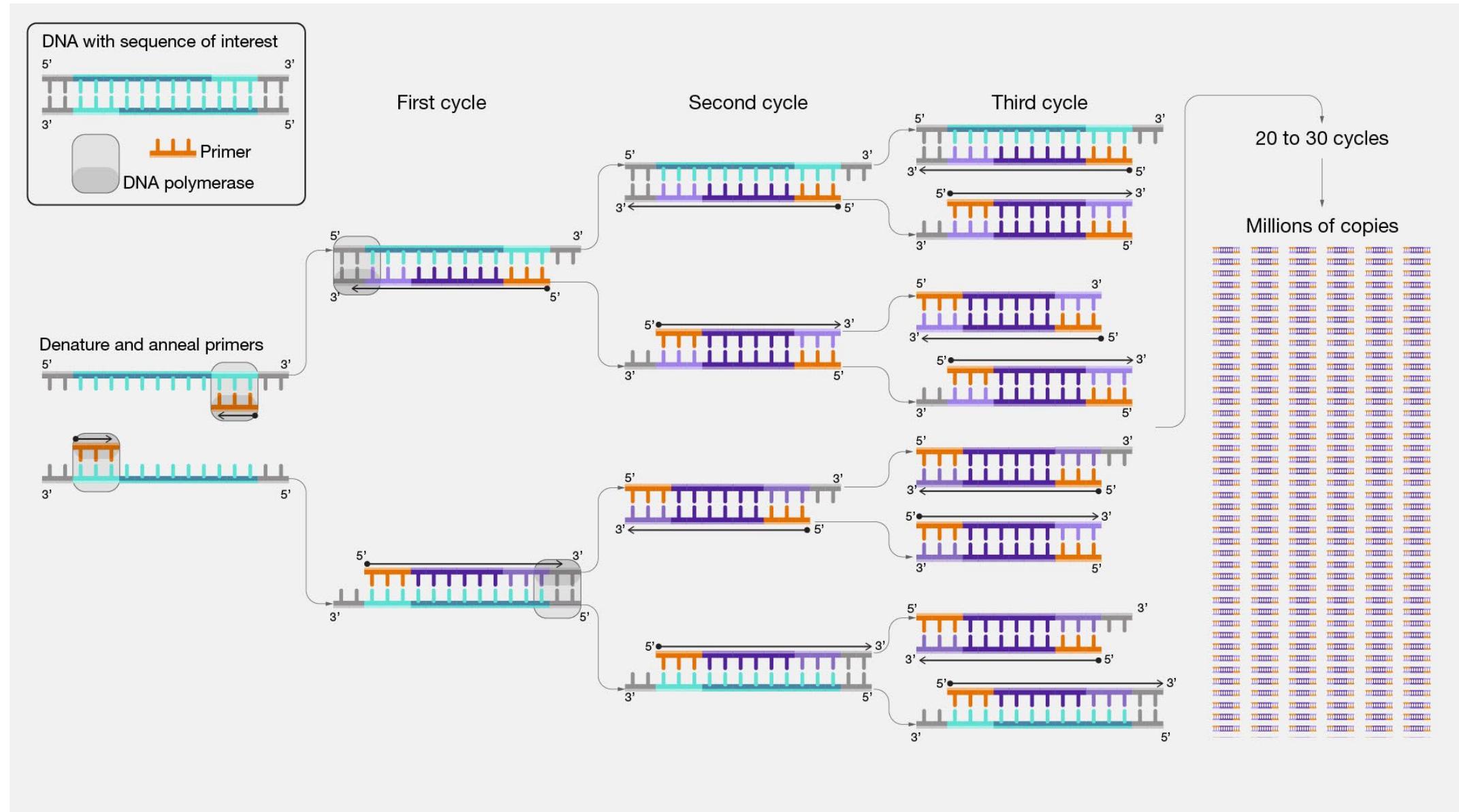
Techniques & Methods



Prof. Sebastian Maerkli

PCR

Polymerase Chain Reaction (PCR)



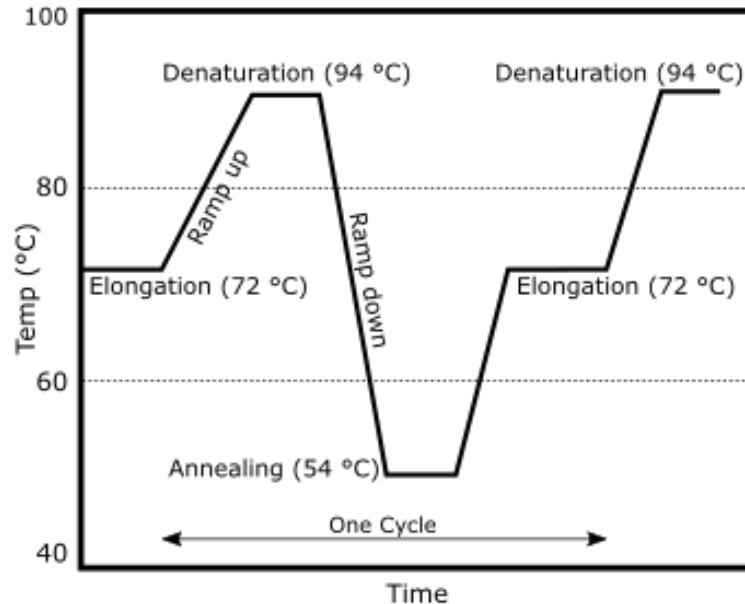
Polymerase Chain Reaction (PCR)

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 Tm)
 - ~10s
- Elongation
 - 72°C or optimal temperature for polymerase)
 - 15-30 s/kb
- Generally 25-35 cycles



Agarose Gels

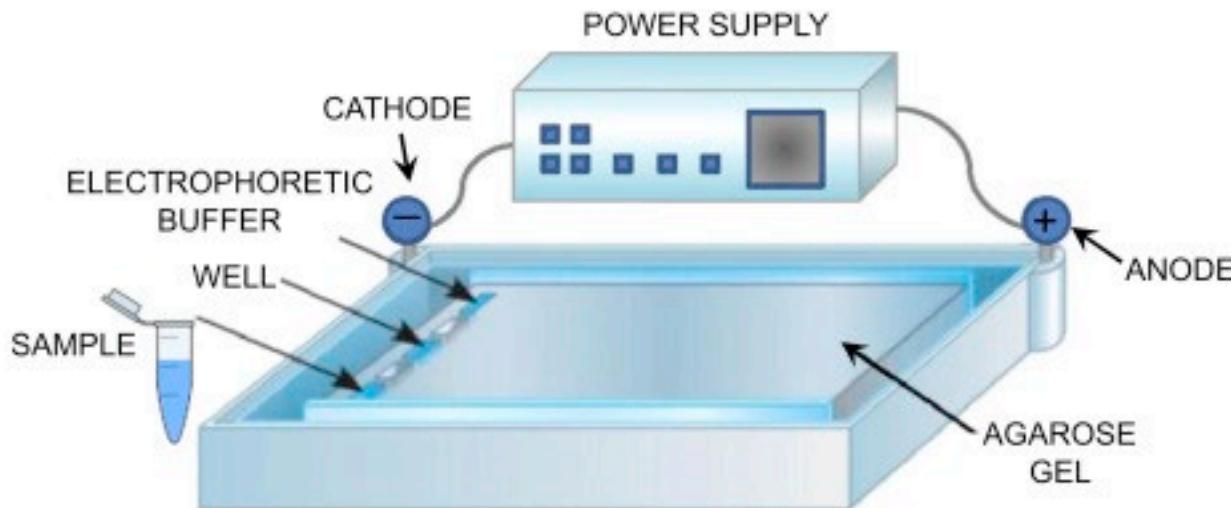
Agarose gel electrophoresis: casting and loading

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

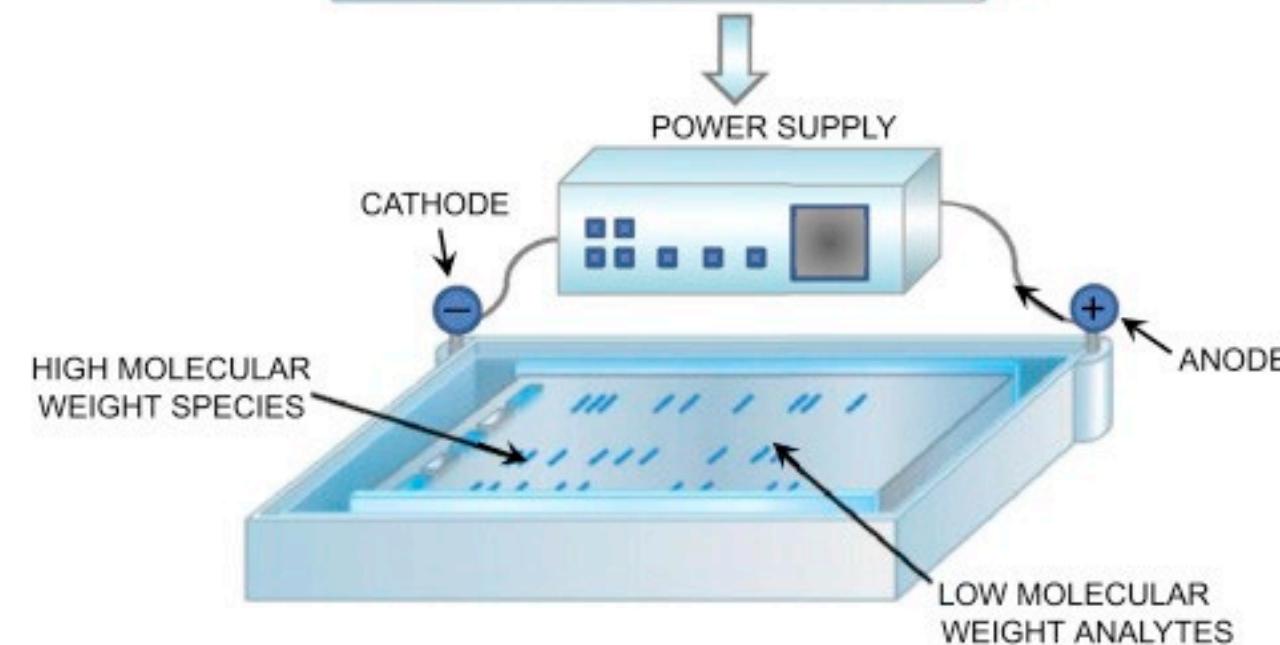


Agarose gel electrophoresis: running gels



Running parameters:

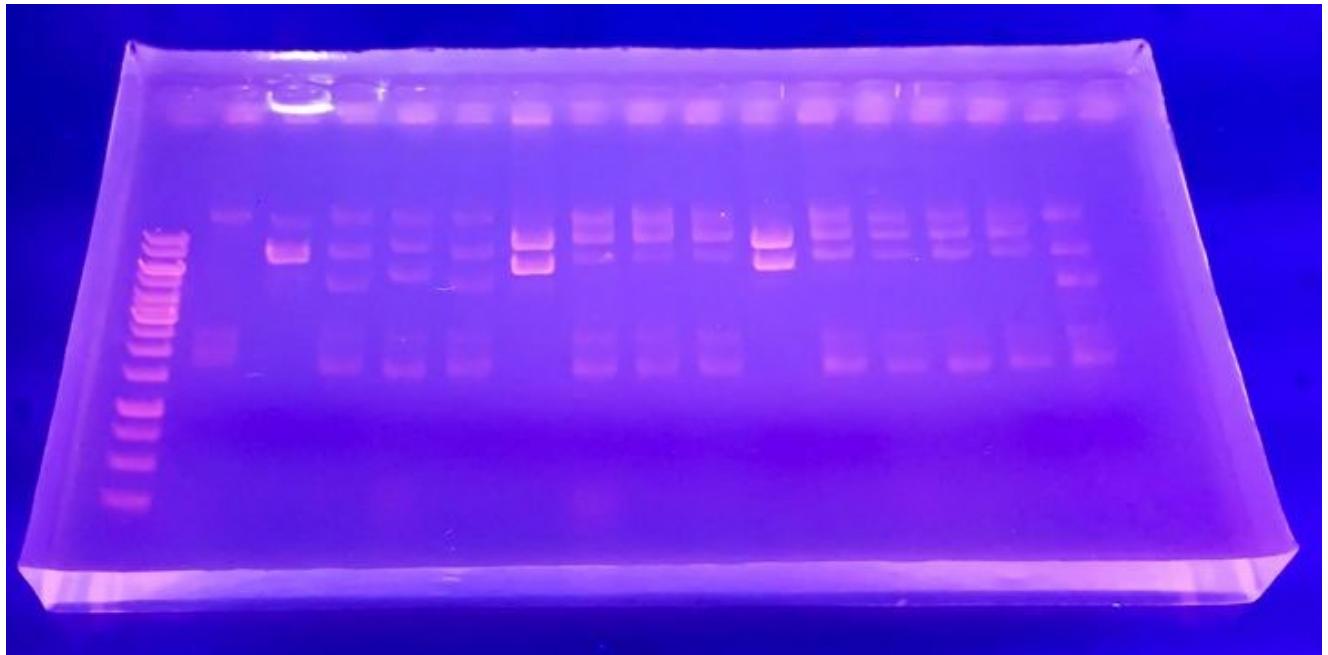
- Voltage:
 - ~100 Volts (10V/cm of gel length)
- Time:
 - Until loading dye reaches bottom of gel



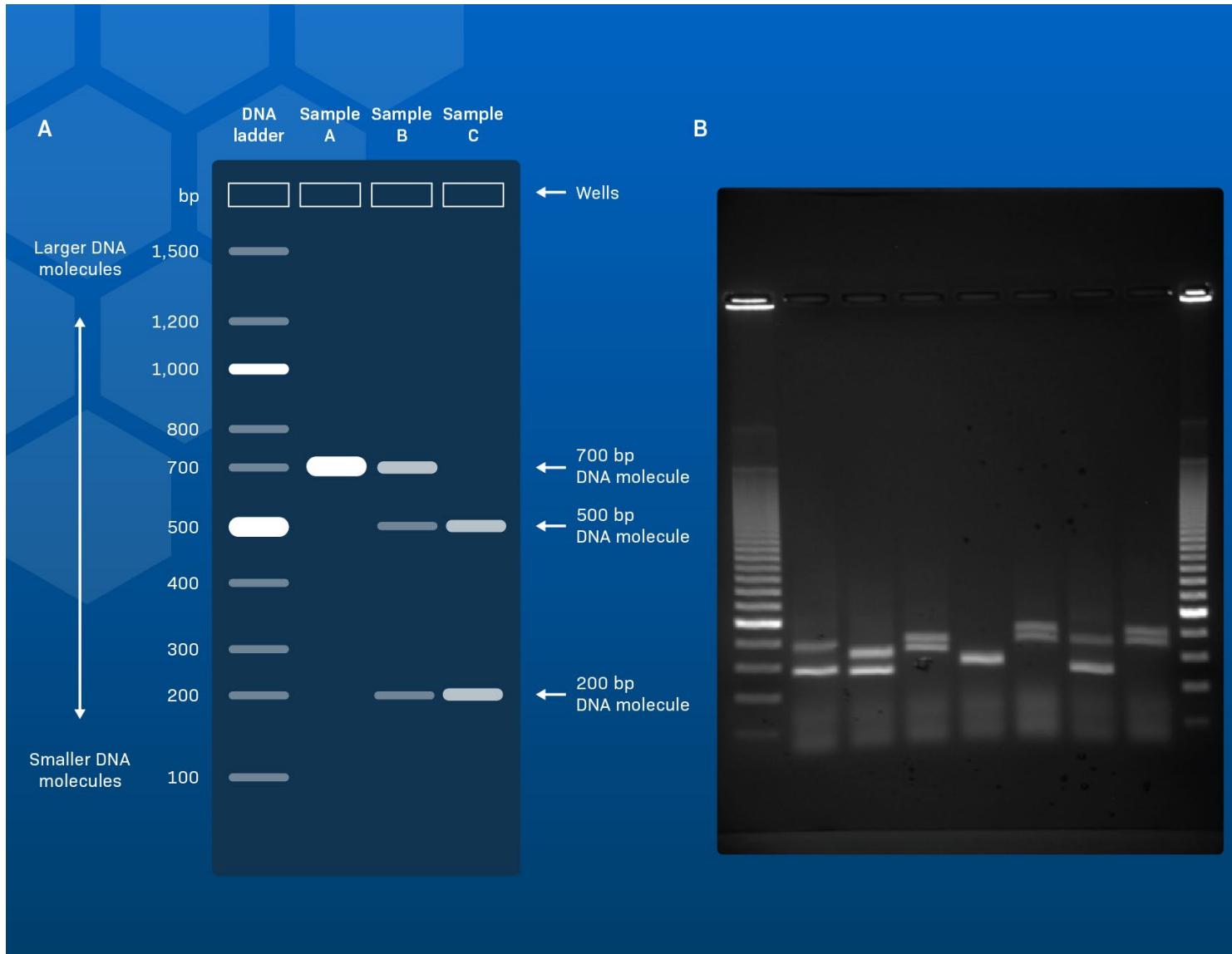
Agarose gel electrophoresis: imaging

Imaging:

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

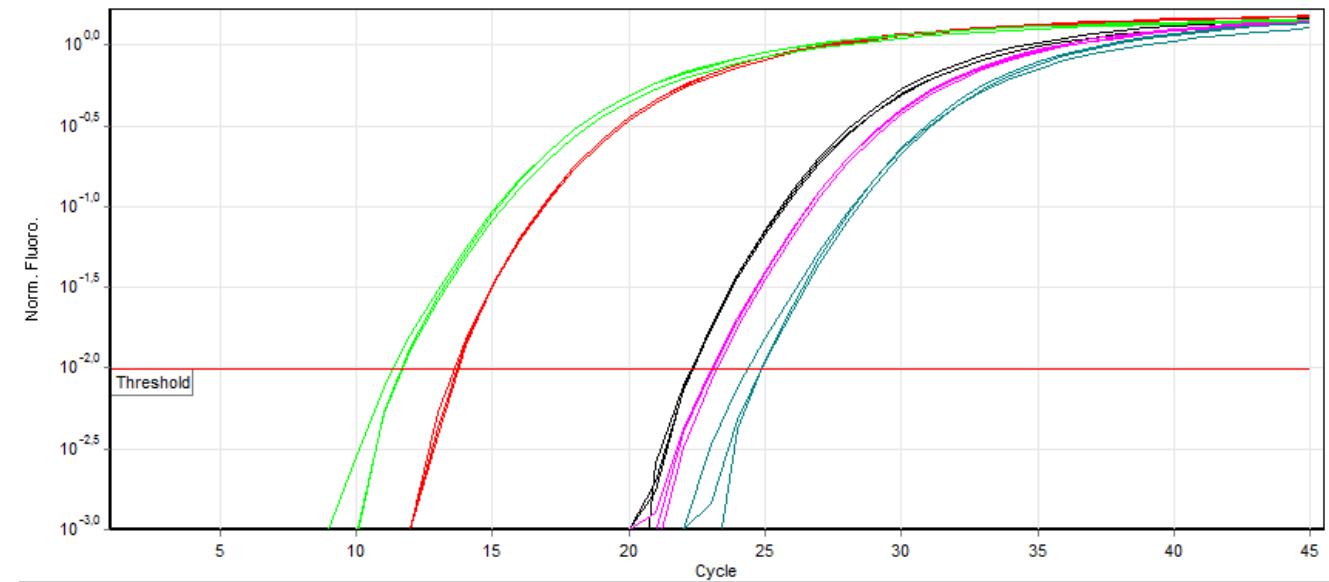
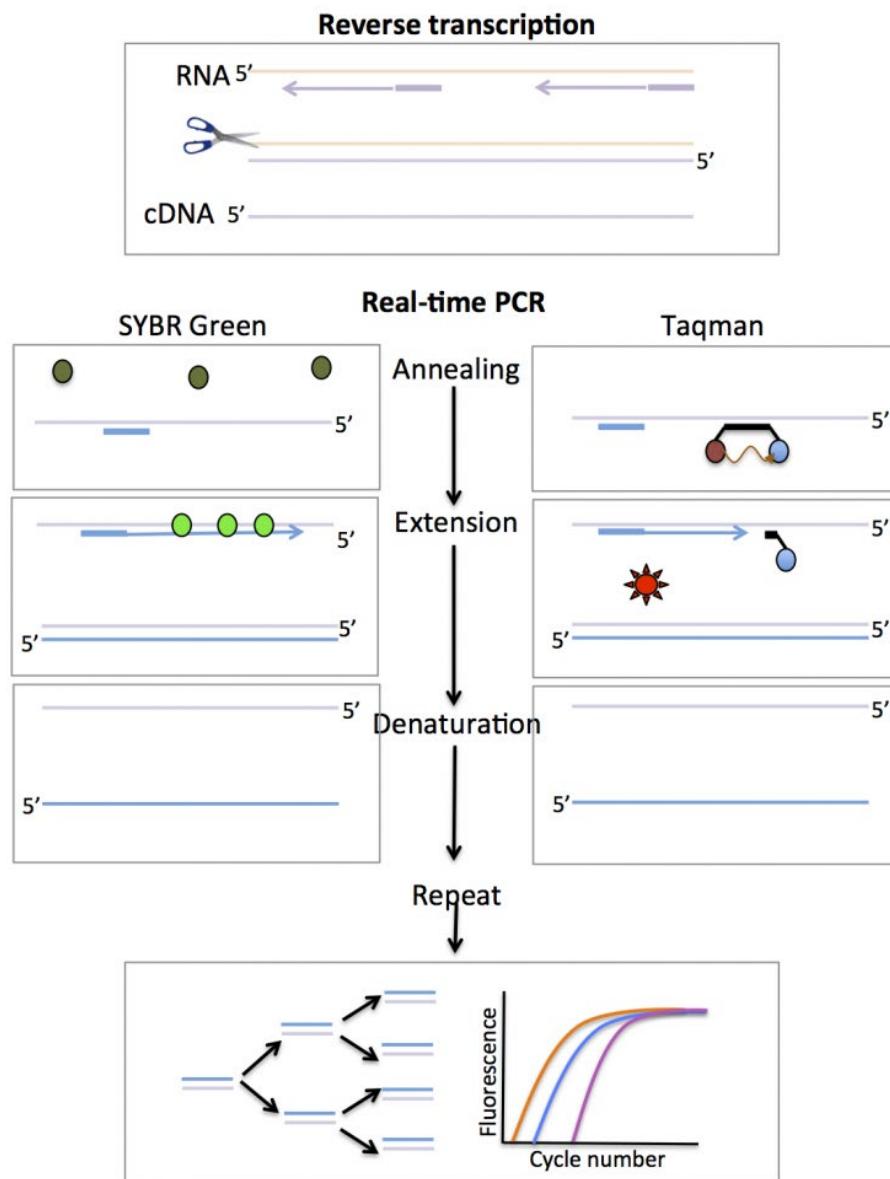


Agarose gel electrophoresis: interpreting results



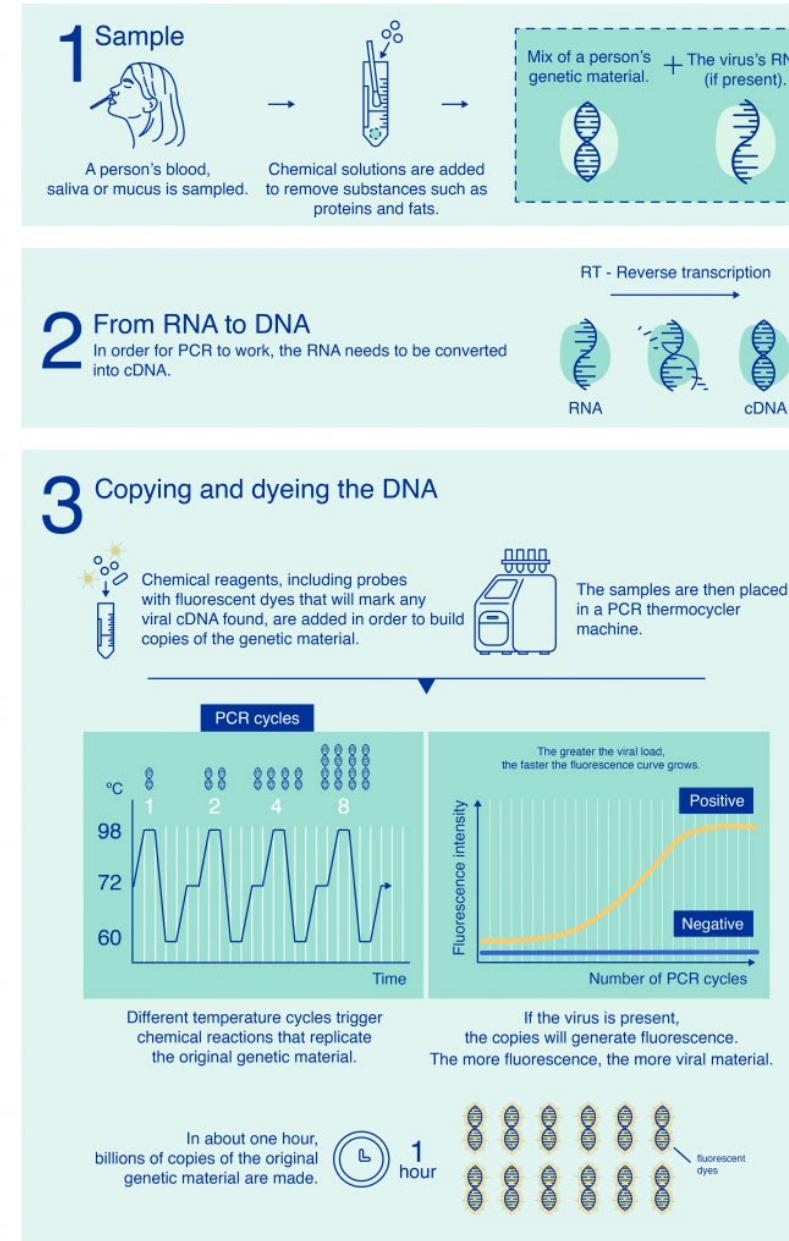
PCR variants

Real-time RT-PCR

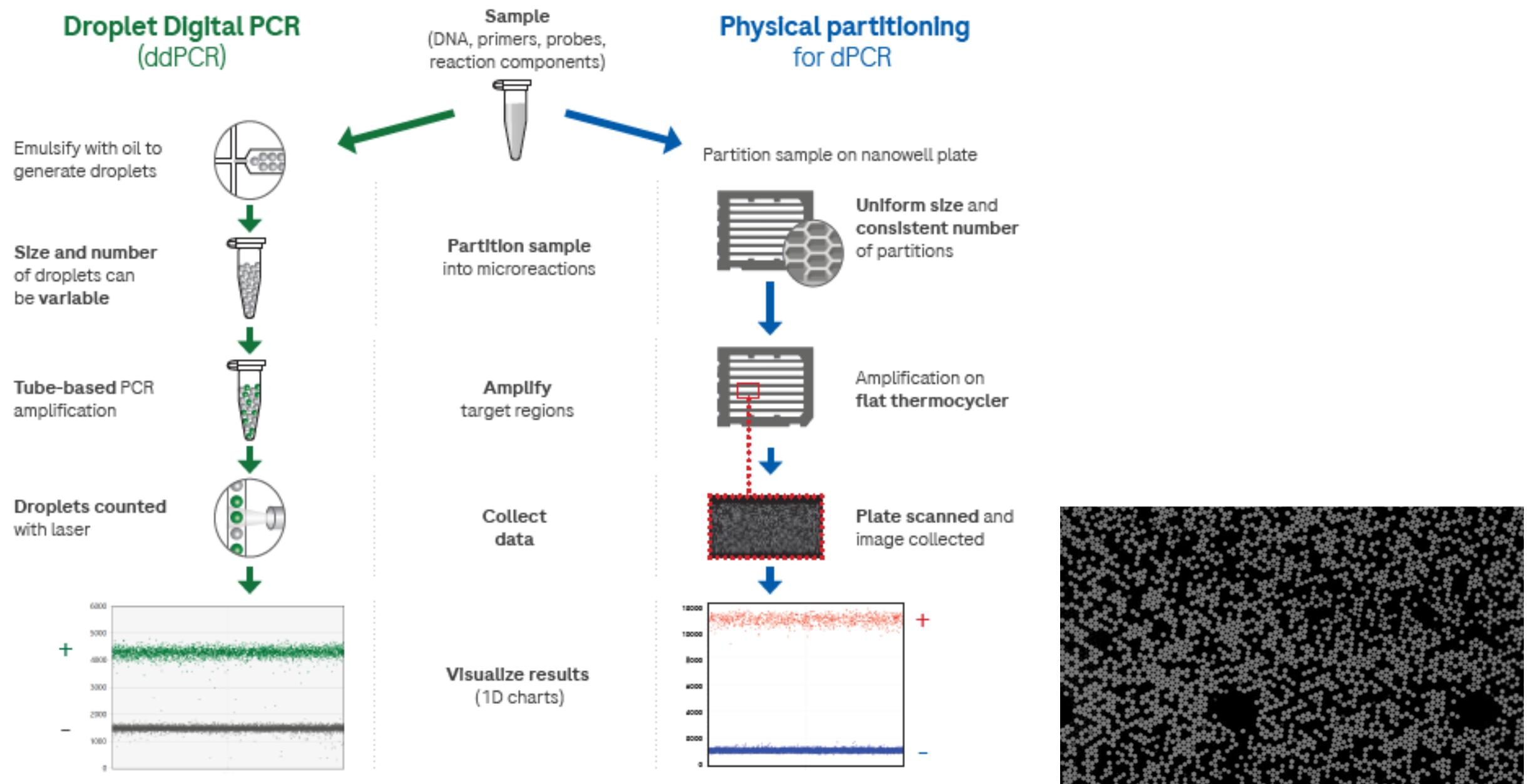


Real-time RT-PCR

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



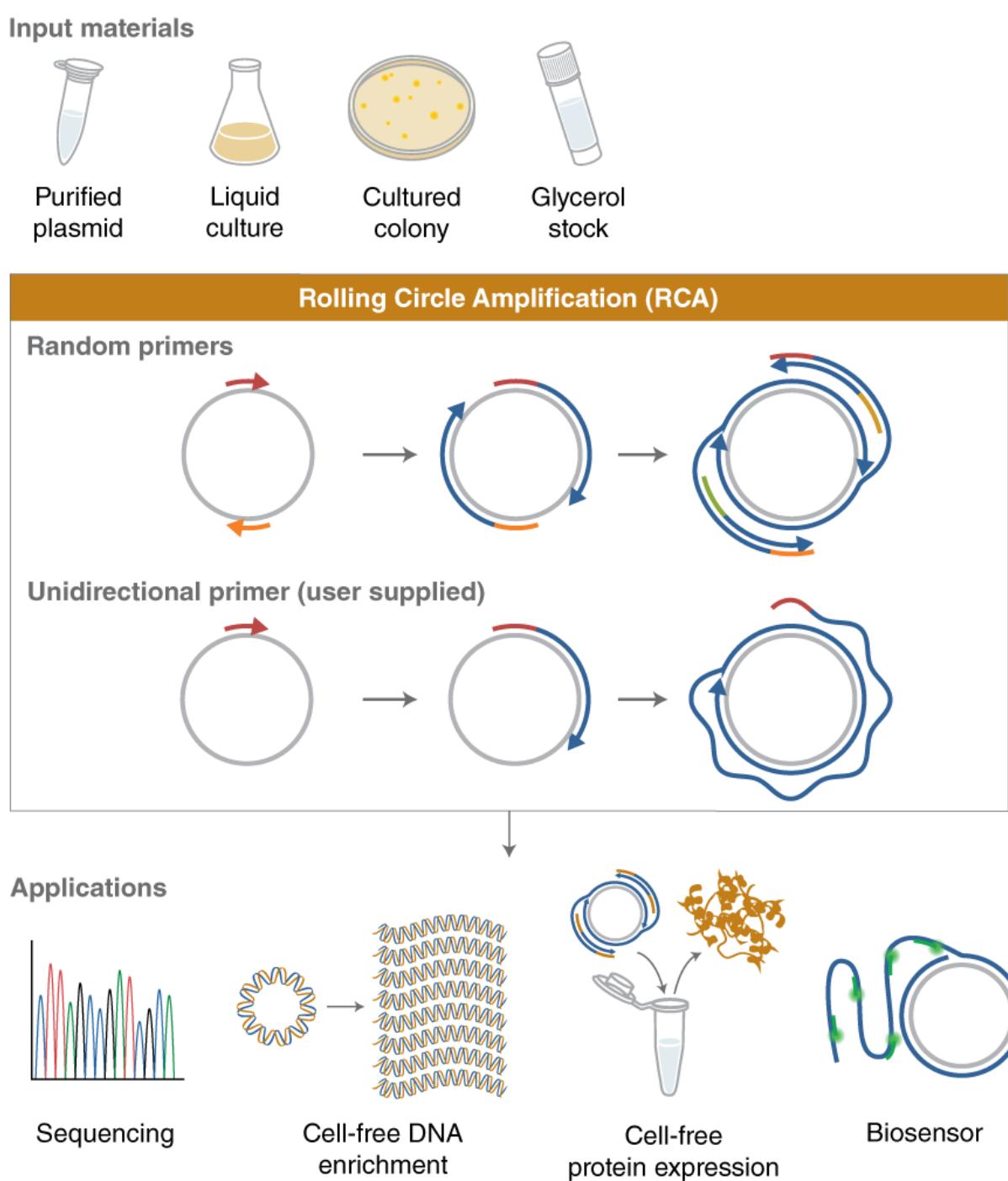
Digital PCR



Isothermal PCRs

RCA

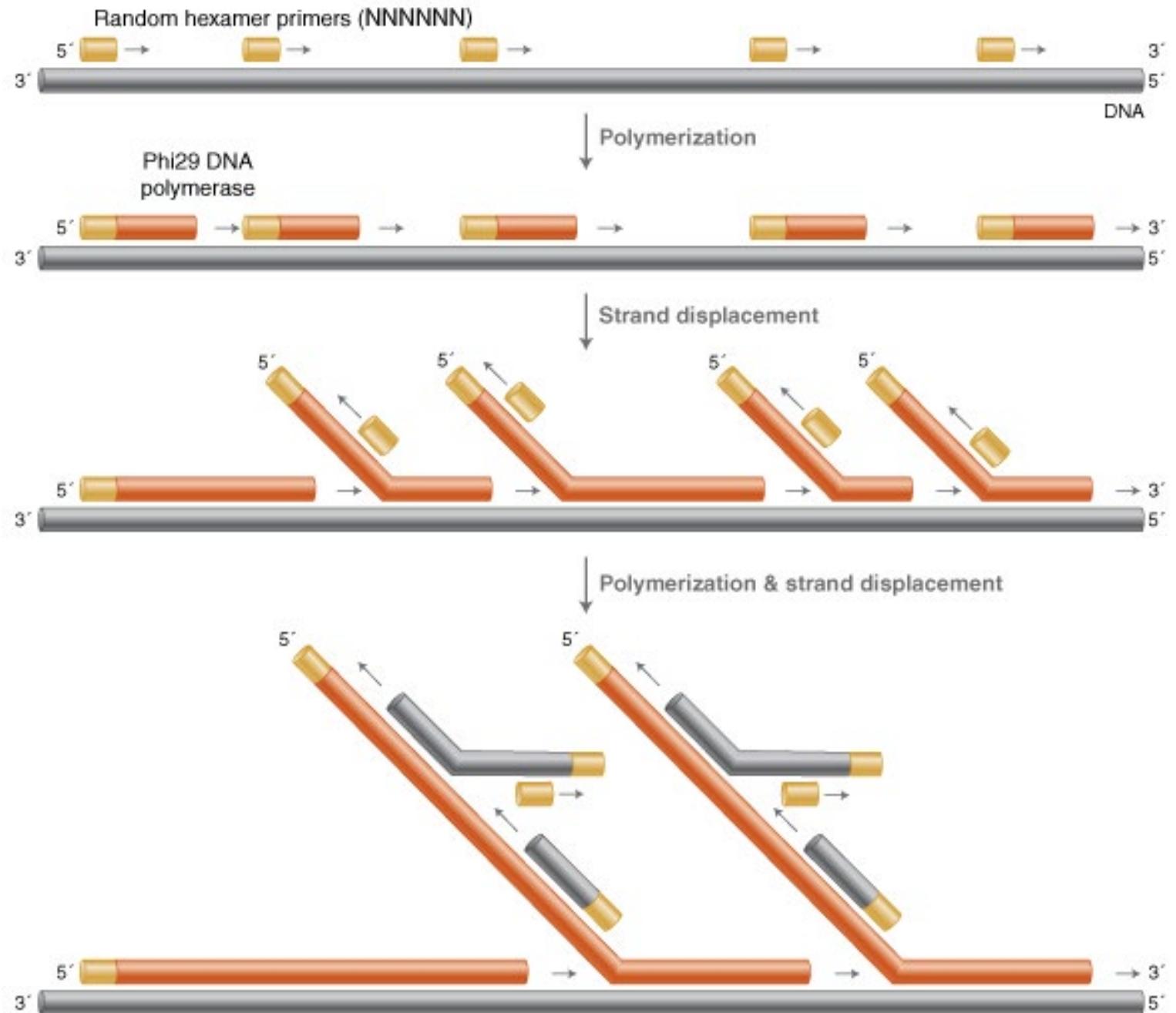
Rolling Circle Amplification



Isothermal PCRs

WGA

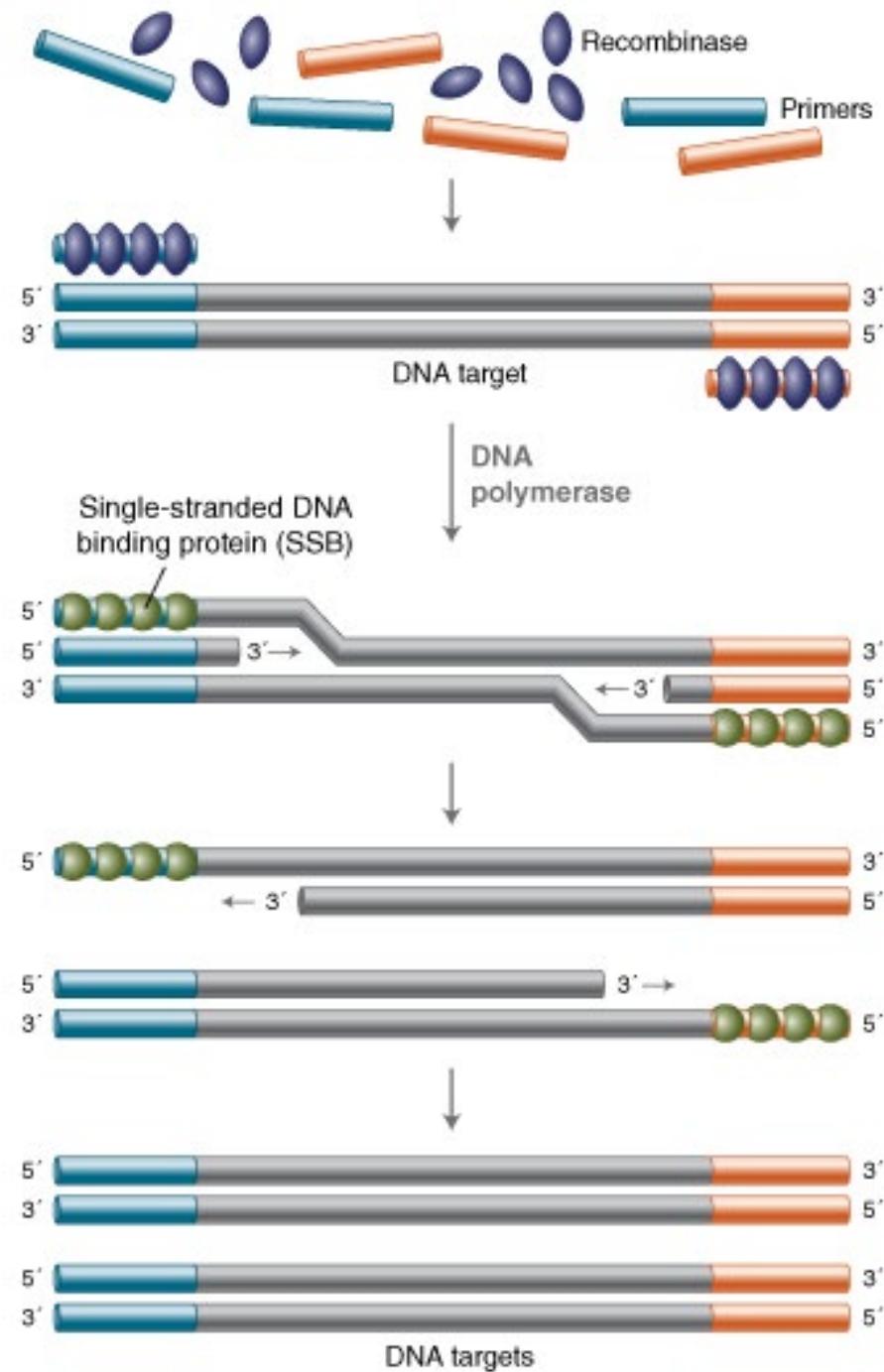
Whole Genome Amplification



Isothermal PCRs

RPA

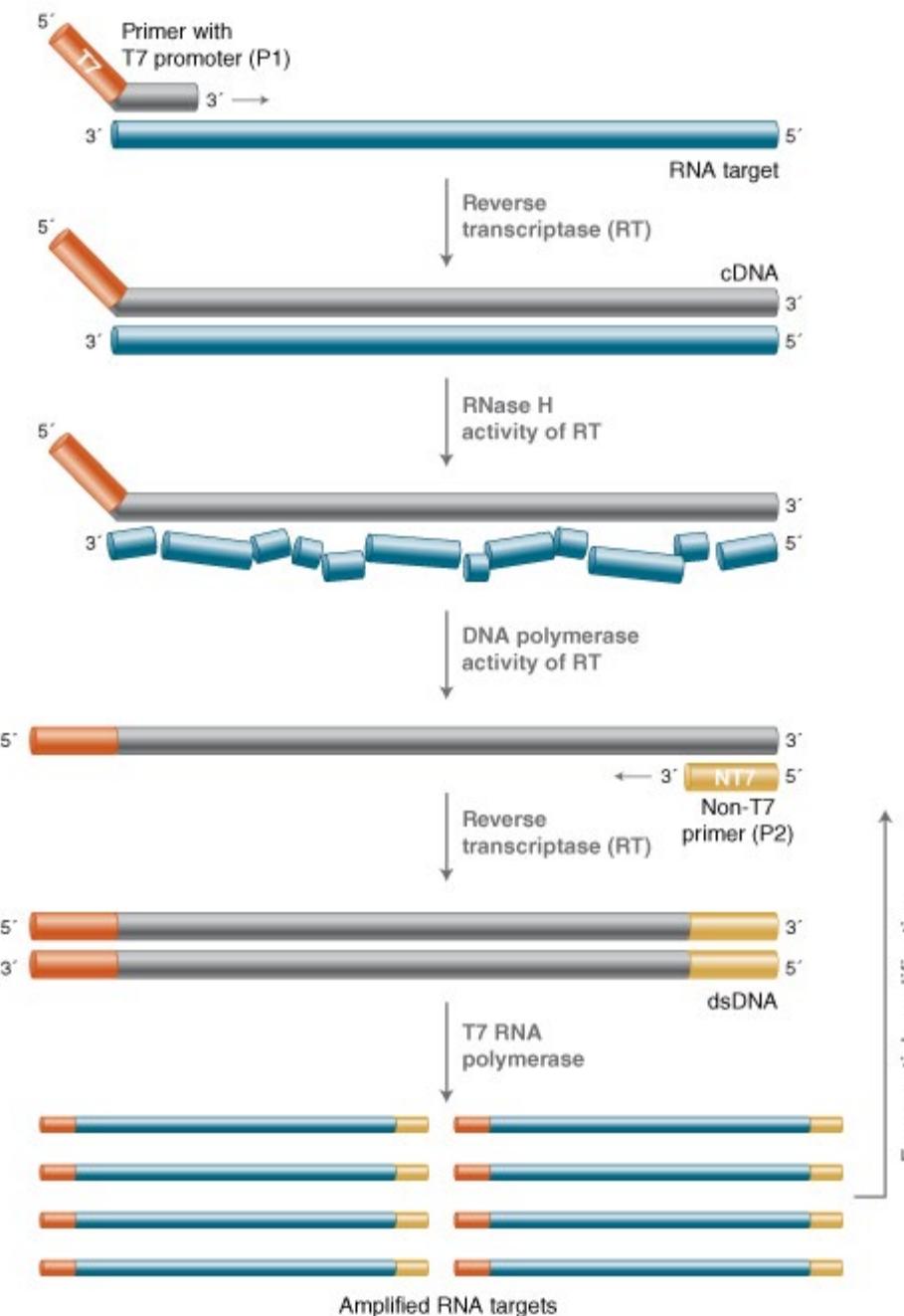
Recombinase Polymerase Amplification



Isothermal PCRs

NASBA

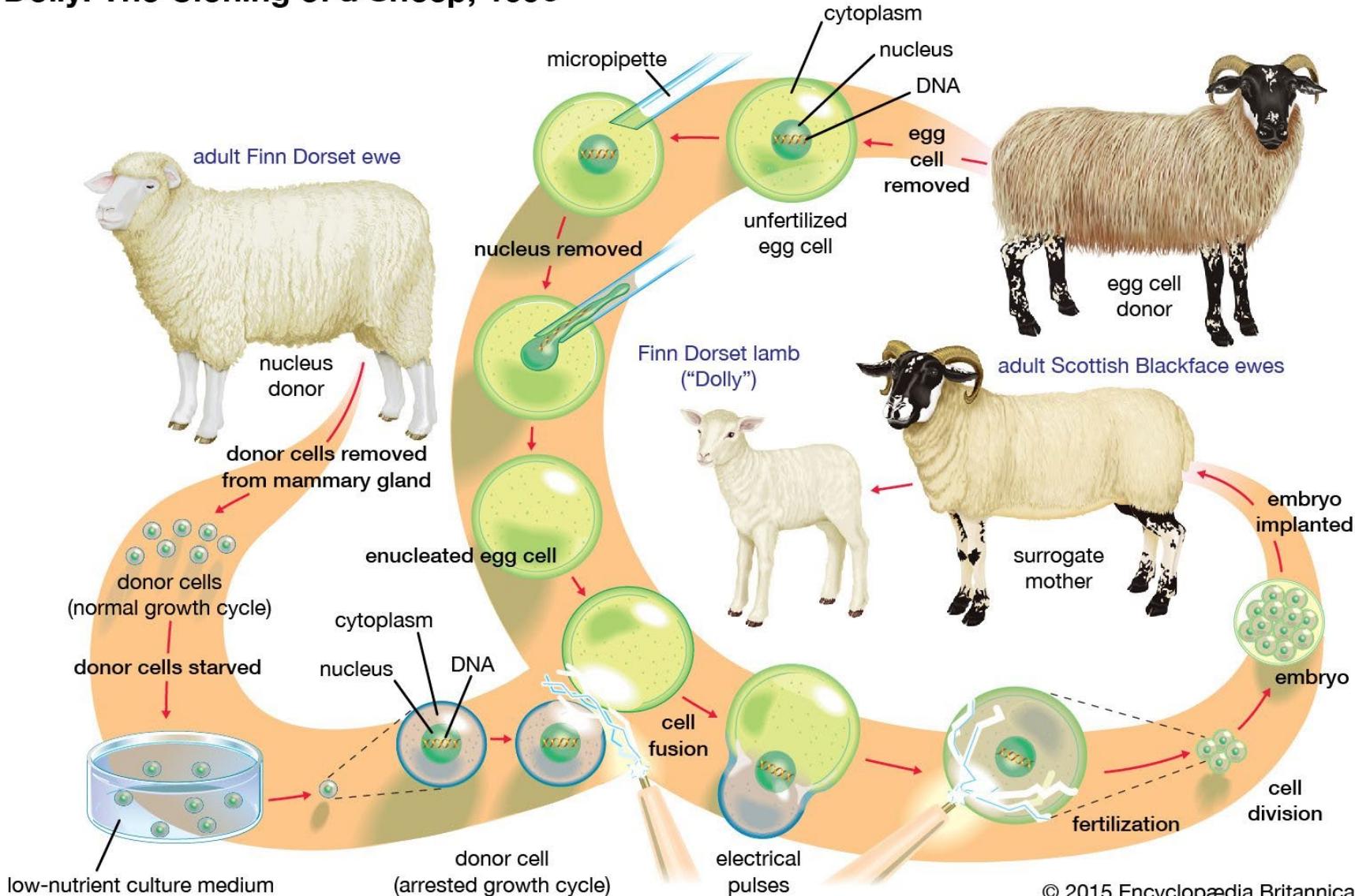
Nucleic Acid Sequenced Based Amplification and Transcription Mediated Amplification



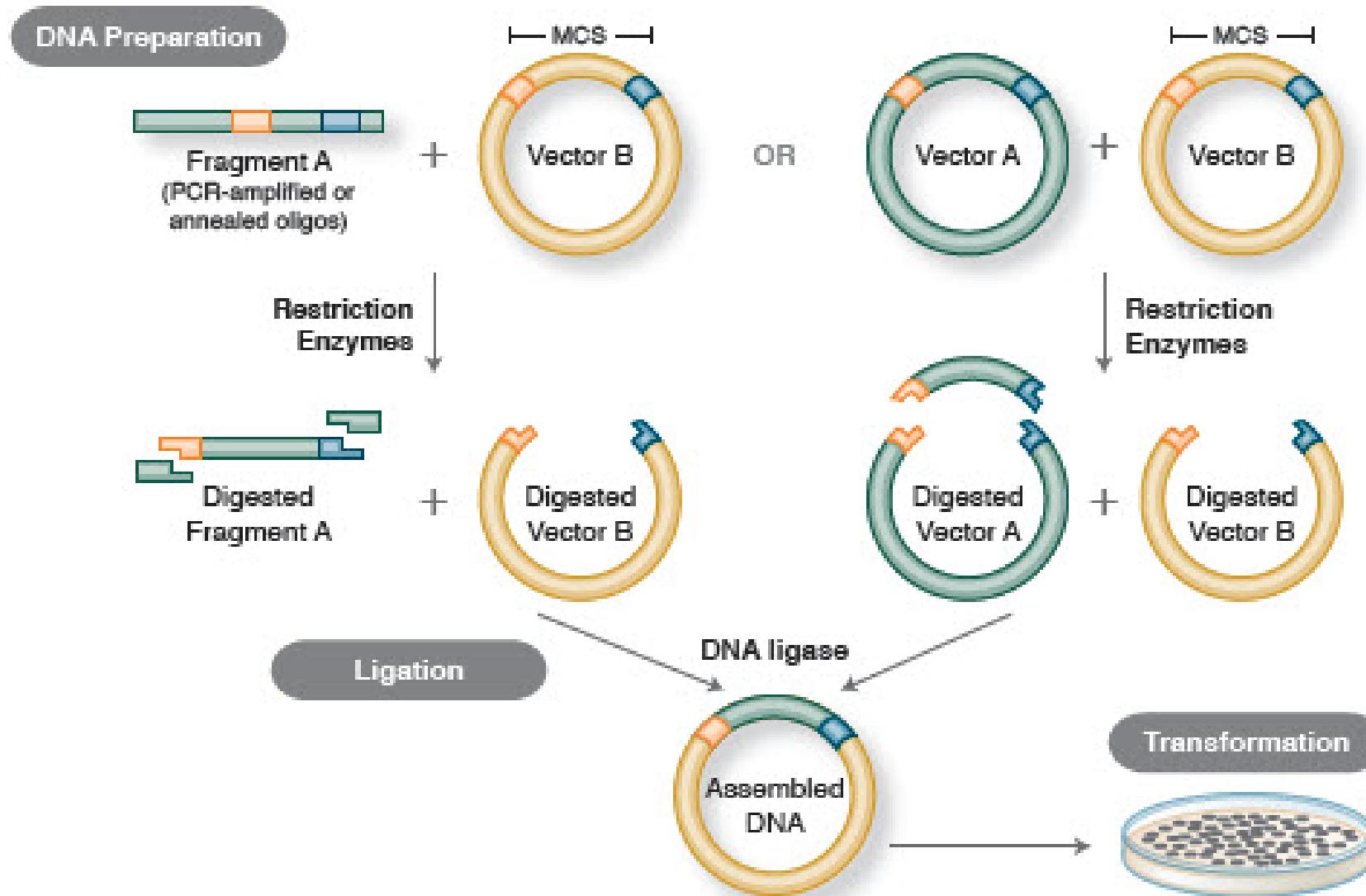
Molecular Cloning

Not this cloning!!!

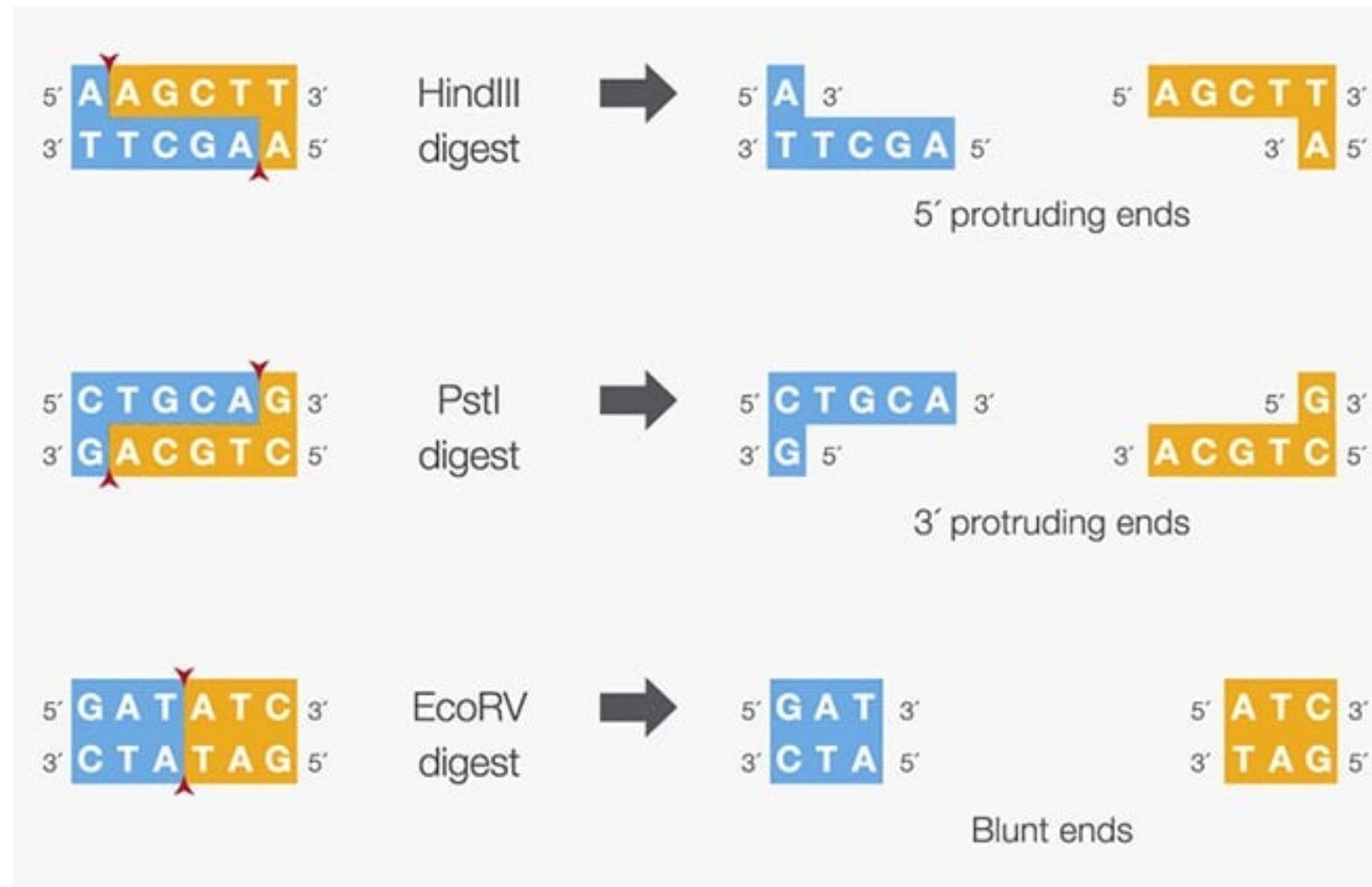
Dolly: The Cloning of a Sheep, 1996



Molecular Cloning

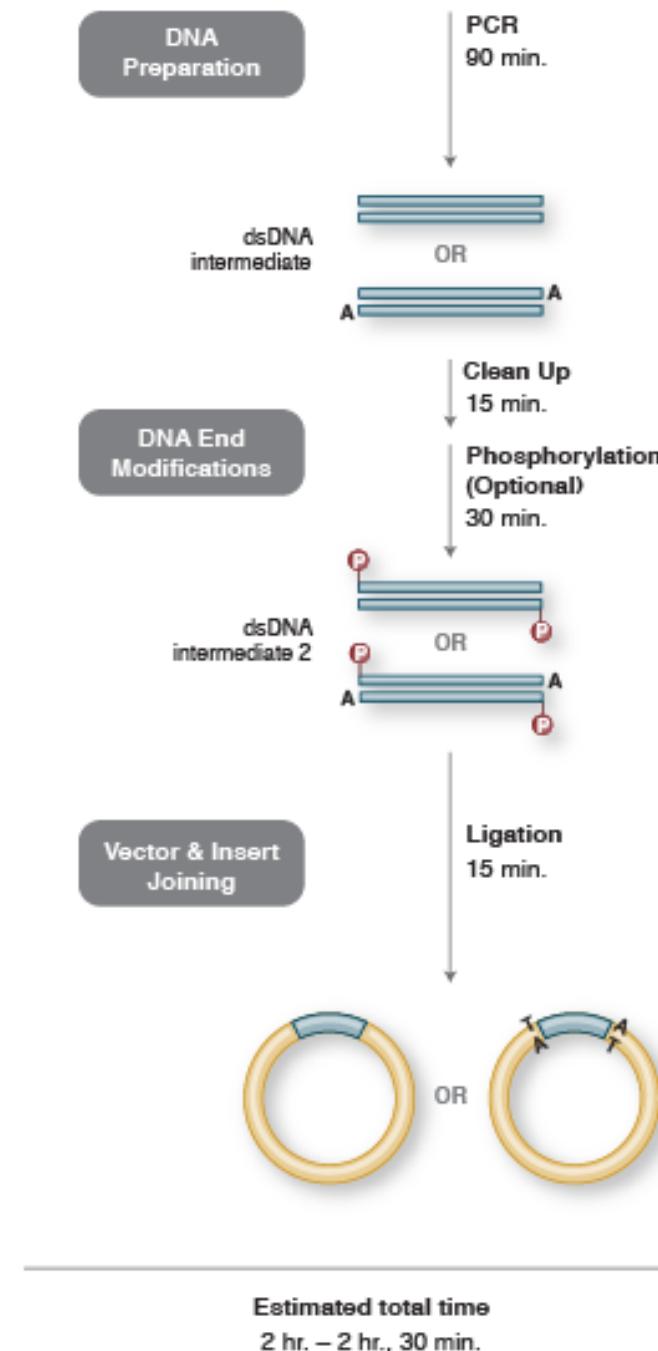


Restriction Enzymes

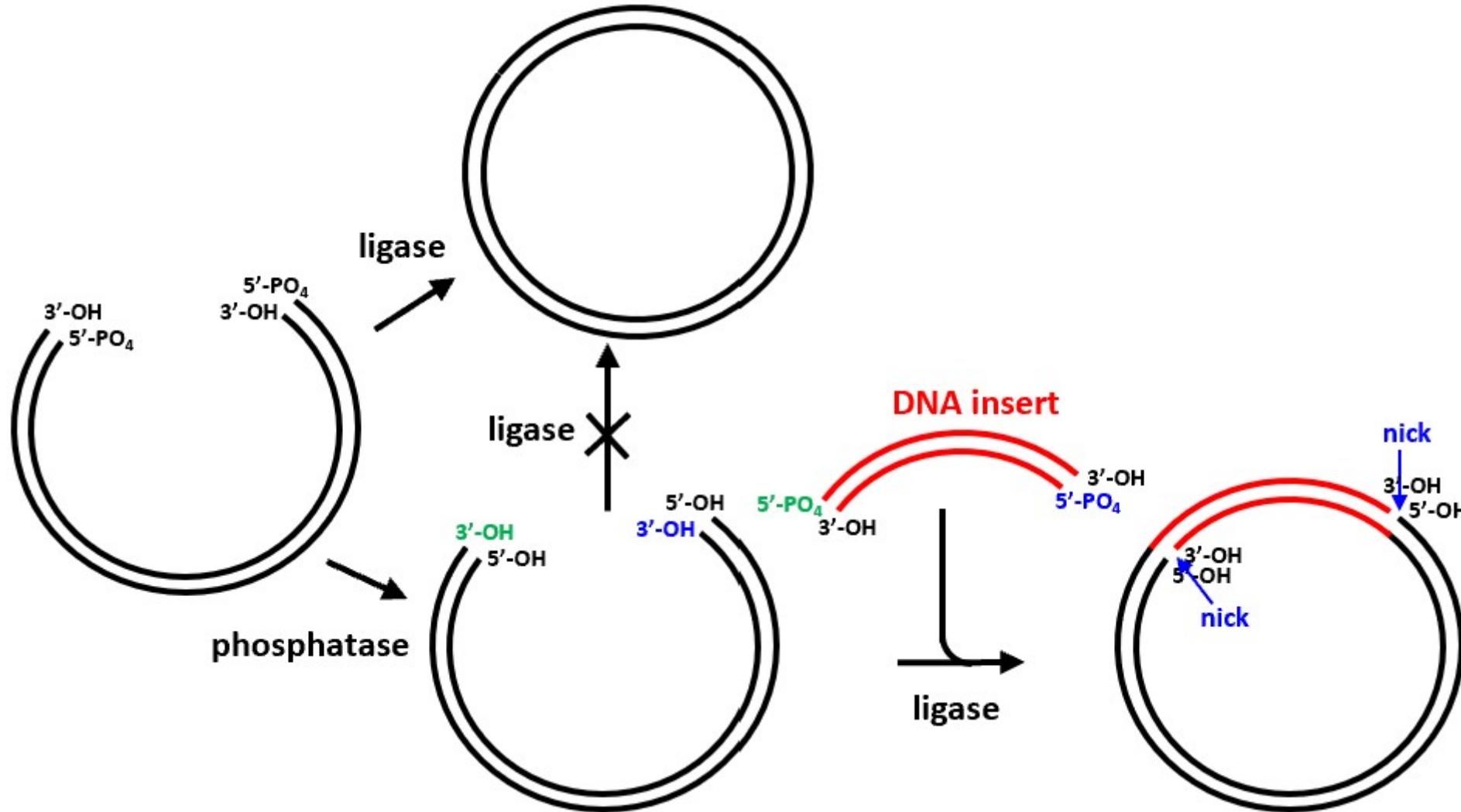


Phosphorylation

(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.

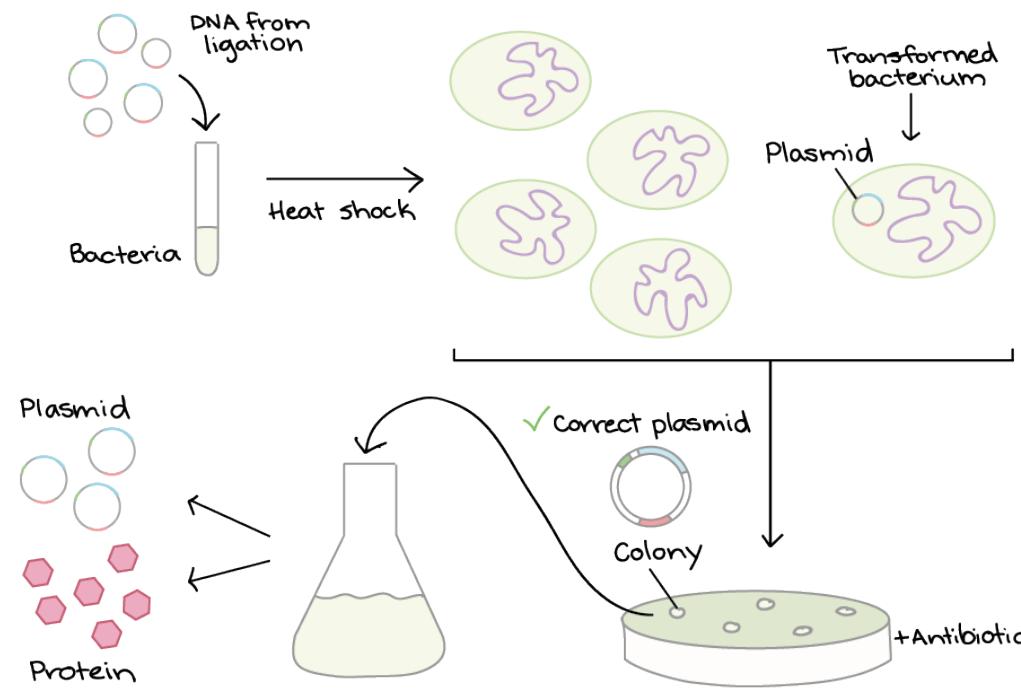
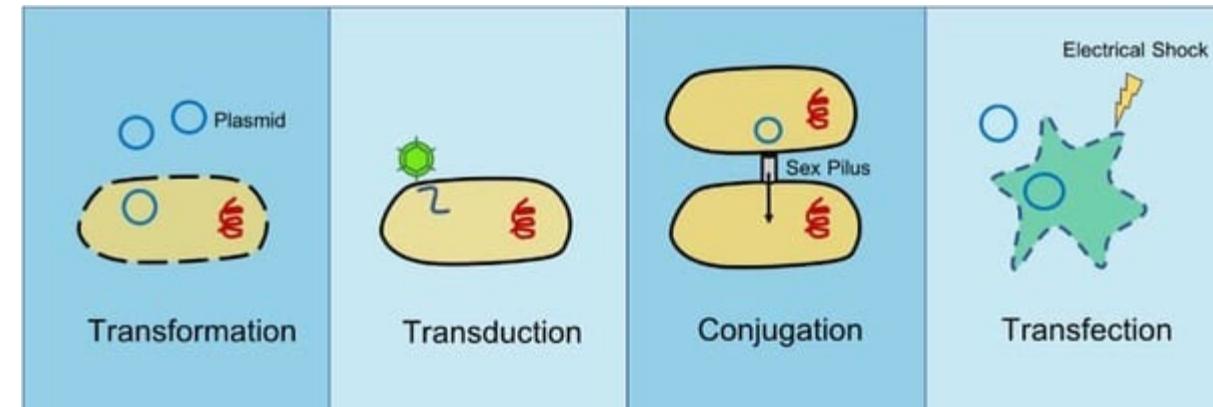
Selection:

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

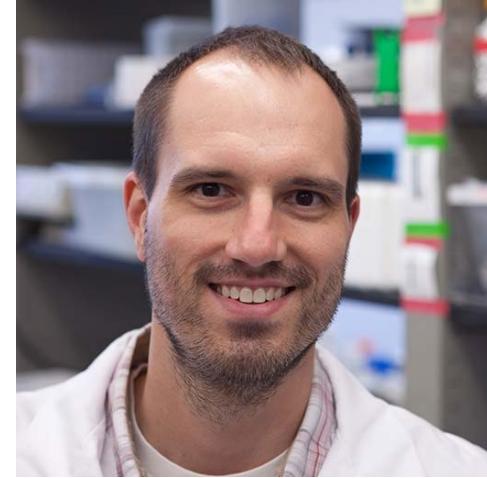
Screening and Confirmation:

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

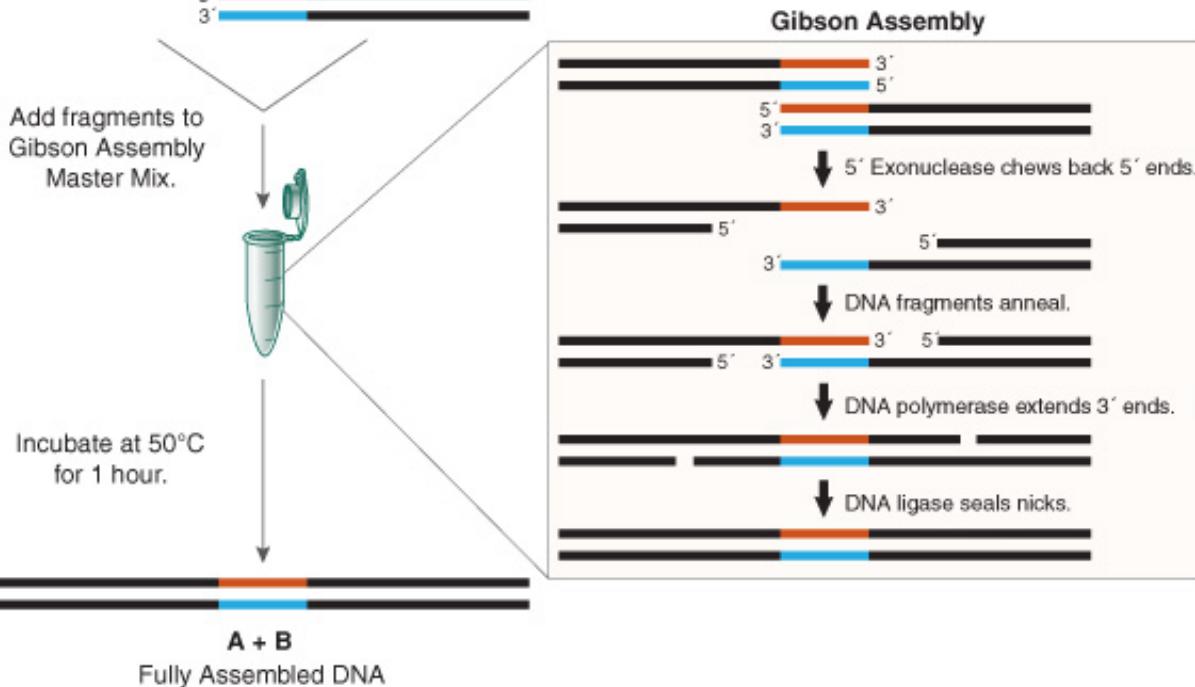
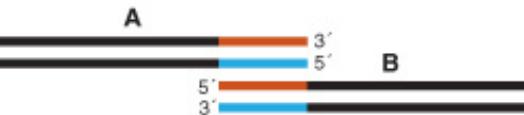
Transformation / Transfection



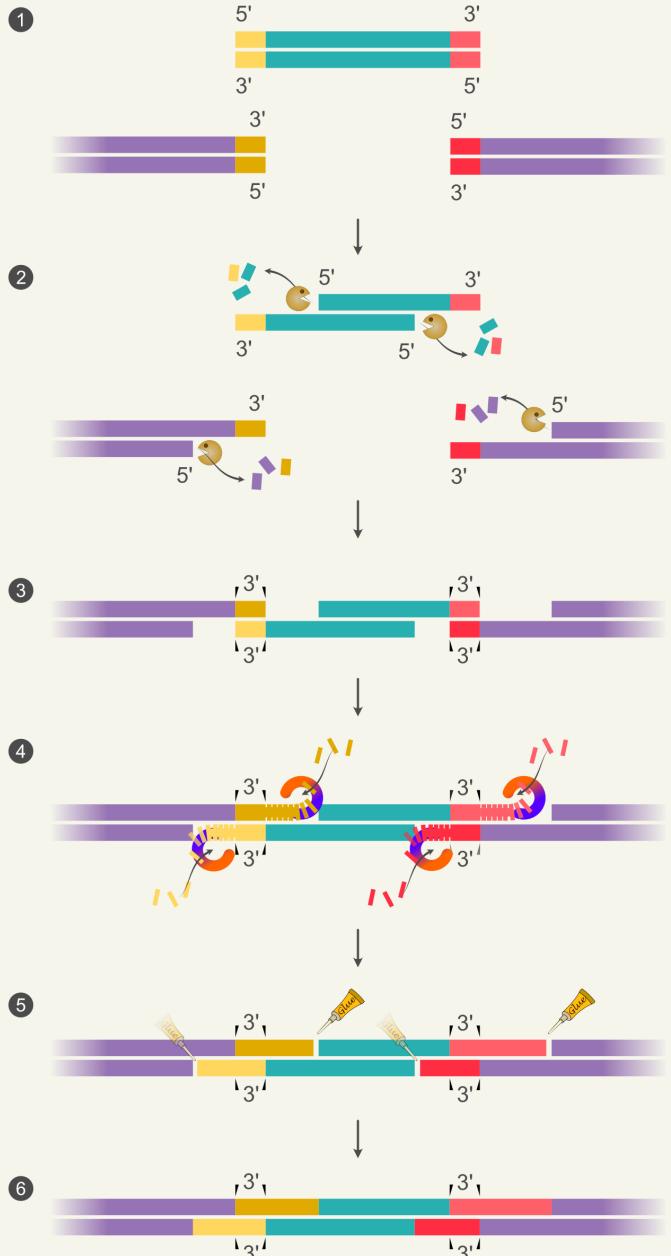
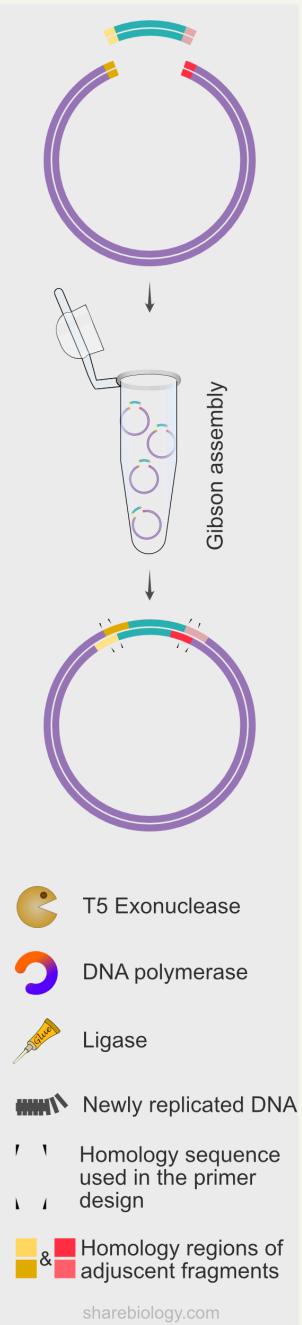
Gibson Assembly



dsDNA fragments with overlapping ends.

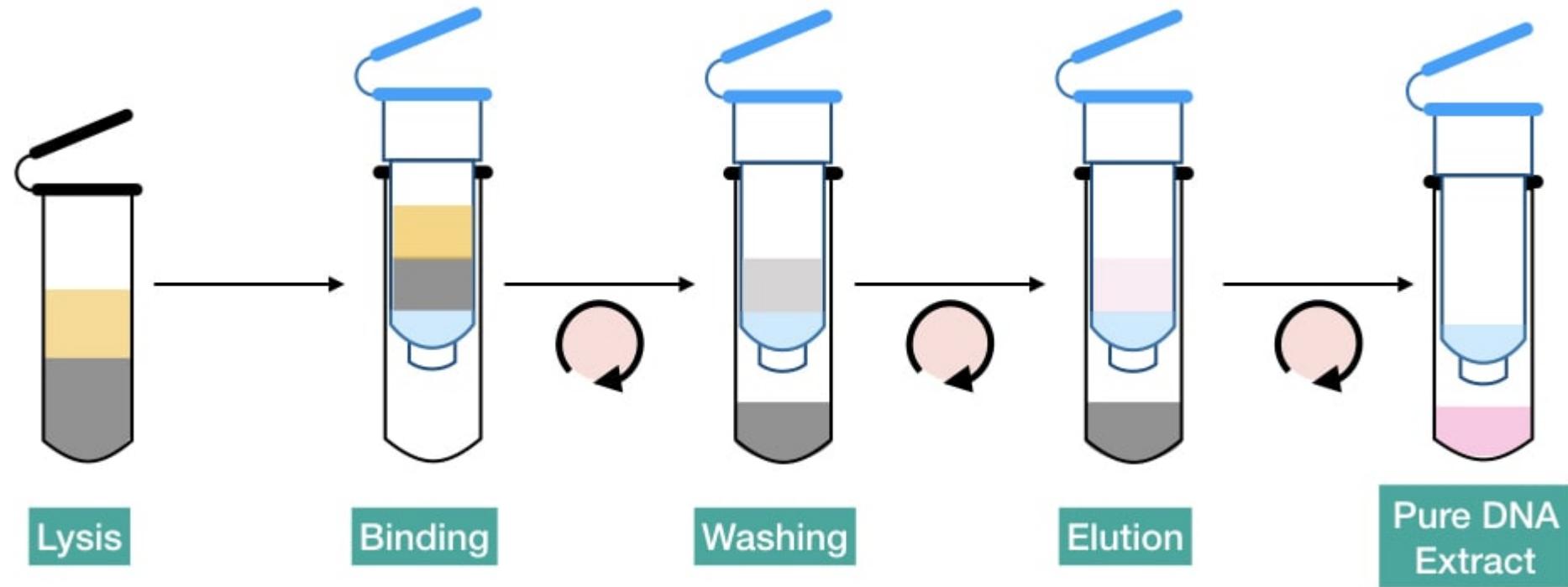
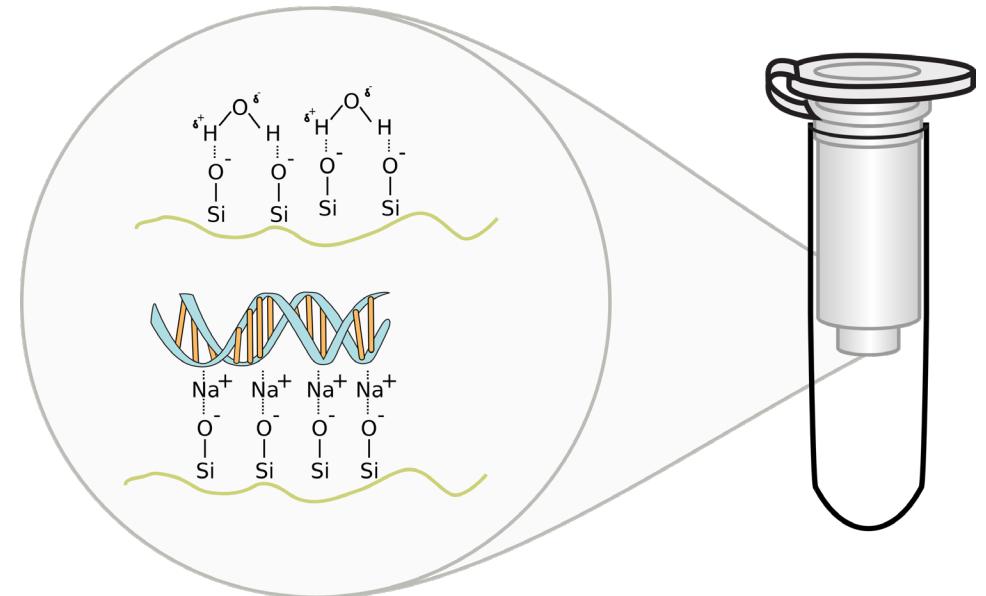


Gibson assembly - Single insert



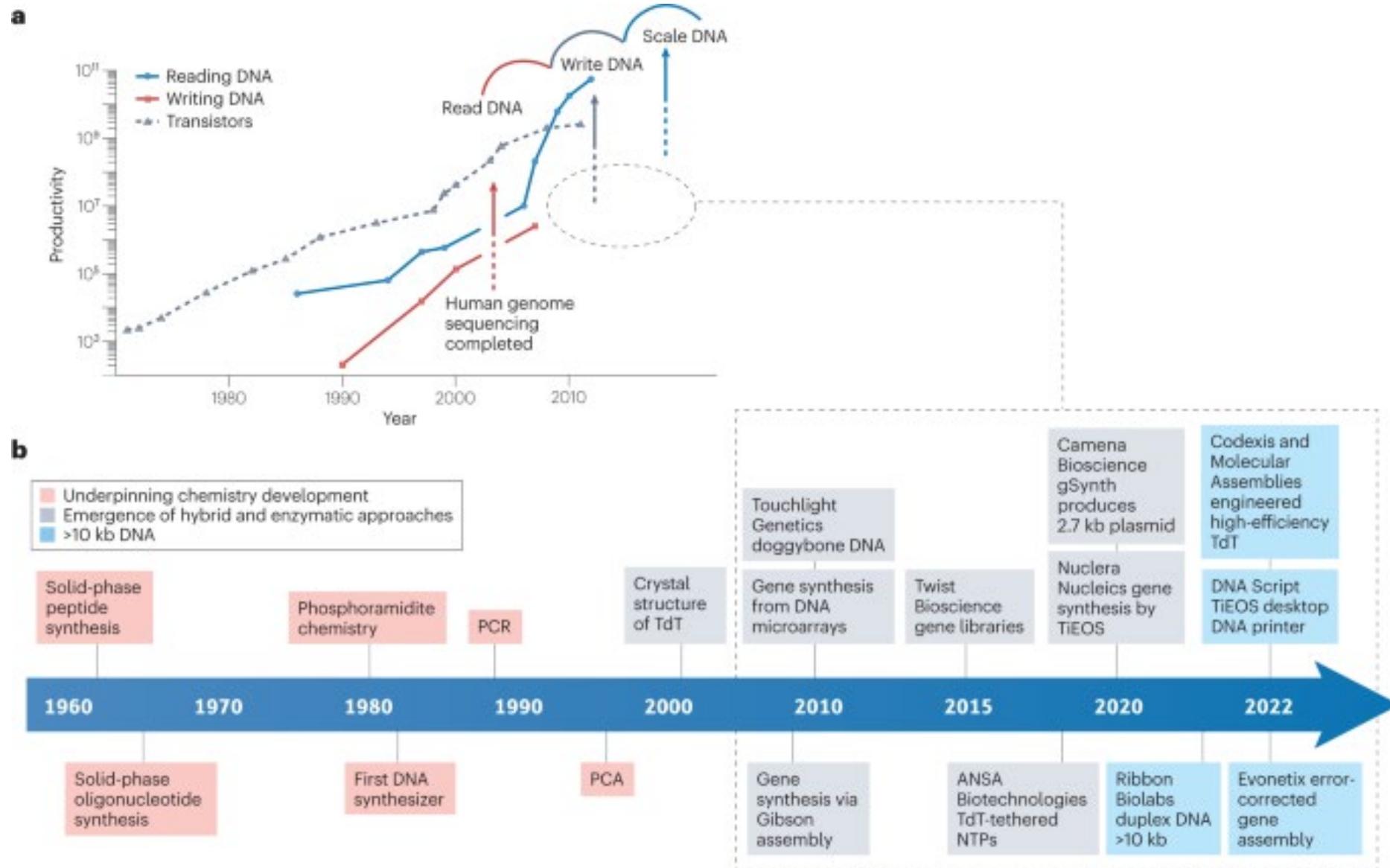
DNA Purification

Spin column DNA purification

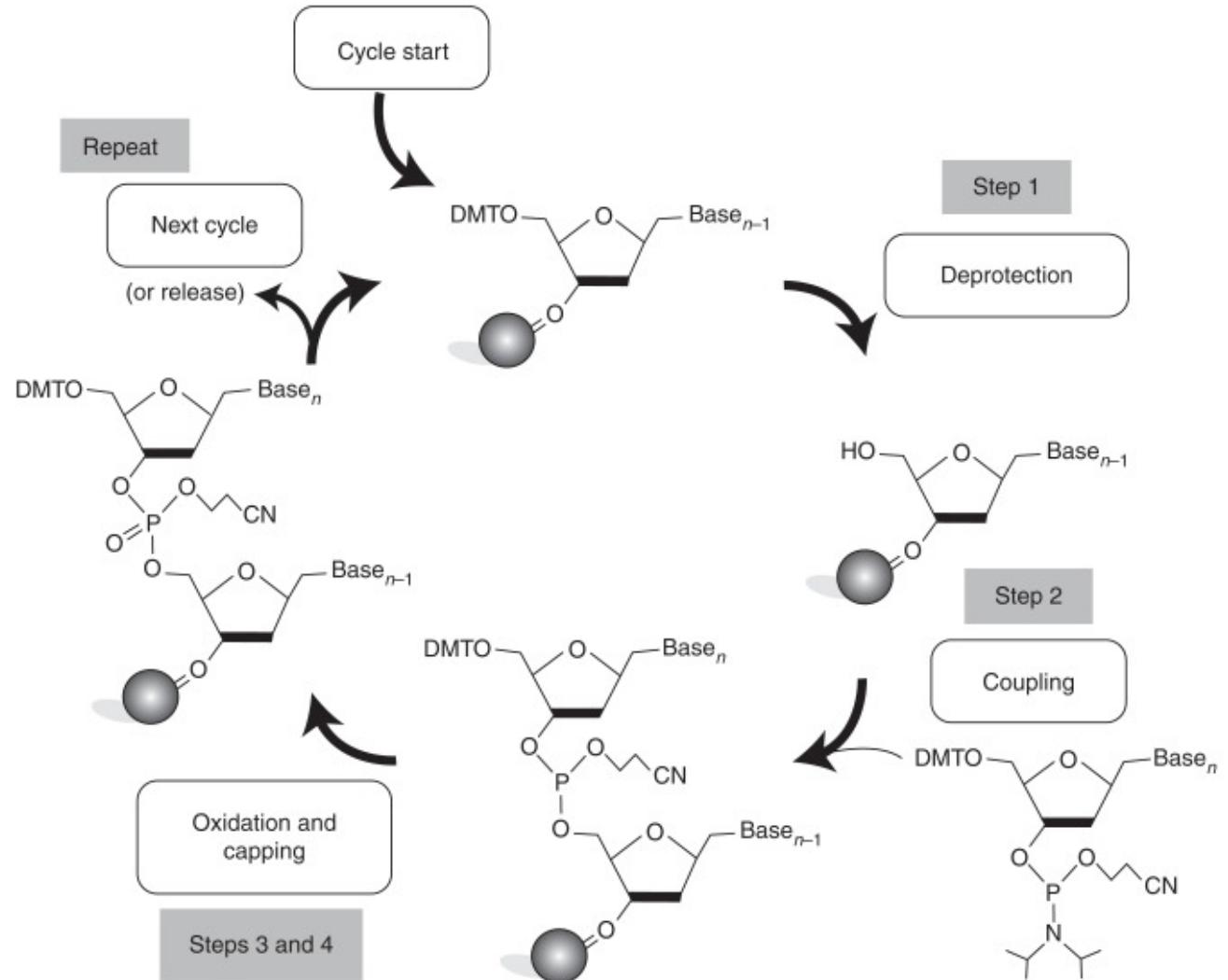


DNA Synthesis

DNA Synthesis History

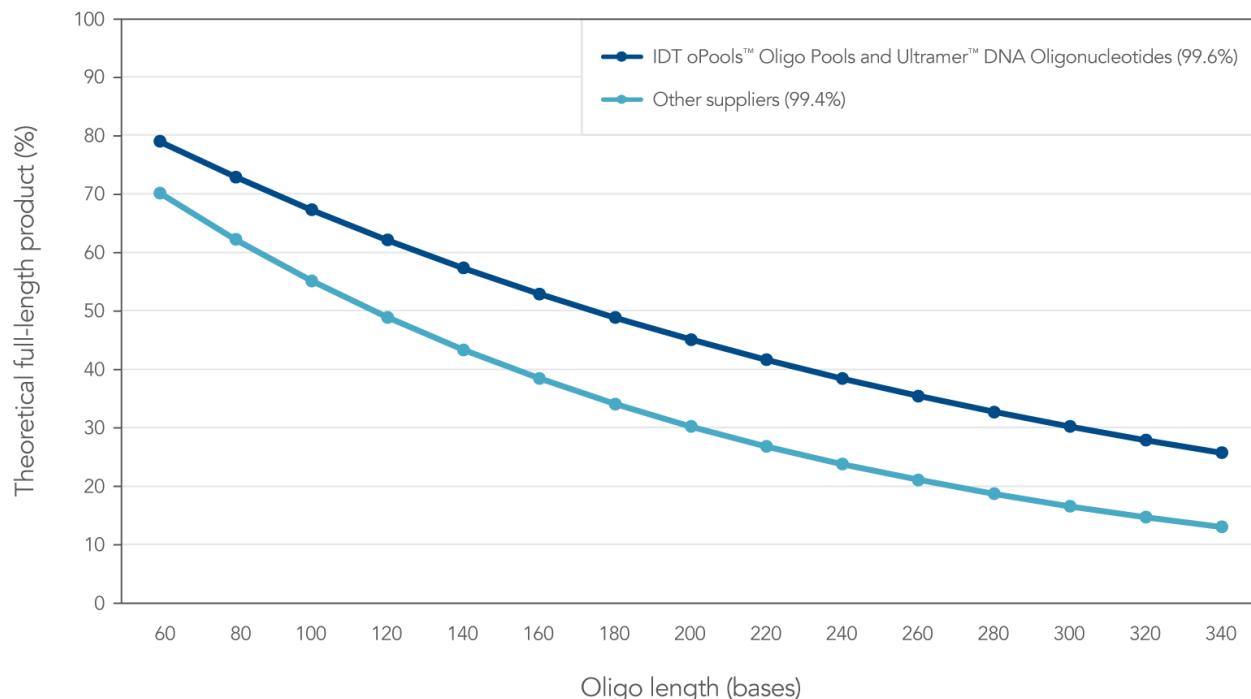


Solid Phase Synthesis



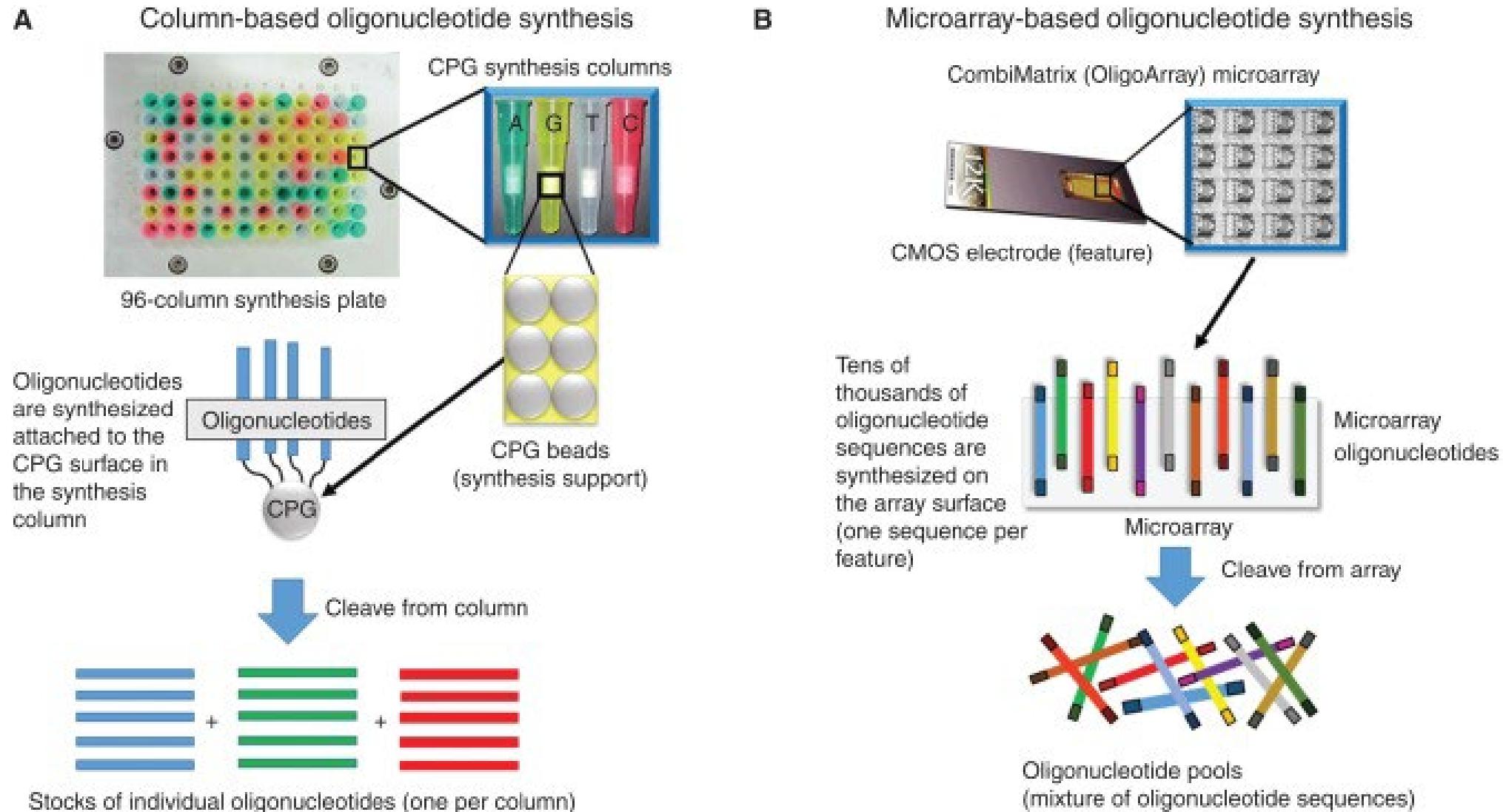
Limits of Solid Phase Synthesis

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

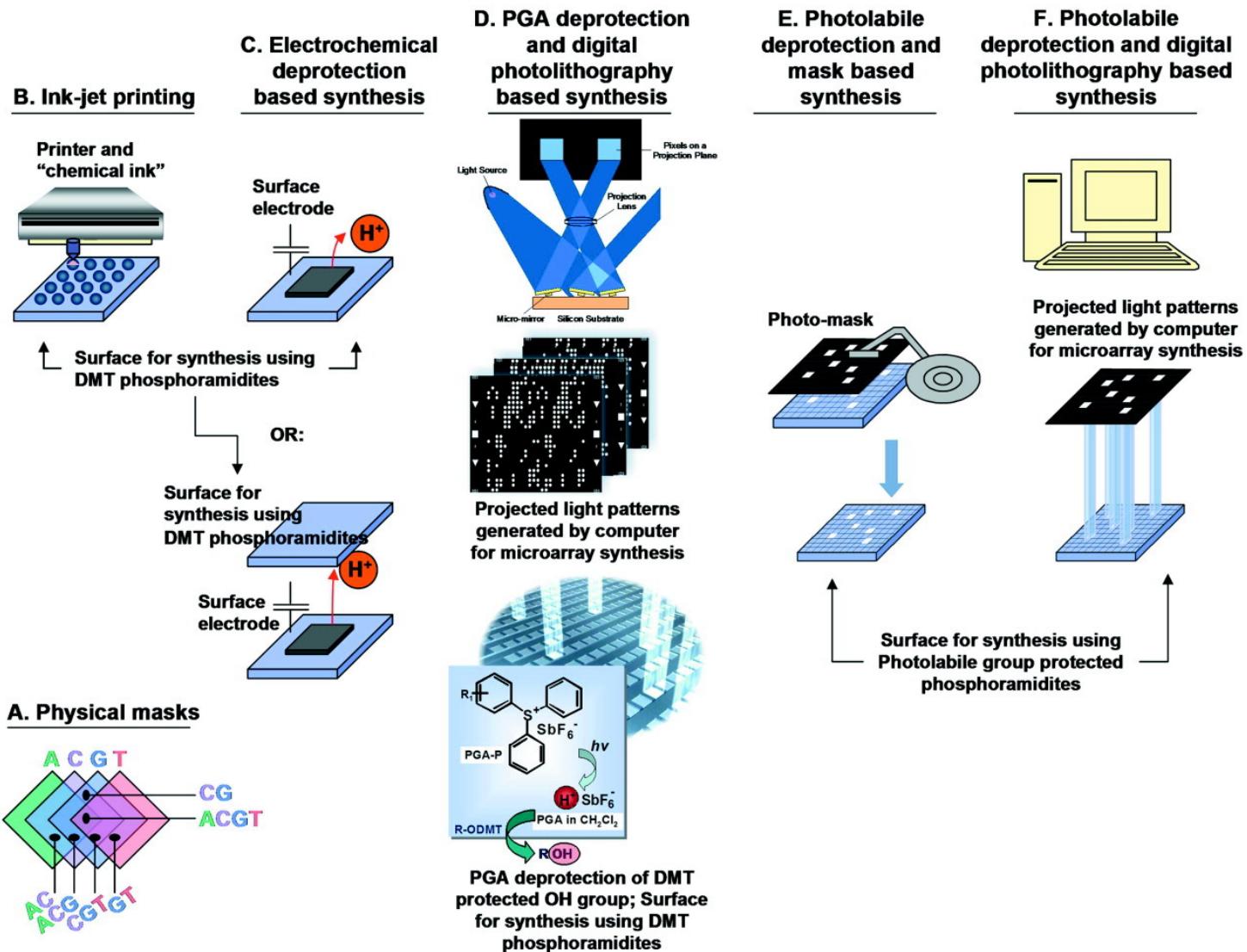


Microarray-based oligo synthesis
(increased throughput)

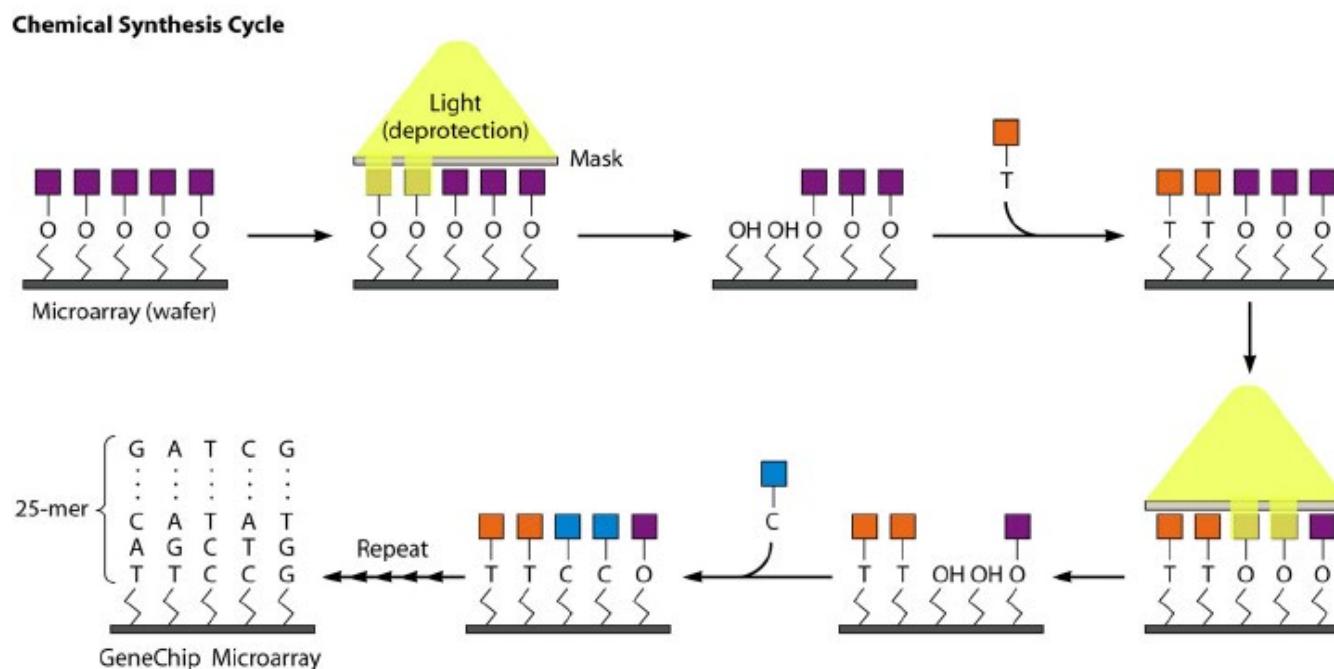
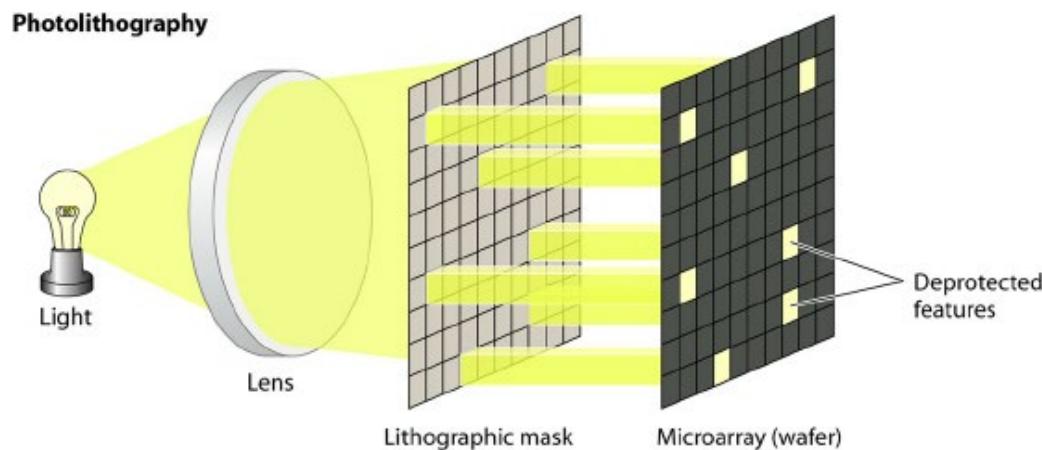
Microarray-based oligo synthesis



Microarray-based oligo synthesis: inkjet and digital photolithography



Digital Photolithography

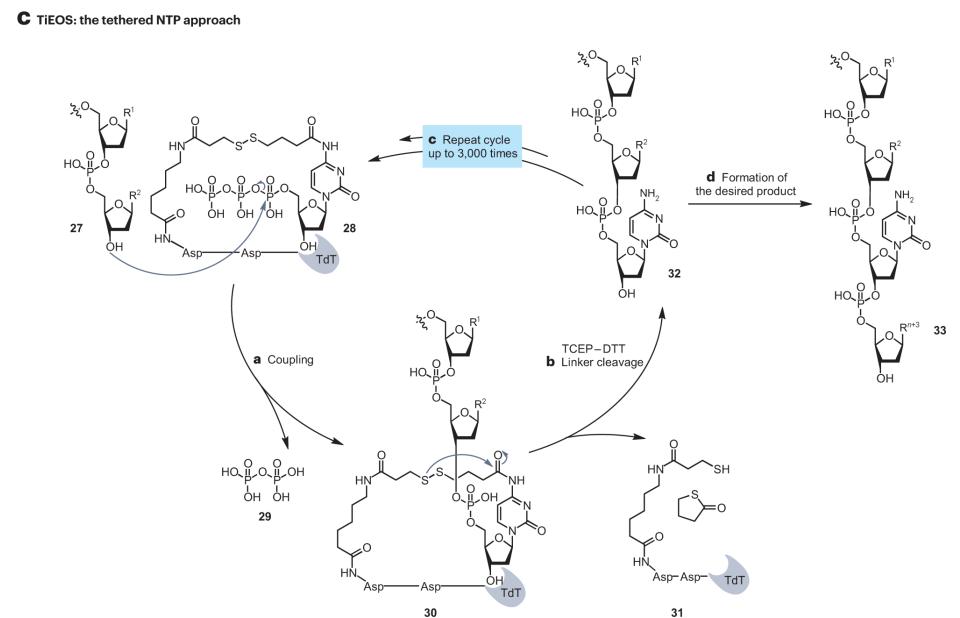
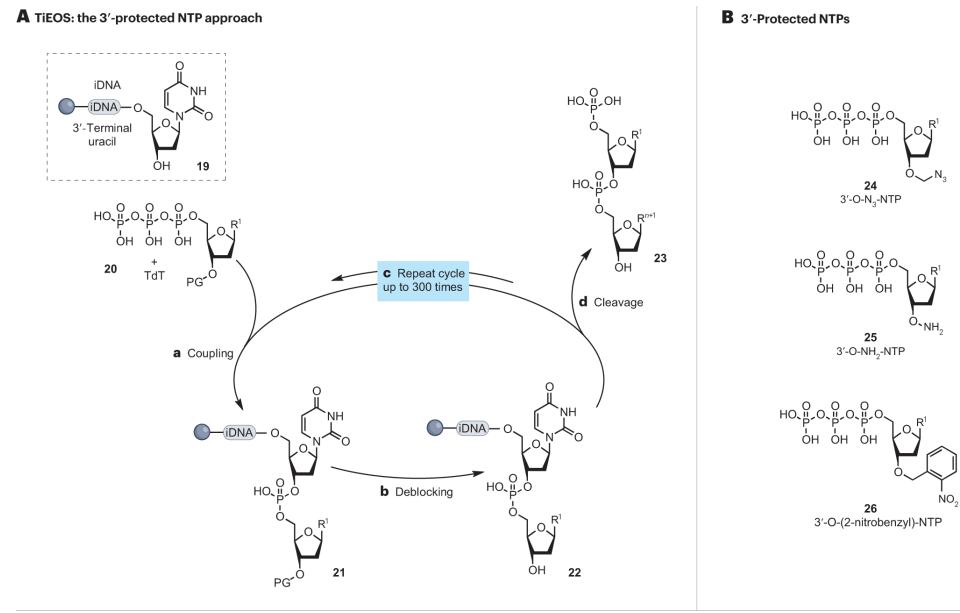
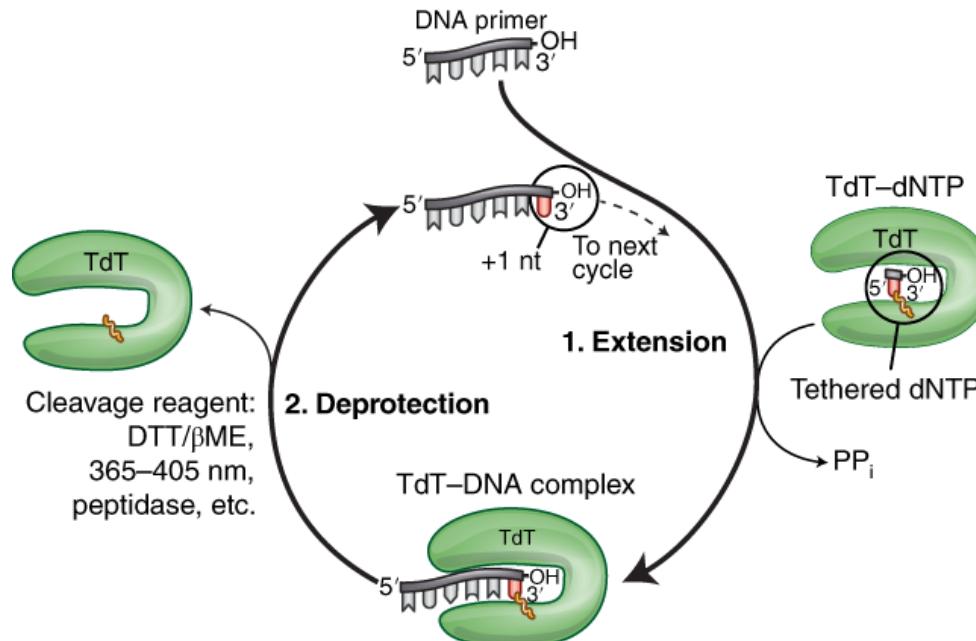


Enzyme Based Oligo Synthesis (increased cycling efficiency)

TiEOS: template-independent enzymatic oligonucleotide oligo synthesis

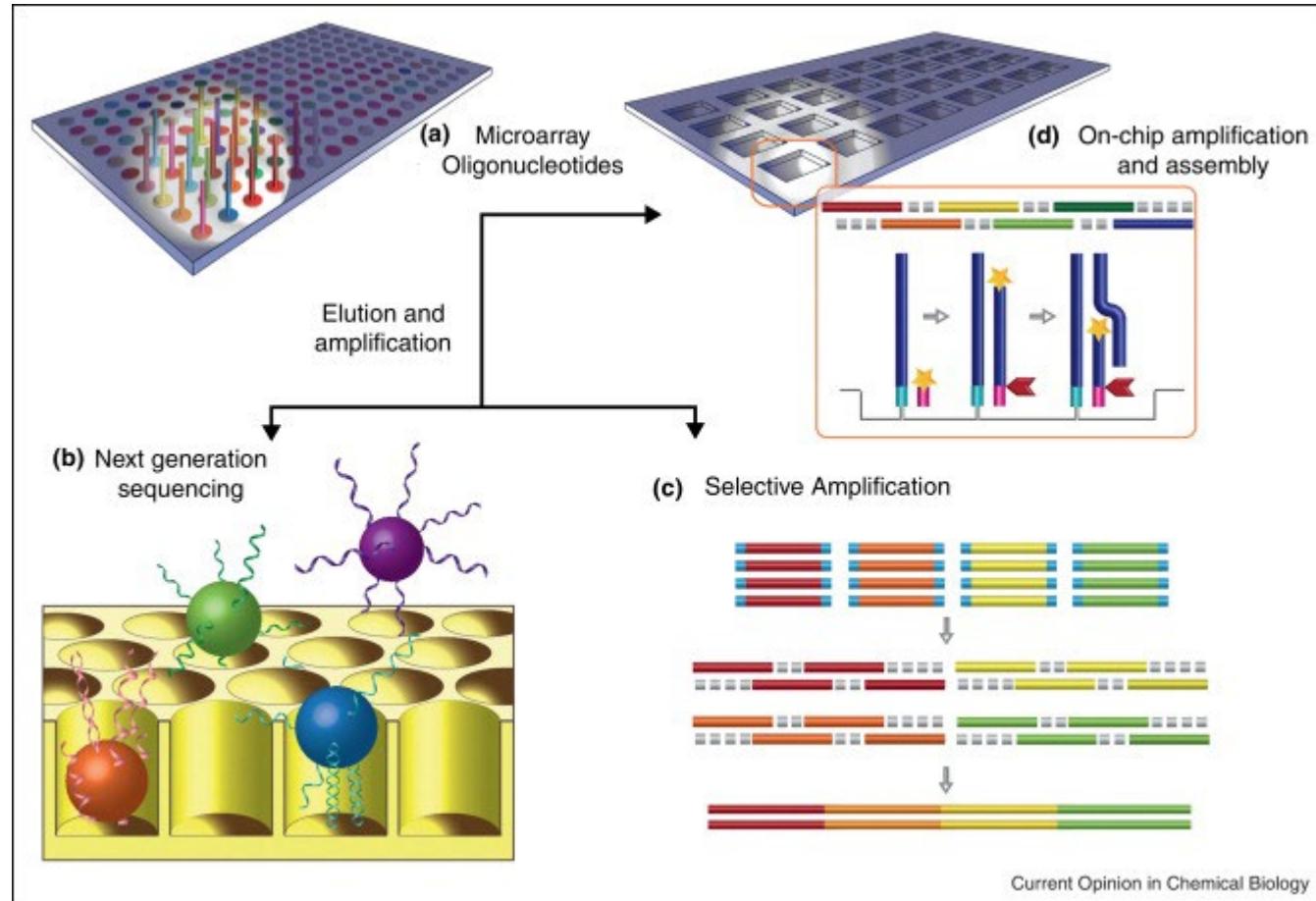
Enzyme:

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

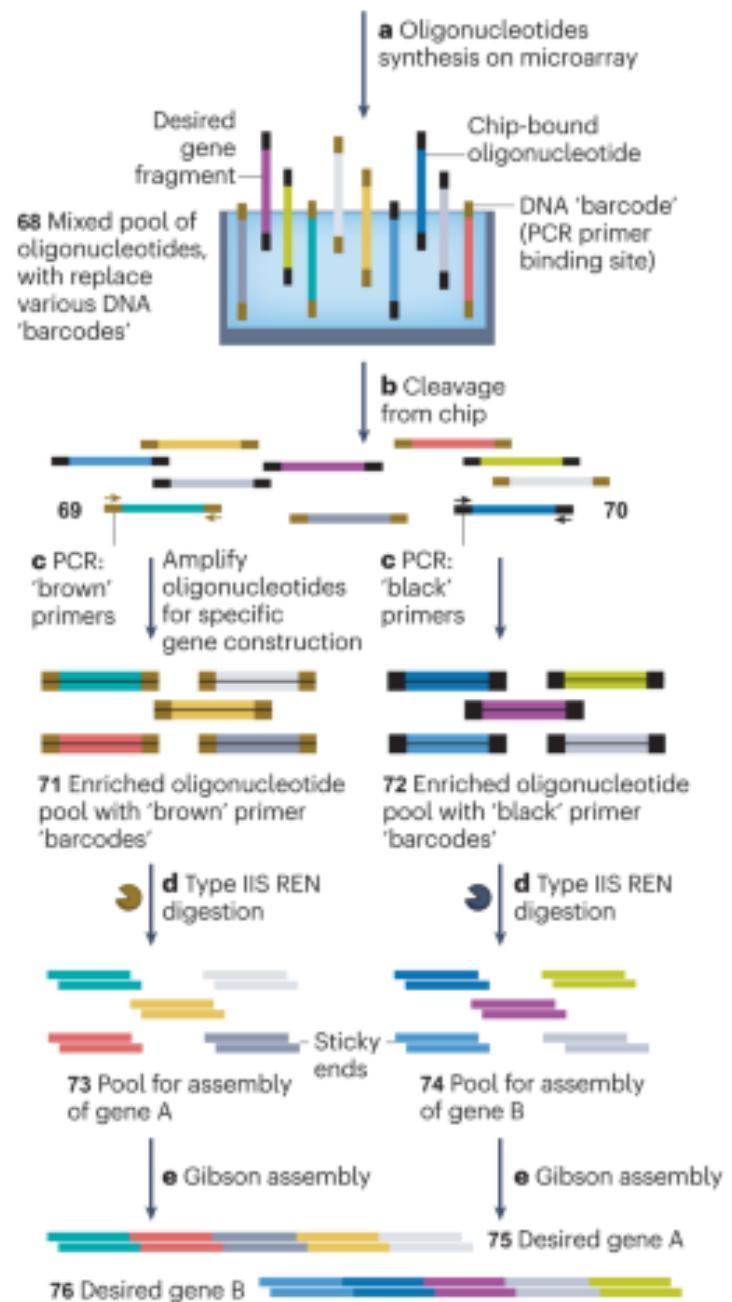


Gene and Genome Synthesis

Gene Synthesis

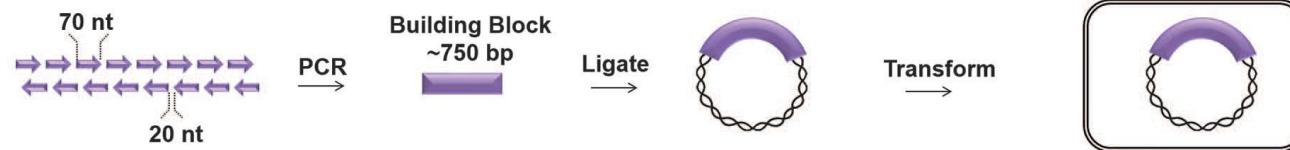


B Gene synthesis from DNA microarrays

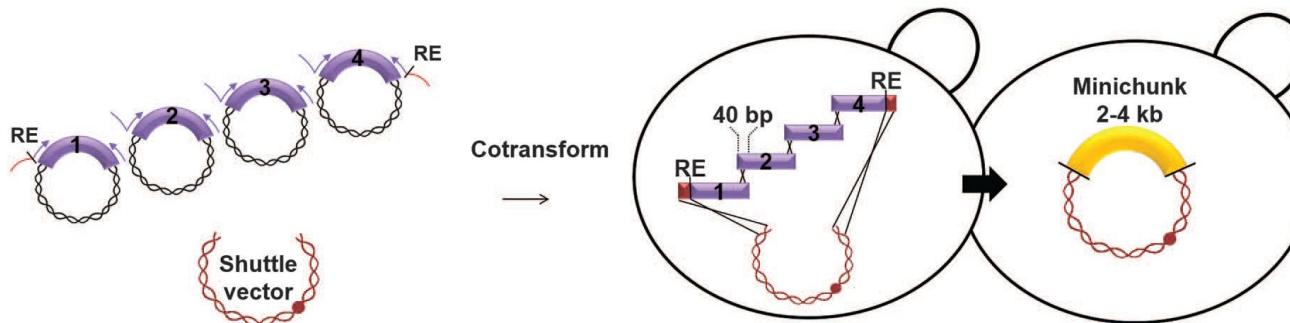


Genome Synthesis

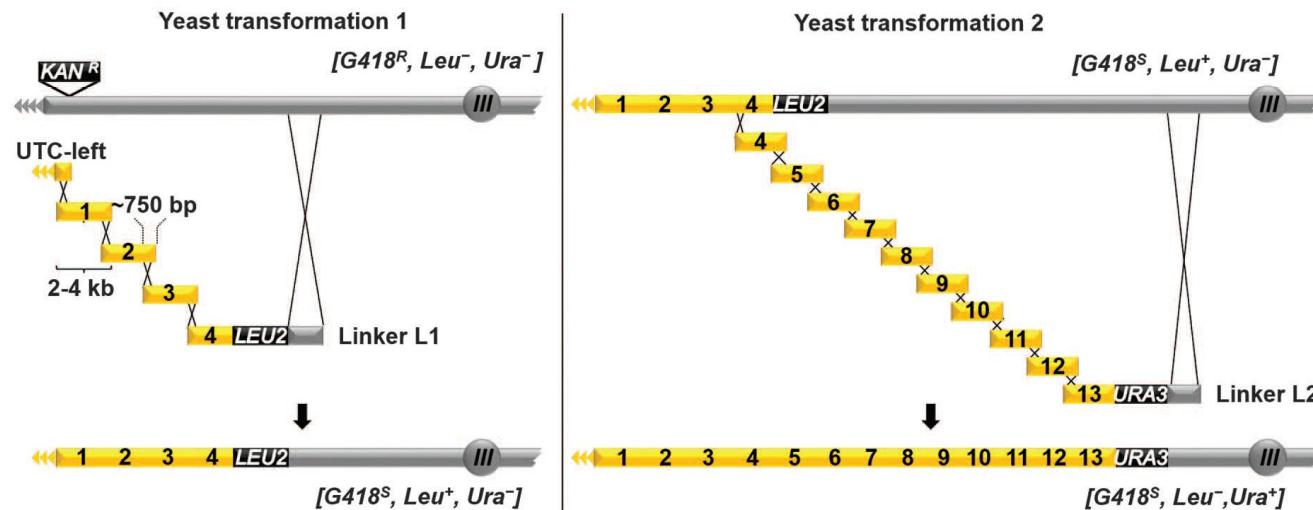
A Step 1: Synthesize Building Blocks (BBs) from oligonucleotides



B Step 2: Assemble 2-4 kb minichunks

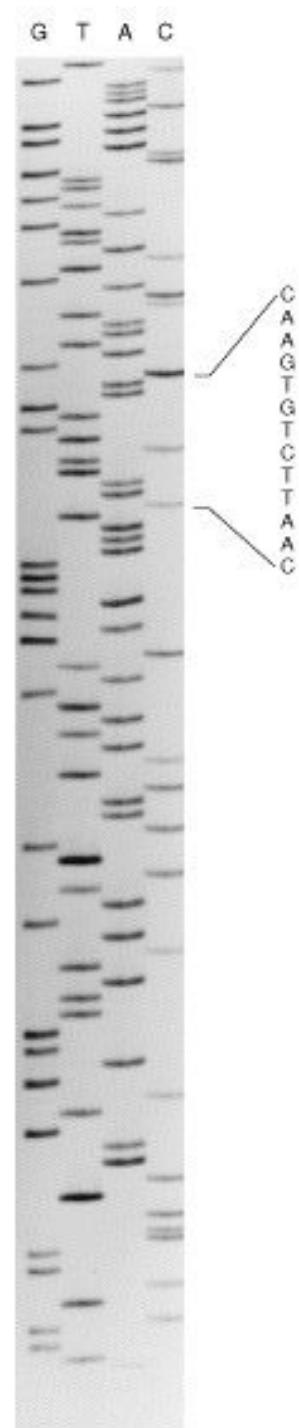
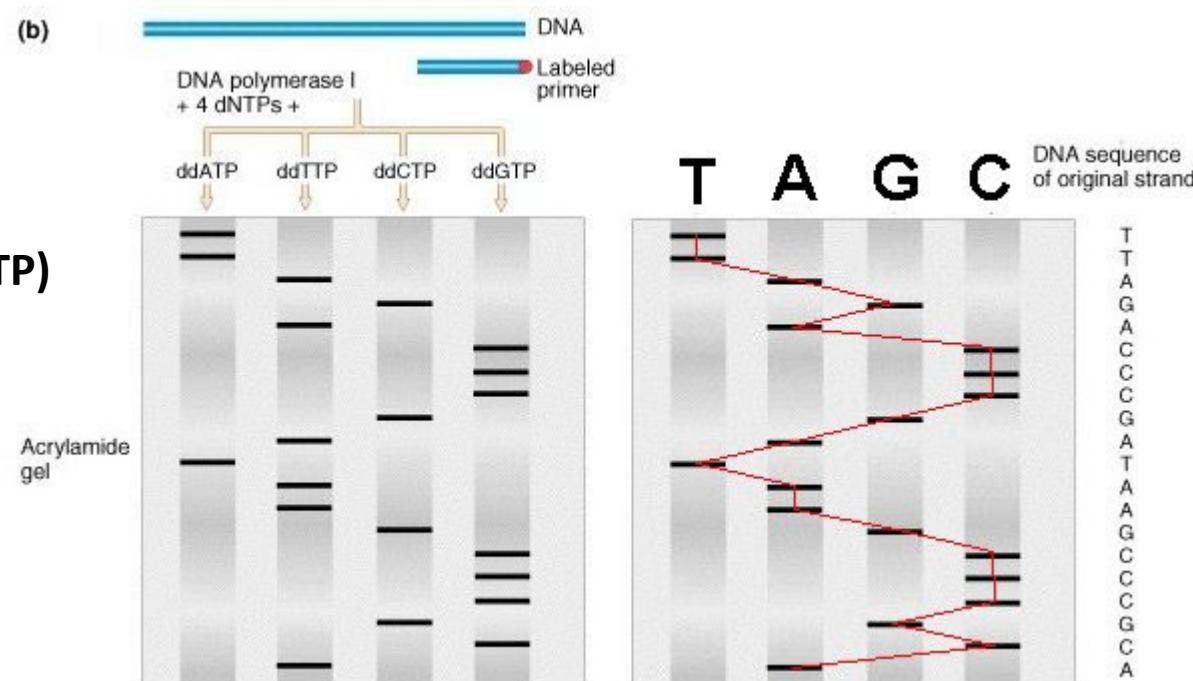
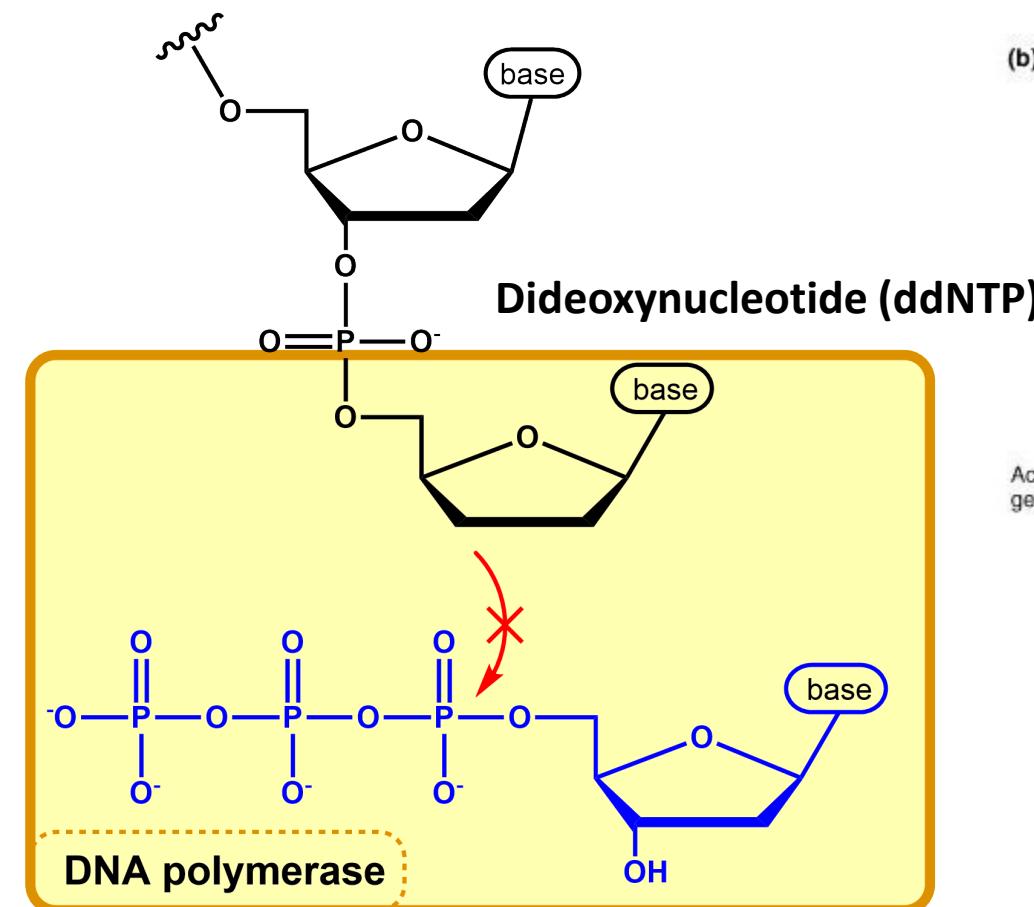


C Step 3: Replace native *III* with minichunks



Sequencing

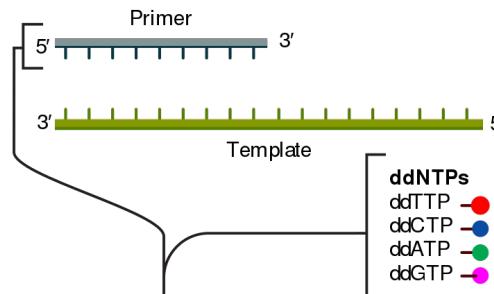
Radioactive DNA Sequencing



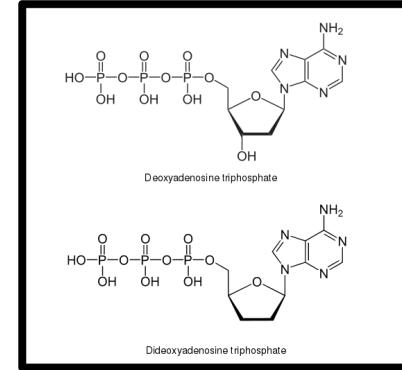
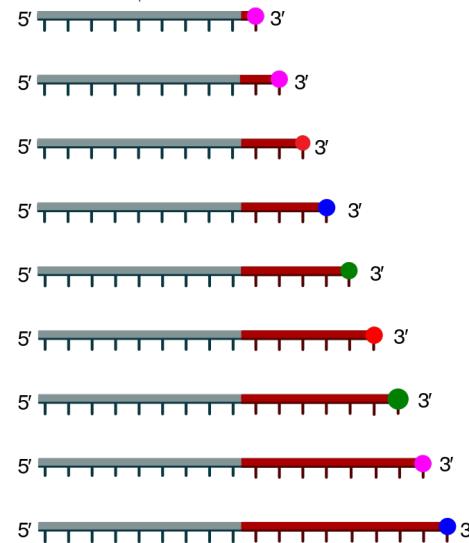
Sanger Sequencing

① Reaction mixture

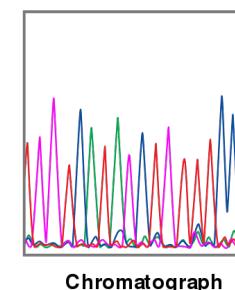
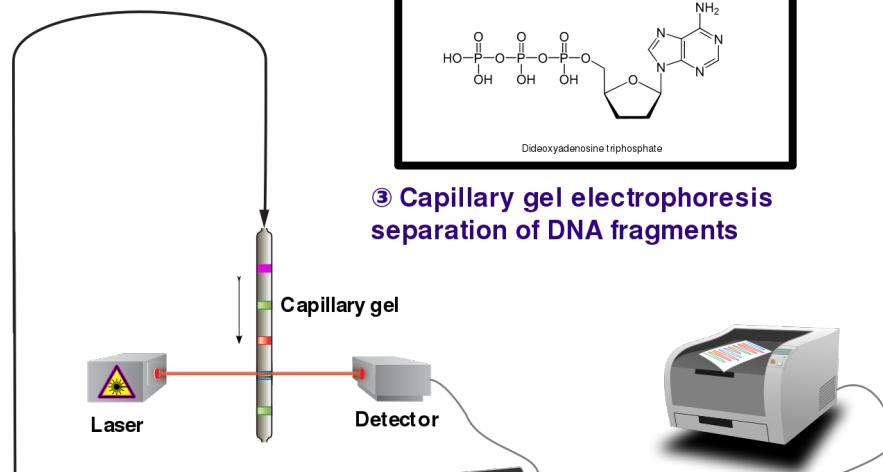
- Primer and DNA template
- DNA polymerase
- ddNTPs with flourochromes
- dNTPs (dATP, dCTP, dGTP, and dTTP)



② Primer elongation and chain termination

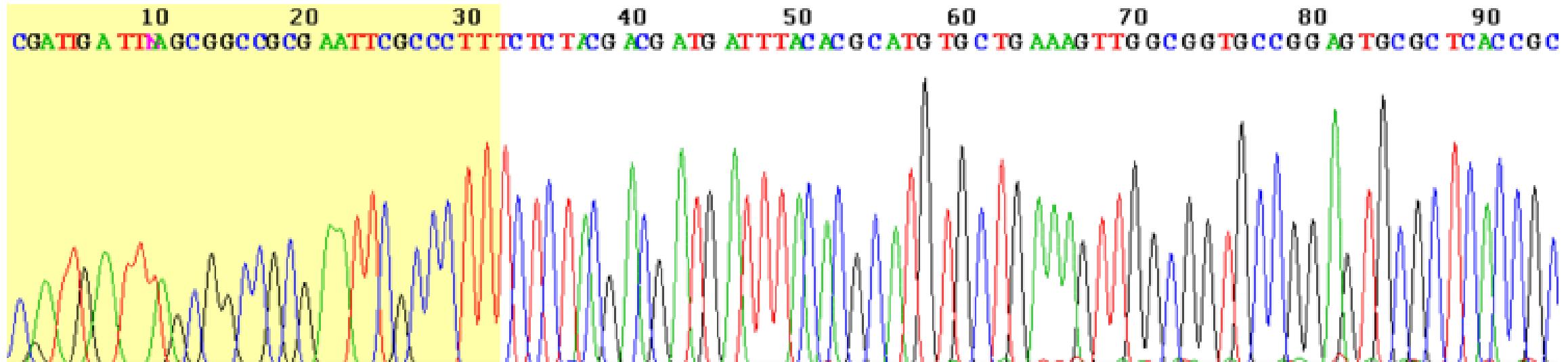


③ Capillary gel electrophoresis separation of DNA fragments

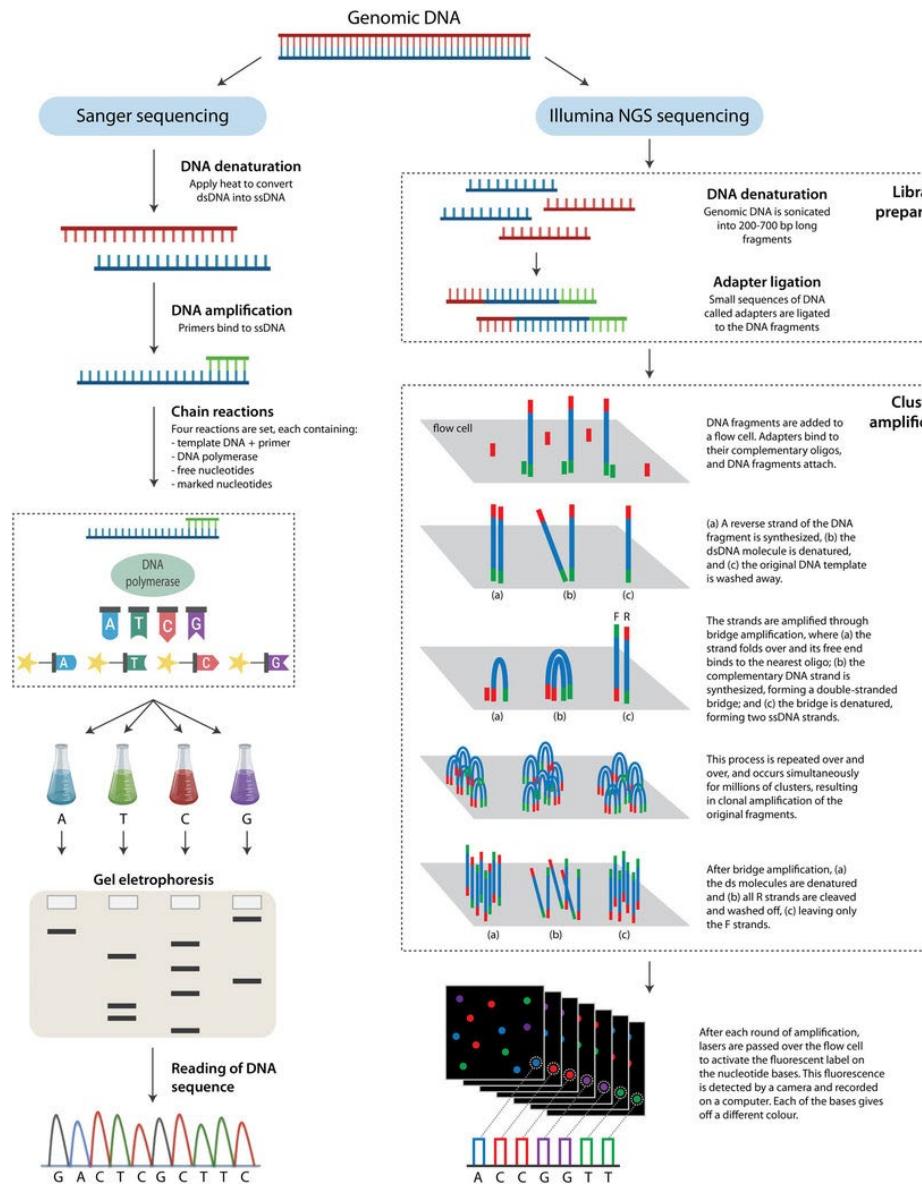


④ Laser detection of flourochromes and computational sequence analysis

Sanger Sequencing Trace



Next Generation Sequencing



News Feature | Published: 12 January 2023

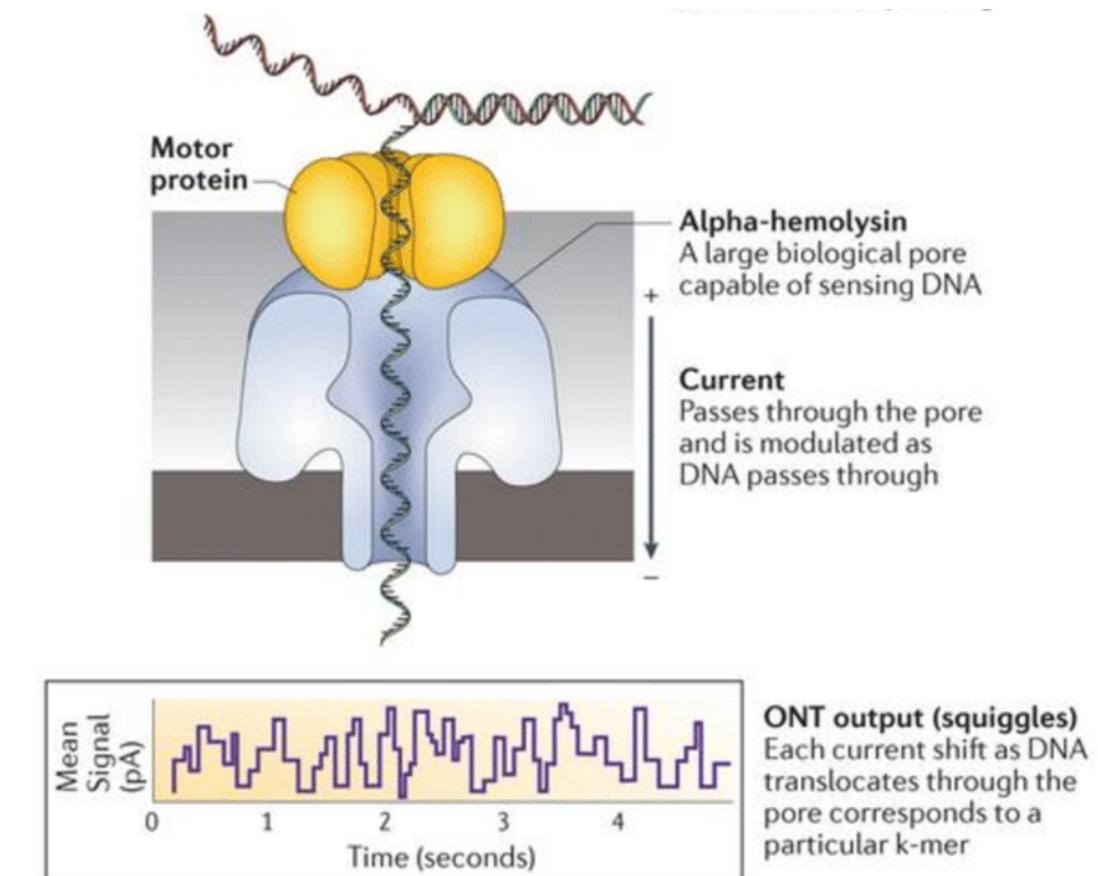
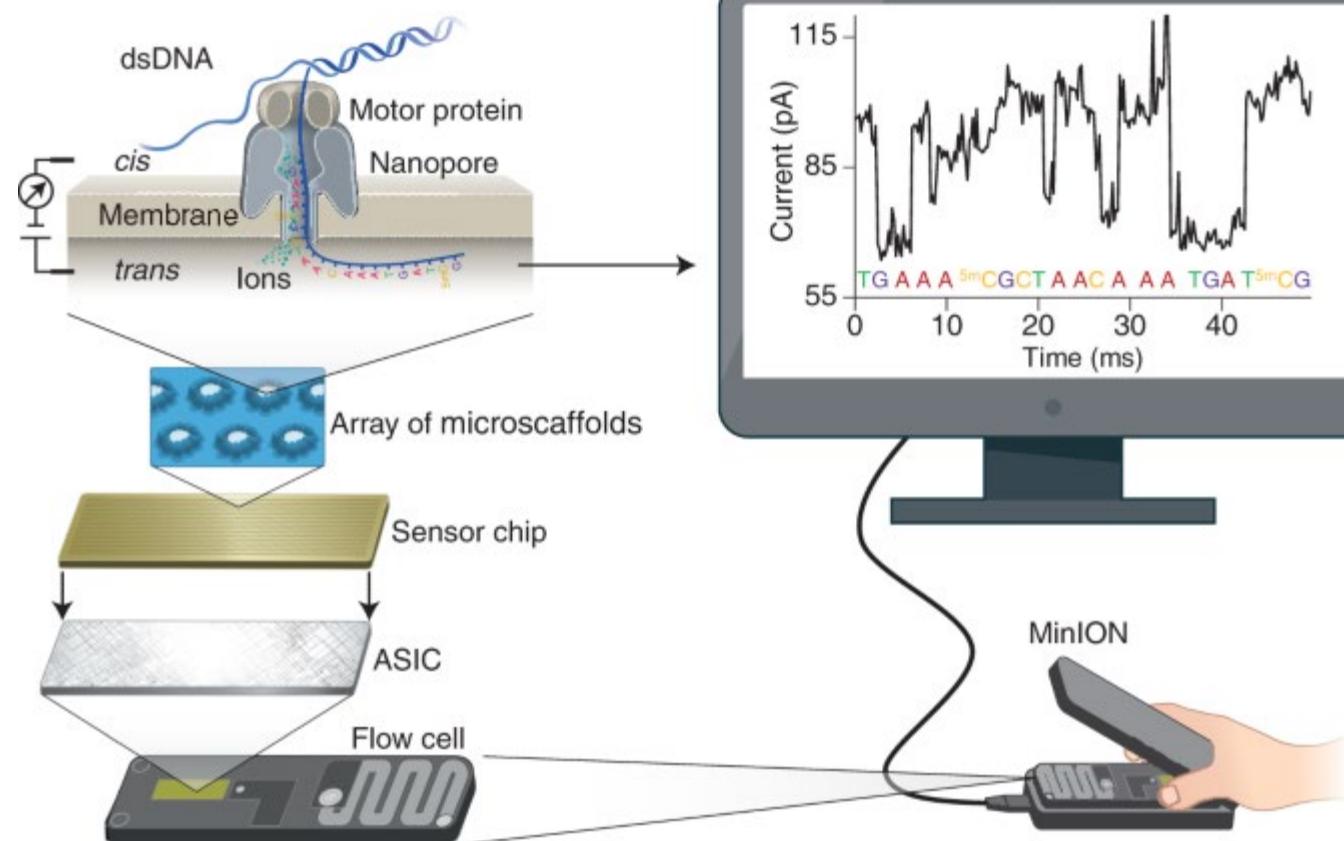
Method of the year: long-read sequencing

[Vivien Marx](#) 

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

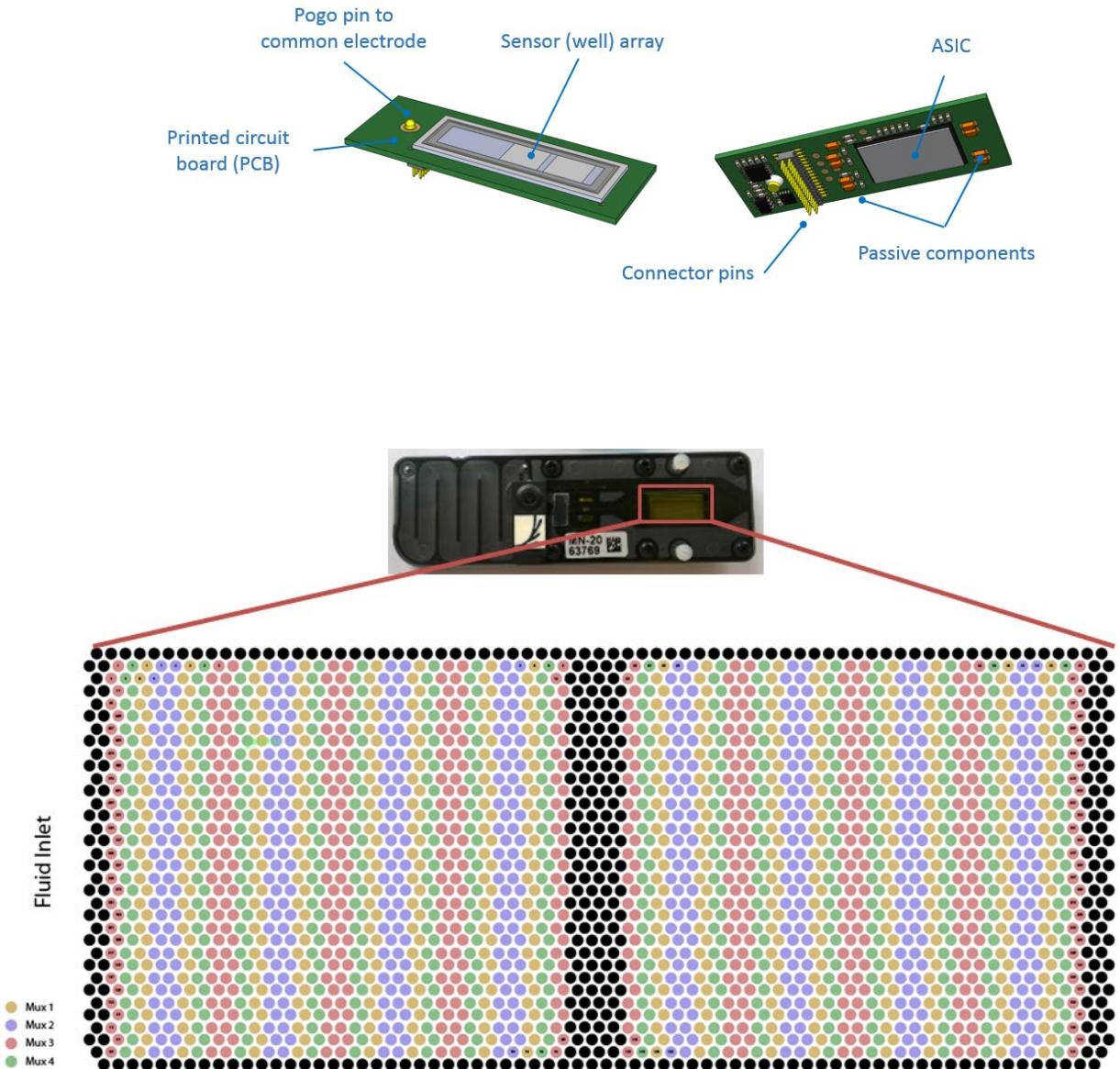
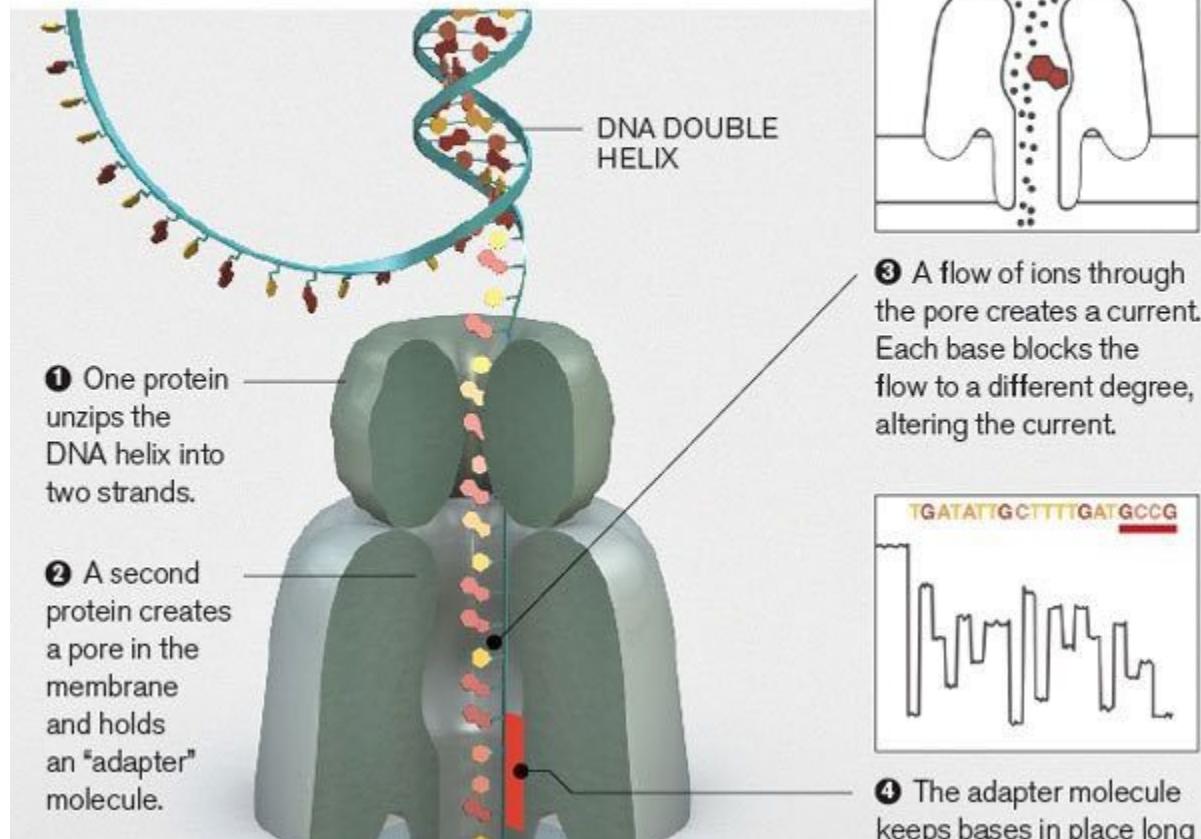
68k Accesses | **50** Citations | **517** Altmetric | [Metrics](#)

Nanopore Sequencing

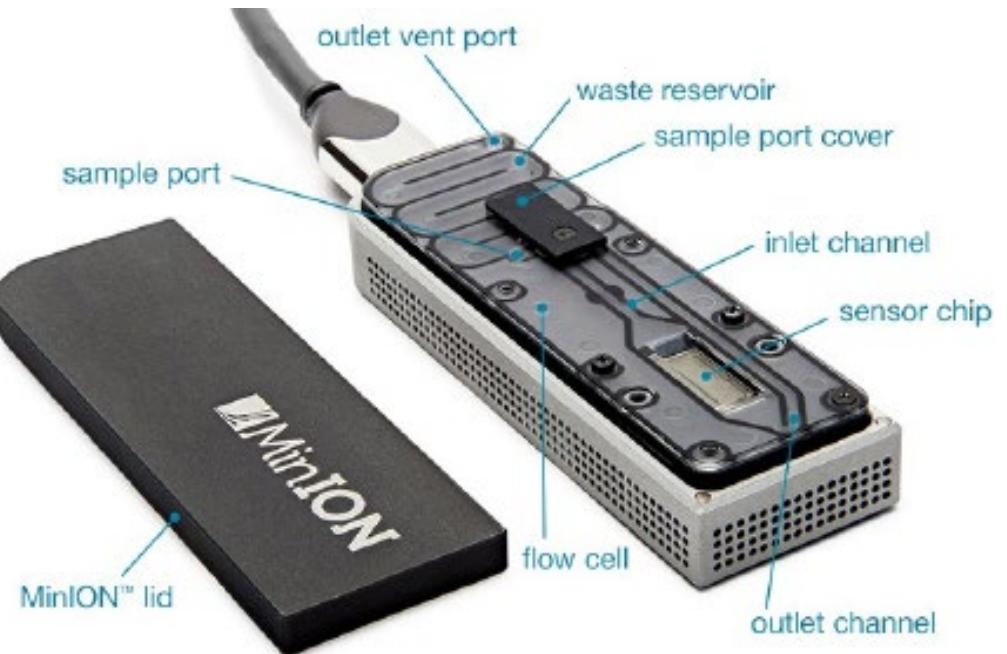
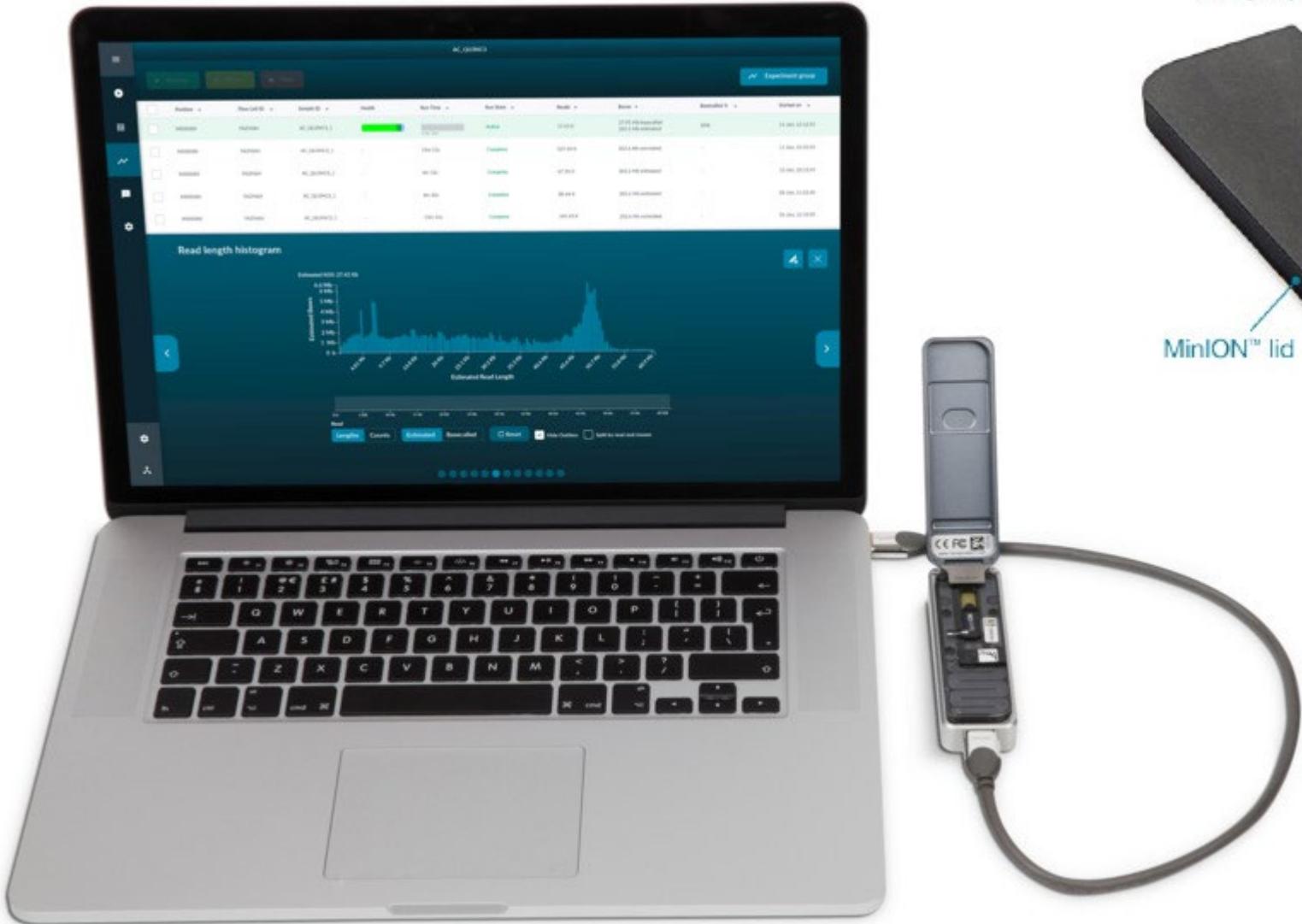


Nanopore Sequencing

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.



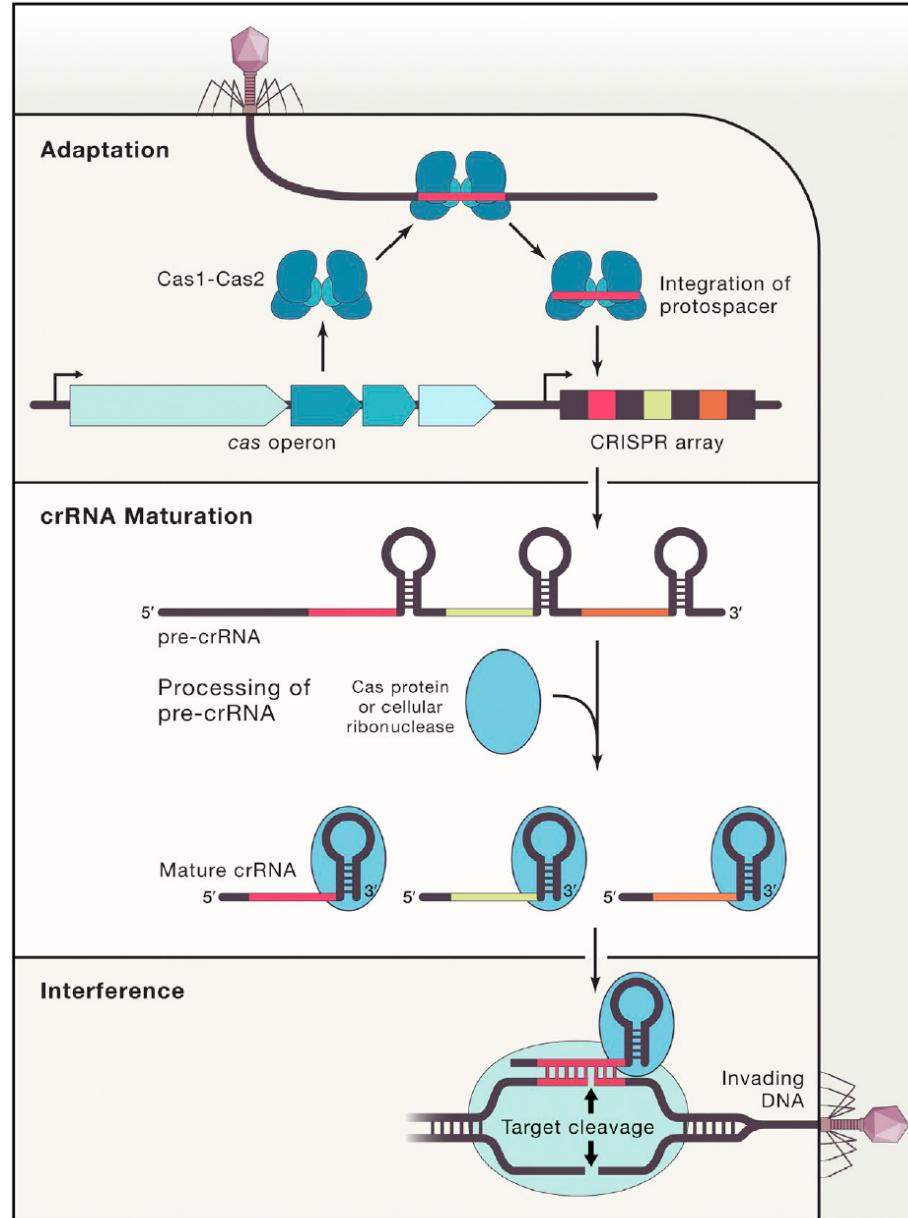
Nanopore Sequencing



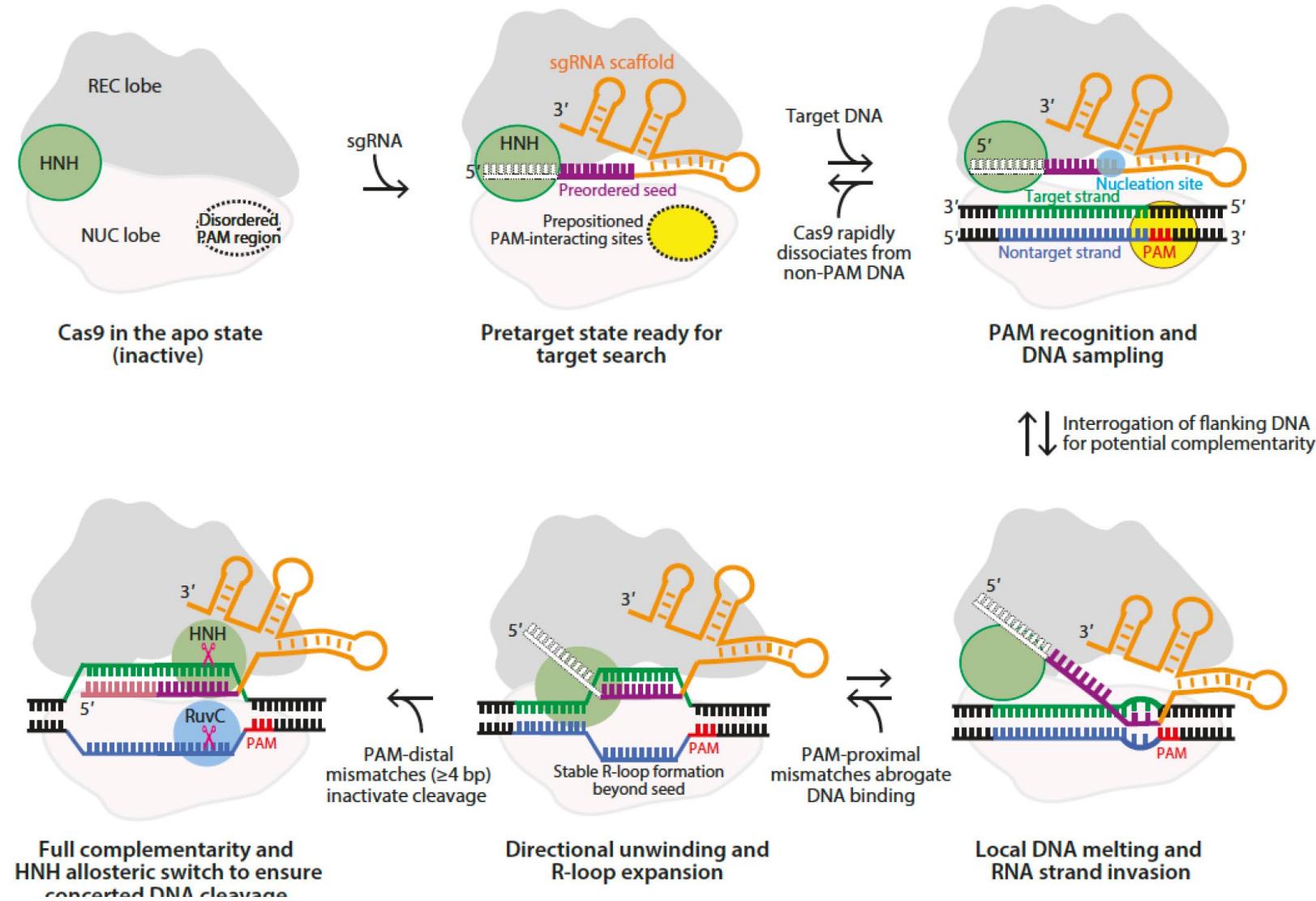
Future: direct single protein sequencing!

CRISPR
Nobel Chemistry 2020:
Emmanuelle Charpentier
and
Jennifer Doudna

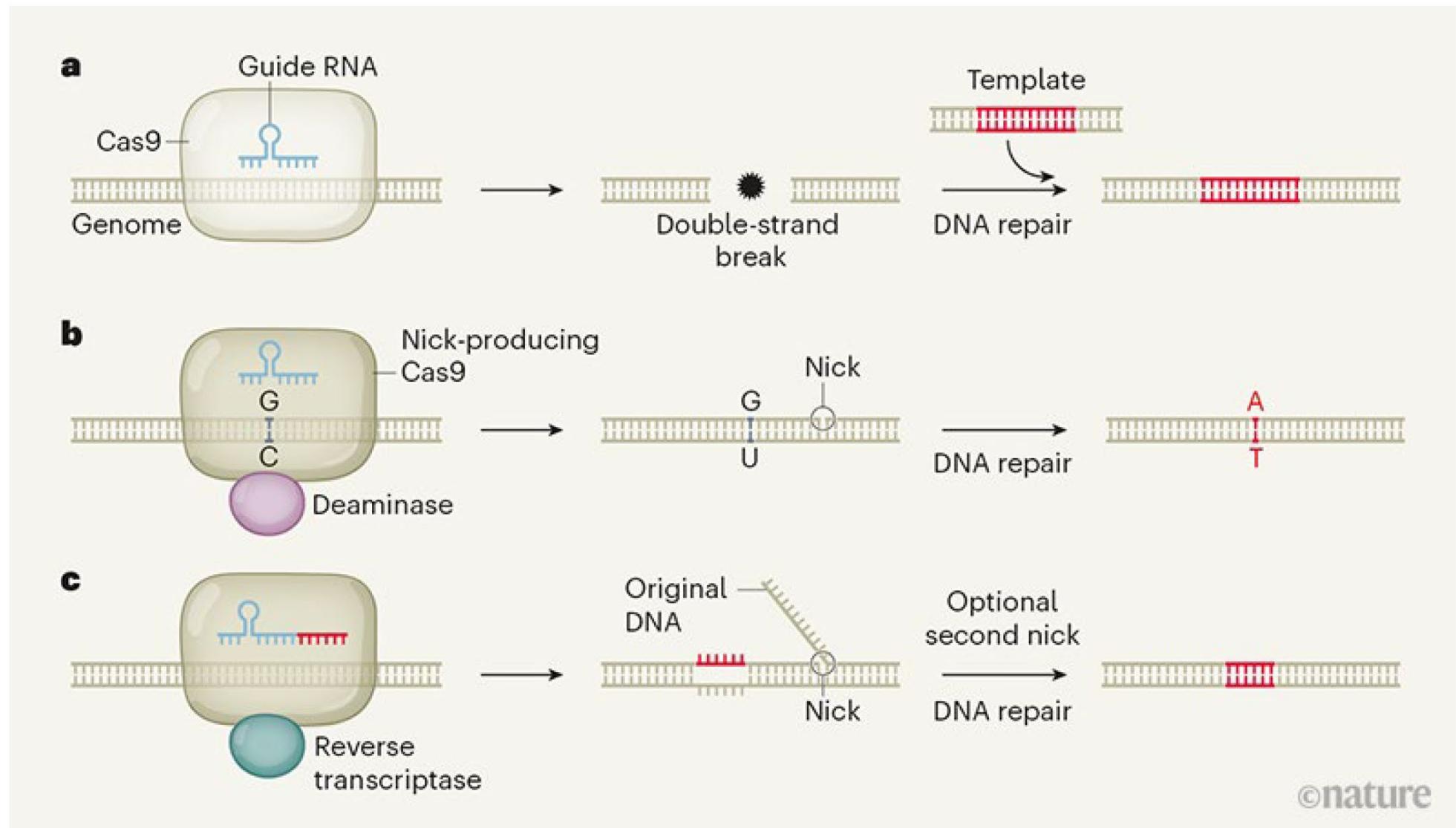
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats



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 *CCR5Δ32* 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.

