

# CHAPTER 3

*Recombinant DNA Technology and DNA  
Cloning*

PART 2 of 4

# Polymerase Chain Reaction

Much more rapid approach to cloning than building and screening a library

Developed in the mid-1980s by Kary Mullis

Technique for making copies, or amplifying, a specific sequence of DNA in a short period of time

# Polymerase Chain Reaction

Reaction mixture contains:

1. Target DNA to be amplified
2. Nucleotides (dATP, dCTP, dGTP, dTTP)
3. Buffer
4. DNA polymerase
5. Paired set of Forward and Reverse **Primers**— short single-stranded DNA oligonucleotides (20–30bp long)

Primers are complementary to nucleotides flanking opposite ends of target DNA

Reaction tube is placed in an instrument called a **thermocycler**

# Polymerase Chain Reaction

Thermocycler will take DNA through a series of reactions called a **PCR cycle**

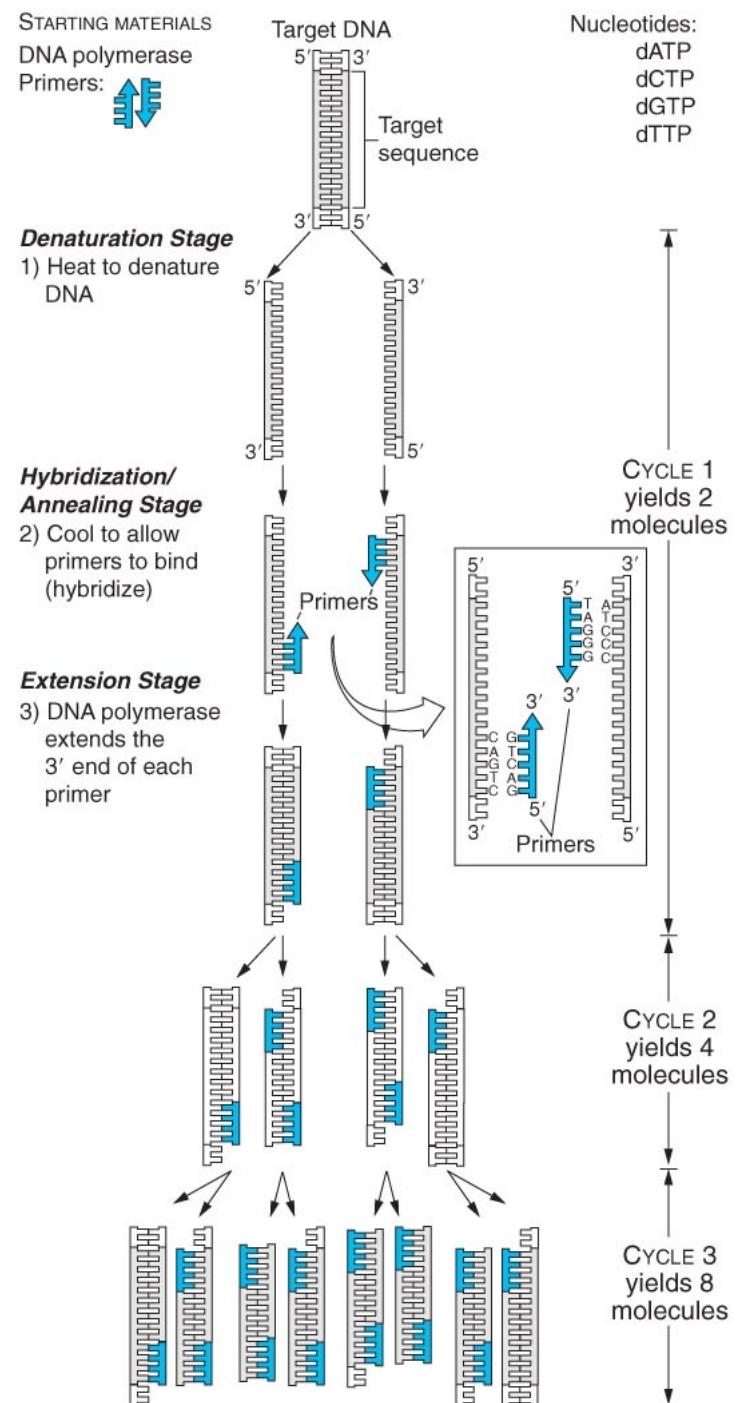
Each cycle consists of three stages

1. **Denaturation** – heat to 94 °C to 96 °C
2. **Annealing** (hybridization) – in which primers H bond with complementary bases at the opposite ends of target sequence at 55 °C to 65 °C
3. **Extension** (elongation) – DNA Pol copies target DNA at 70 to 75 °C

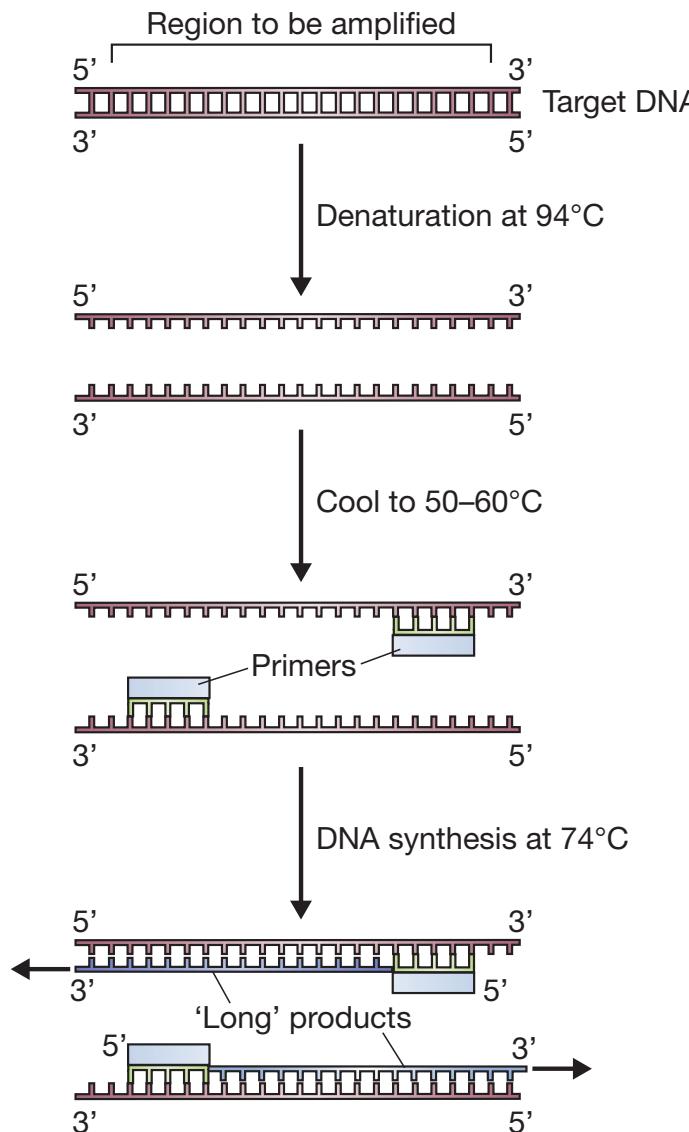
At the end of one cycle, the amount of DNA has doubled

Cycles are repeated 20–35 times

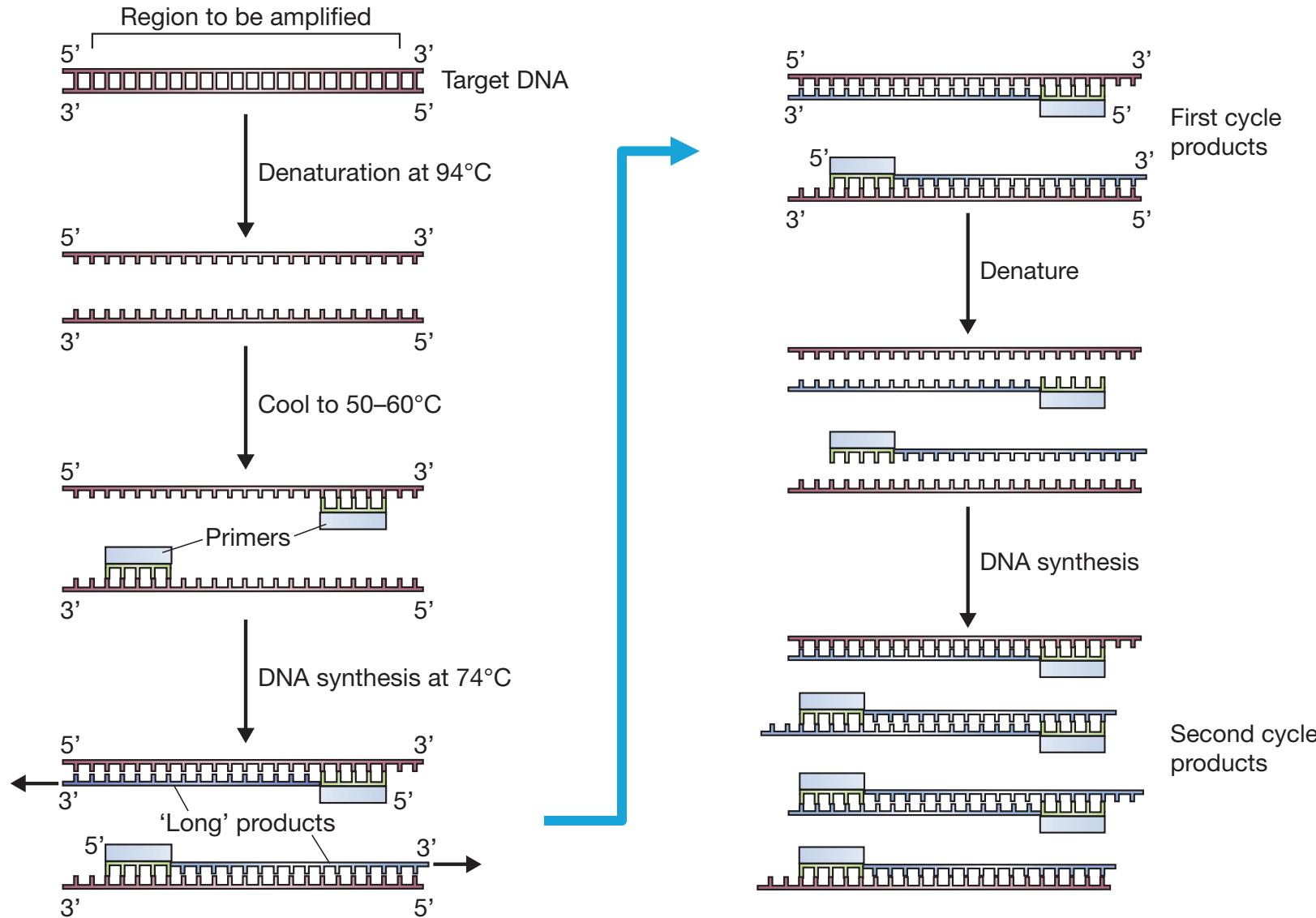
# Polymerase Chain Reaction



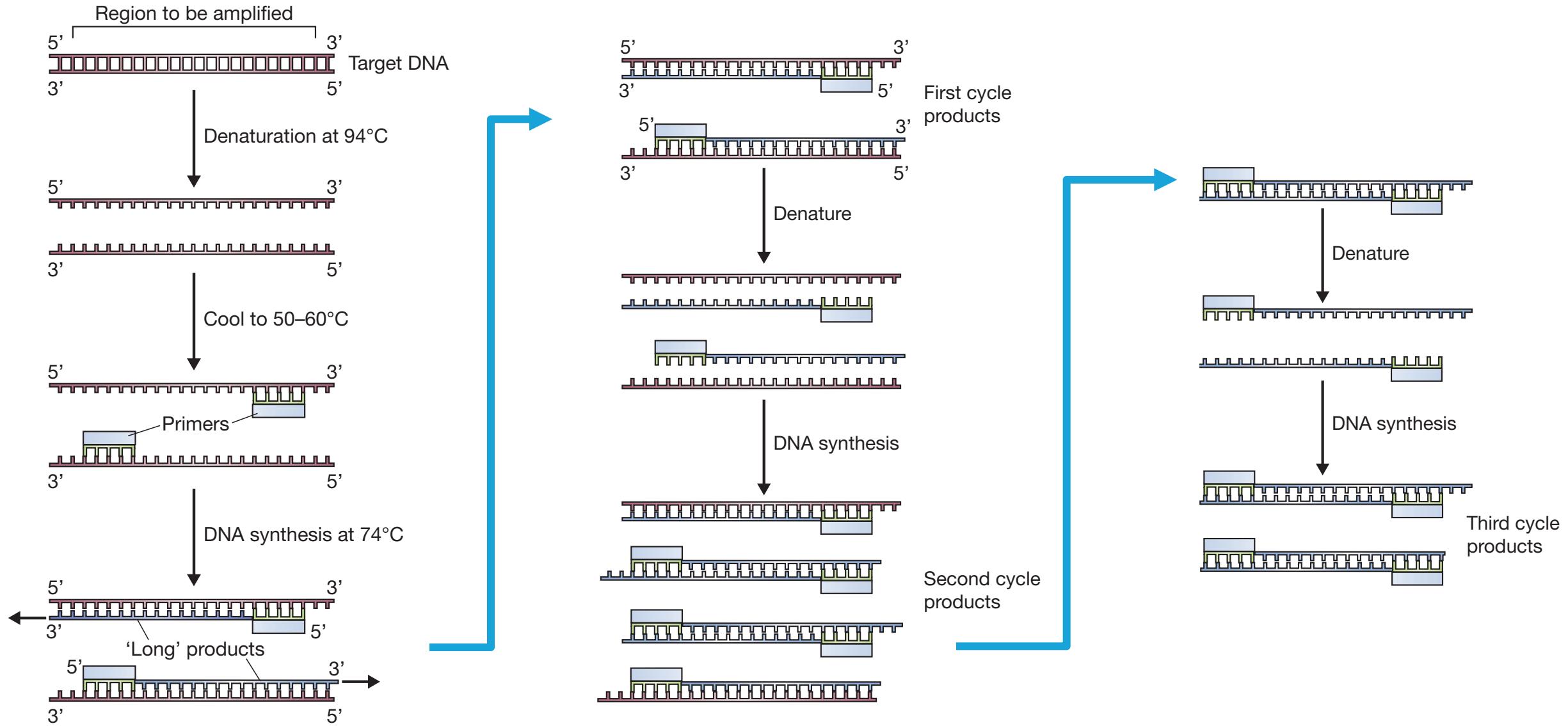
# Polymerase Chain Reaction (First 3 cycles)



# Polymerase Chain Reaction (First 3 cycles)



# Polymerase Chain Reaction (First 3 cycles)



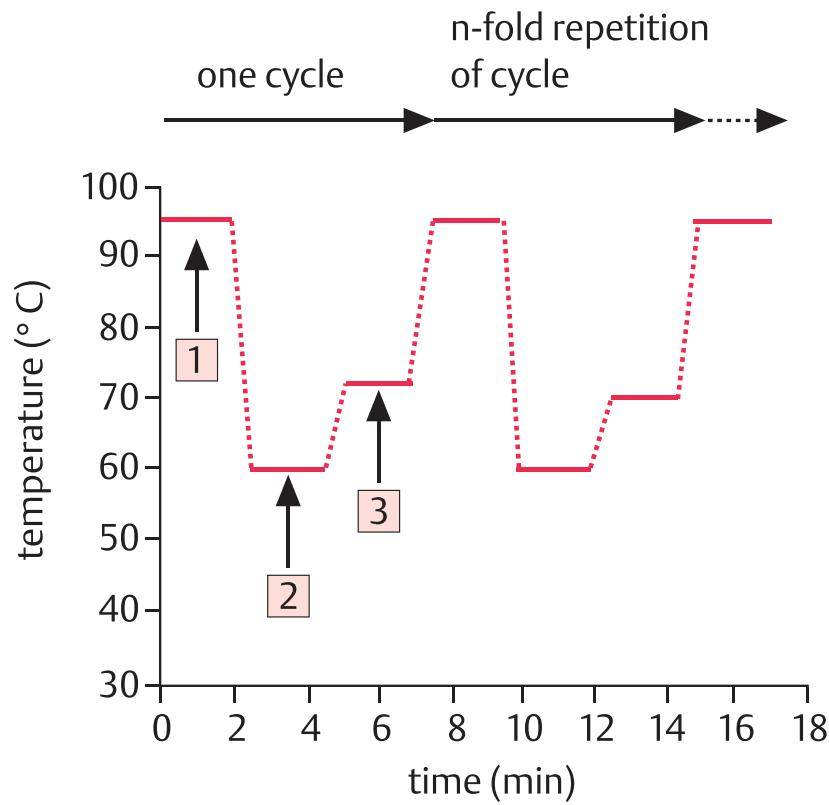
# Polymerase Chain Reaction

Can amplify millions of copies of target DNA from small amount of starting material in short period of time

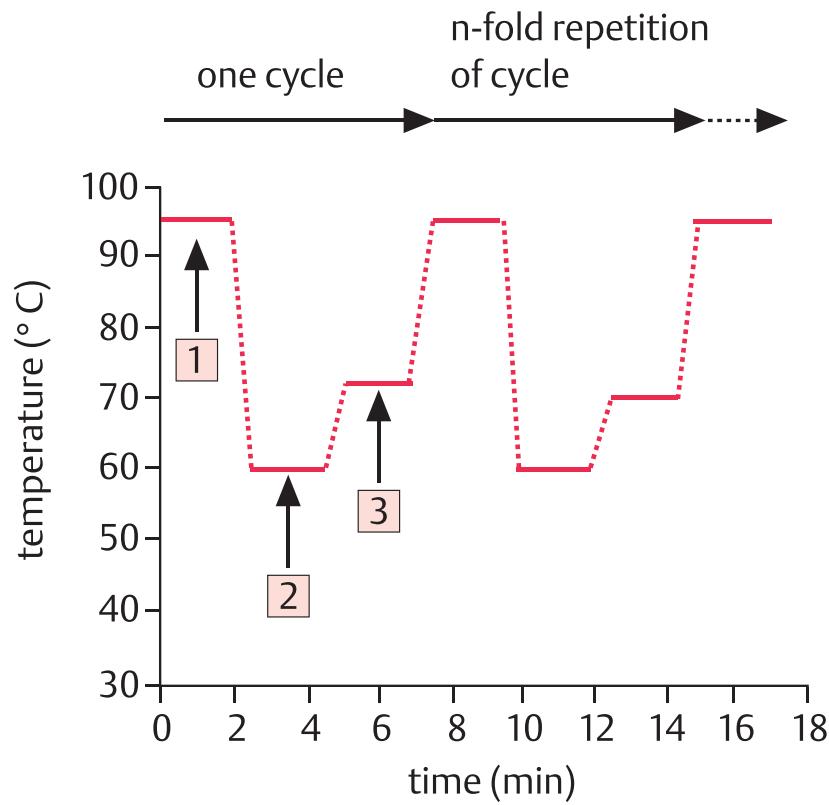
The type of DNA polymerase used is very important

*Taq* DNA polymerase – isolated from a species known as *Thermus aquaticus* that thrives in hot springs

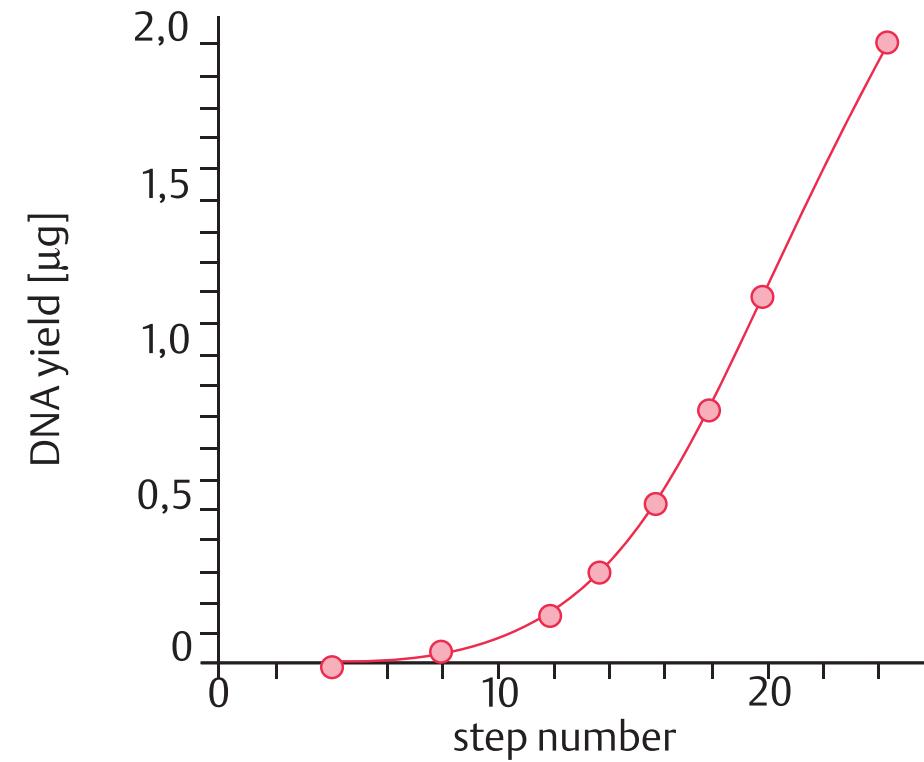
Pfu polymerase from the archaeal hyperthermophile *Pyrococcus furiosus* found in hydrothermal environments



- 1 step 1: denaturation
- 2 step 2: annealing of primers
- 3 step 3: extension of primers



- [1] step 1: denaturation
- [2] step 2: annealing of primers
- [3] step 3: extension of primers



by incorporation of ethidium bromide or SYBR Green  
the PCR yield can be determined in real time (Light Cycler<sup>TM</sup>)

# Primer design

## Base composition

G plus C should be between 40 and 60%

## Length

Between 18 to 30 nucleotides in length

## Internally repeated and self complementary structures

No inverted repeat sequences or self complementary sequences more than three base pairs in length

## Complementarity between members of a primer pair

The 3' end sequence of one primer should not be able to bind to any site on the other timer

## Melting temperature

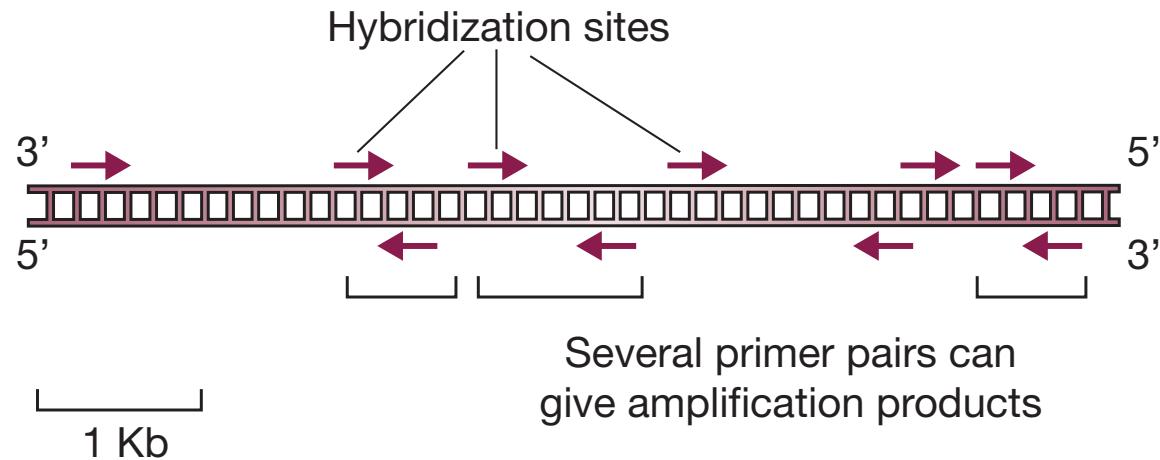
The  $T_m$  can be determined experimentally but is more usually calculated from the simple formula :  $T_m = (4 \times [G+C]) + (2 \times [A+T])^{\circ}\text{C}$   
The melting temperatures of the primers in the PCR should not differ by more than 2 to 3 degrees

## GC clamp

The presence of G and C bases within the last five pieces from the three prime end of the primer will help the tight binding of the primer to the target

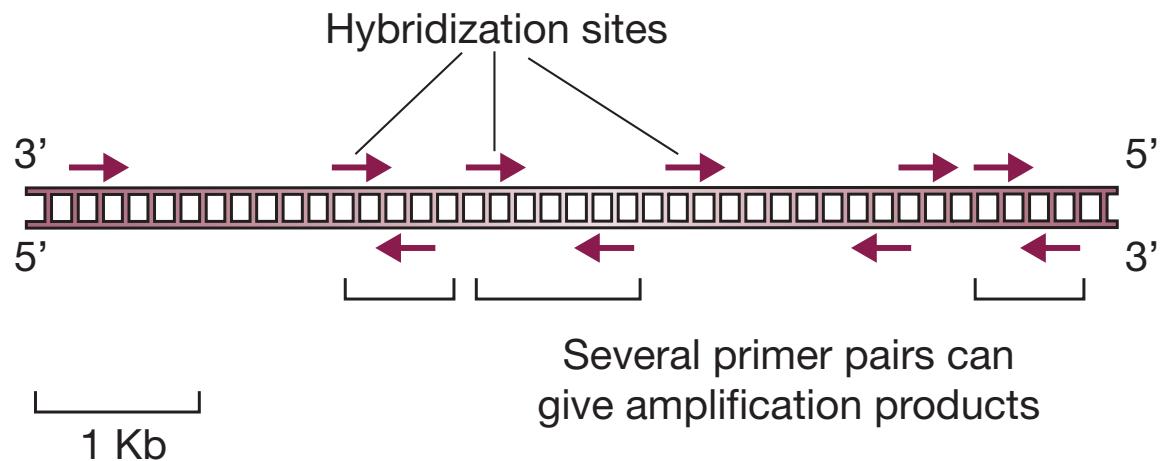
# Primers

(a) PCR of human DNA with 8-mer primers

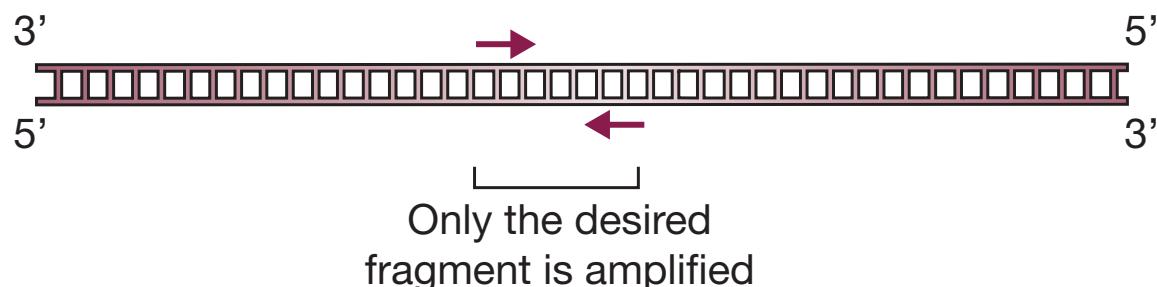


# Primers

(a) PCR of human DNA with 8-mer primers



(b) PCR of human DNA with 17-mer primers



# Primer Dimers

A GCATCA 3'  
T |||||  
C TGTAGCATGCAT 5'

Hairpin

3' GTCGTATA GTGGGTATCCCC 5'  
||||| |||||  
5' CCCCTATGGGGTGATATGCTG 3'

Self-dimer

5' TGTGATGCAGCATCATTACAC 3'  
||||| |||||  
3' CTACGACACCTCAGAGTAGTACG 5'

Cross-dimer

# Primer annealing temperature

The primer annealing temperature ( $T_a$ ) is the temperature needed for the annealing step of the PCR reaction to allow the primers to bind to the template DNA.

The theoretical annealing temperature can be calculated as follows:

$$T_a = 0.3 \times T_{m(\text{primer})} + 0.7 \times T_{m(\text{product})} - 14.9$$

$T_a$ : primer annealing temperature

$T_{m(\text{primer})}$ : lower melting temperature of the primer pair

$T_{m(\text{product})}$ : melting temperature of the PCR product

# DNA polymerases

- Thermal stability** DNA polymerase must be robust enough to tolerate high-temperature cycles without compromising activity, a factor dependent on buffer composition and pH
- Extension rate** This refers to the speed at which nucleotides are added, per second, per molecule of DNA polymerase, a factor determined by extension temperature, DNA template sequence and buffer composition.
- Fidelity** Fidelity is an inherent DNA polymerase property defining the frequency of insertion of an incorrect nucleotide per kb of DNA. For standard polymerases, fidelity refers to the ability to discriminate correct vs. incorrect nucleotide incorporation and can be influenced by the buffer composition. High-fidelity polymerases are more accurate because of the ability to "proofread" and excise incorrectly incorporated mononucleotides, replacing them with the correct base.
- Processivity** The probability that a polymerase will detach from DNA during extension, indicating the average number of nucleotides the enzyme adds in a single binding event, is known as its processivity. Like extension rate, processivity depends on buffer composition (salt concentrations) and the sequence of a DNA template. High processivity is important when amplifying long amplicons.

# DNA polymerases

## Standard thermostable DNA polymerases

These polymerases are suitable for routine PCR. Standard Taq produces fragments with a single-based 'A' overhang at the 3'-end, enabling direct insertion into T/A cloning vectors. Newer Taq DNA polymerases exhibit good processivity and fast extension rates but lack proofreading capabilities and so cannot be used for amplifying fragments for cloning and expression, or for mutagenesis studies.

## Hot-start (HS) polymerases

Hot-start DNA polymerases are used to suppress nonspecific product amplification during setup to increase yield of the desired product.

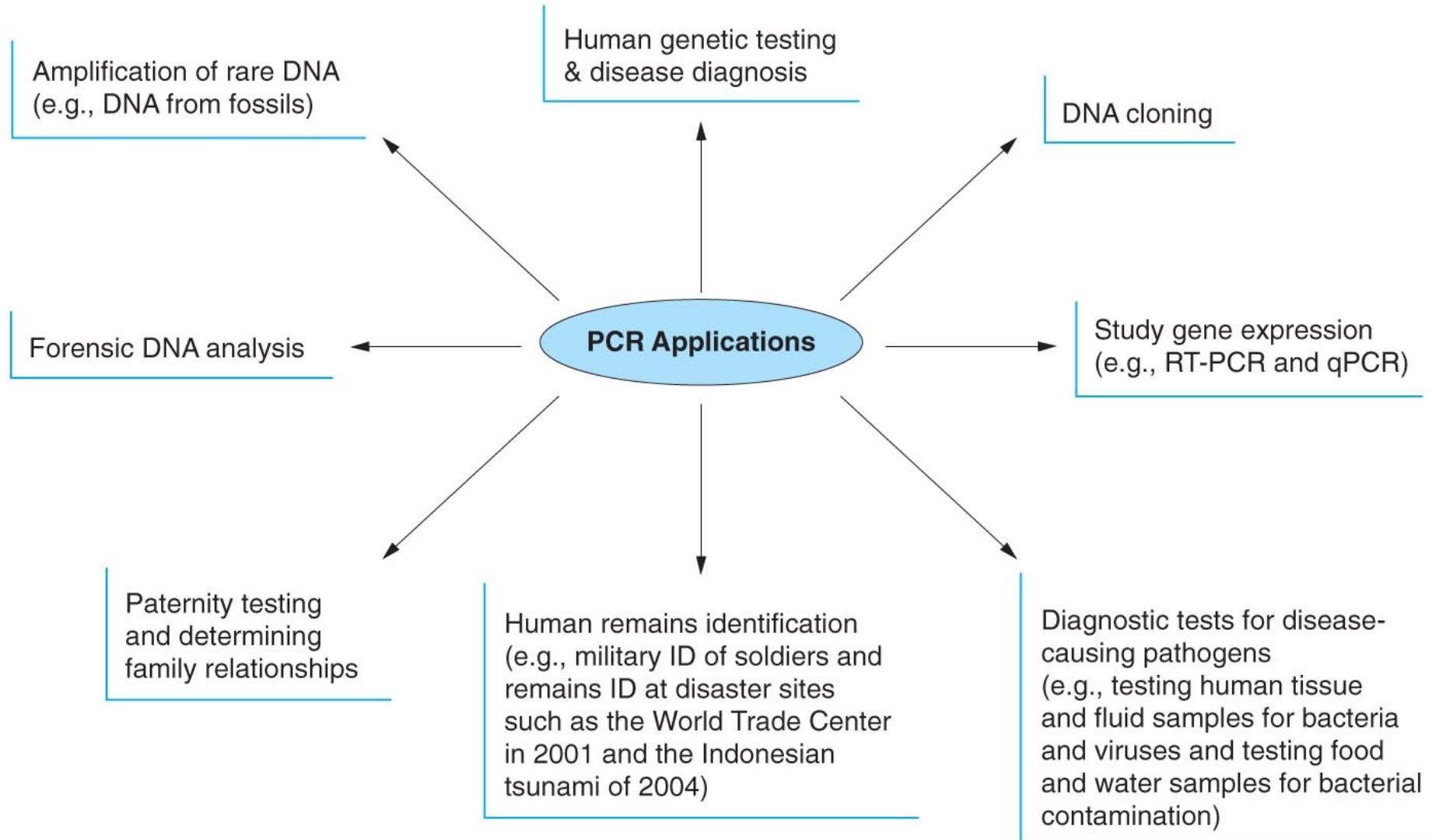
## High-fidelity polymerases (Hi-Fi)

Proofreading DNA polymerases possess a 3'-to-5' exonuclease activity and remove erroneously attached bases when incorporated in the growing DNA strand. This increases the accuracy of DNA synthesis from template DNA, resulting in a better DNA copy. For cloning and expression of amplified product, mutagenesis studies and related applications, proofreading enzymes should be used.

## Polymerases for amplification of long amplicons

Amplification of long amplicons requires combining the processivity of standard DNA polymerases with the accuracy of a proofreading polymerase. This is achieved by blending the thermostable polymerase with a proofreading enzyme

# PCR applications



## Cloning PCR Products (TA cloning)

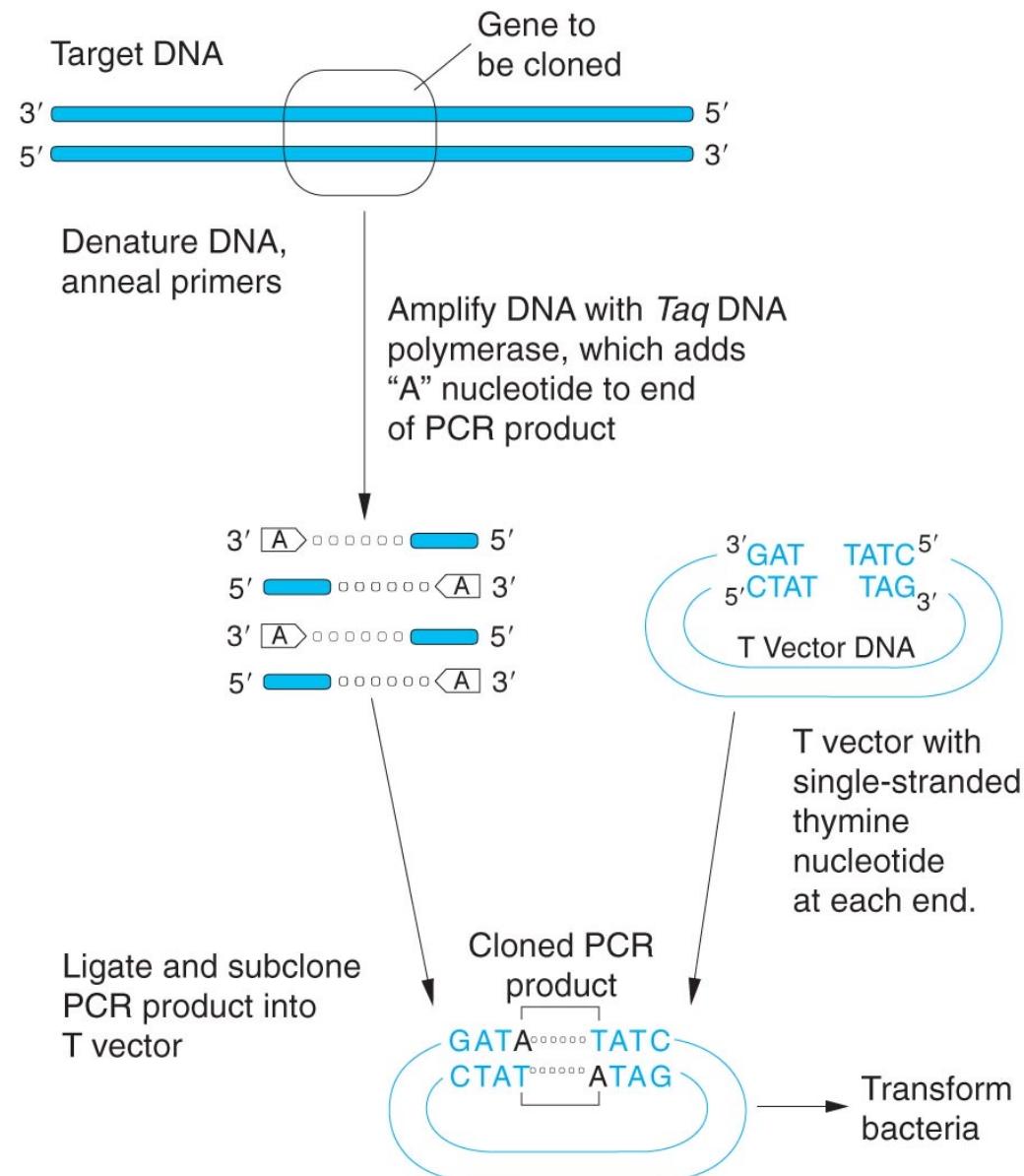
*Taq* polymerase puts a single adenine nucleotide on the 3' end of all PCR products

T vector that has single stranded thymine on each end so can complementary base pair with the adenine in the PCR products

### **Disadvantage**

Need to know something about the DNA sequence that flanks the gene of interest to design primers

# Cloning PCR Products (TA cloning)



# Cloning PCR Products (TOPO cloning)

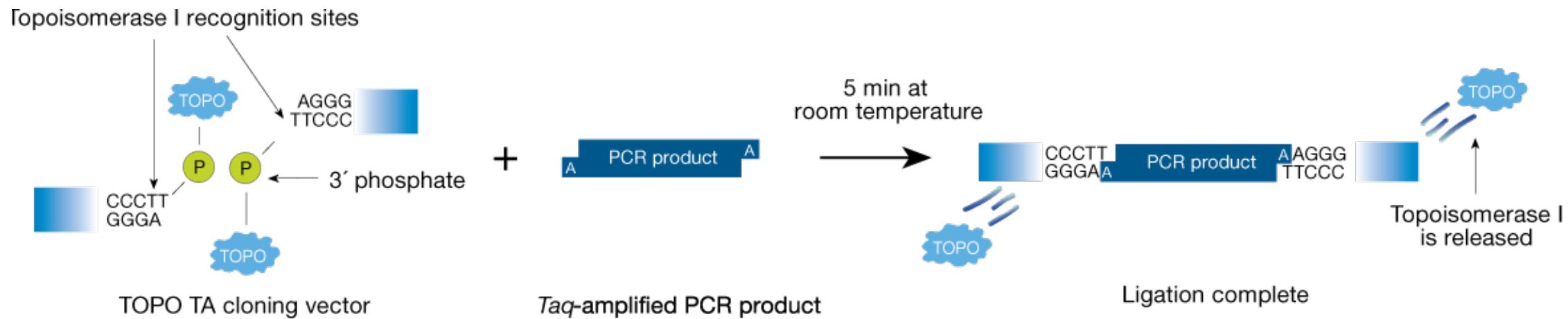
The enzyme DNA topoisomerase I can function both as a restriction enzyme and as a ligase.

Vaccinia virus topoisomerase I specifically recognizes the pentameric sequence 5'-(C/T)CCTT-3' and forms a covalent bond with the phosphate group attached to the 3' thymidine.

It cleaves one DNA strand, enabling the DNA to unwind. The enzyme then religates the ends of the cleaved strand and releases itself from the DNA.

To harness the religating activity of topoisomerase, TOPO vectors are provided linearized with topoisomerase I covalently bound to each 3' phosphate

# Cloning PCR Products (TOPO cloning)



# Cloning PCR Products (Ligation Independent Cloning)

Relies on the 3'-5' exonuclease activity of T4 DNA polymerase, which removes nucleotides from the end of a DNA strand, creating "chewed-back" overhangs of 10-12 base pairs on the 5' end of both the vector and the DNA insert.

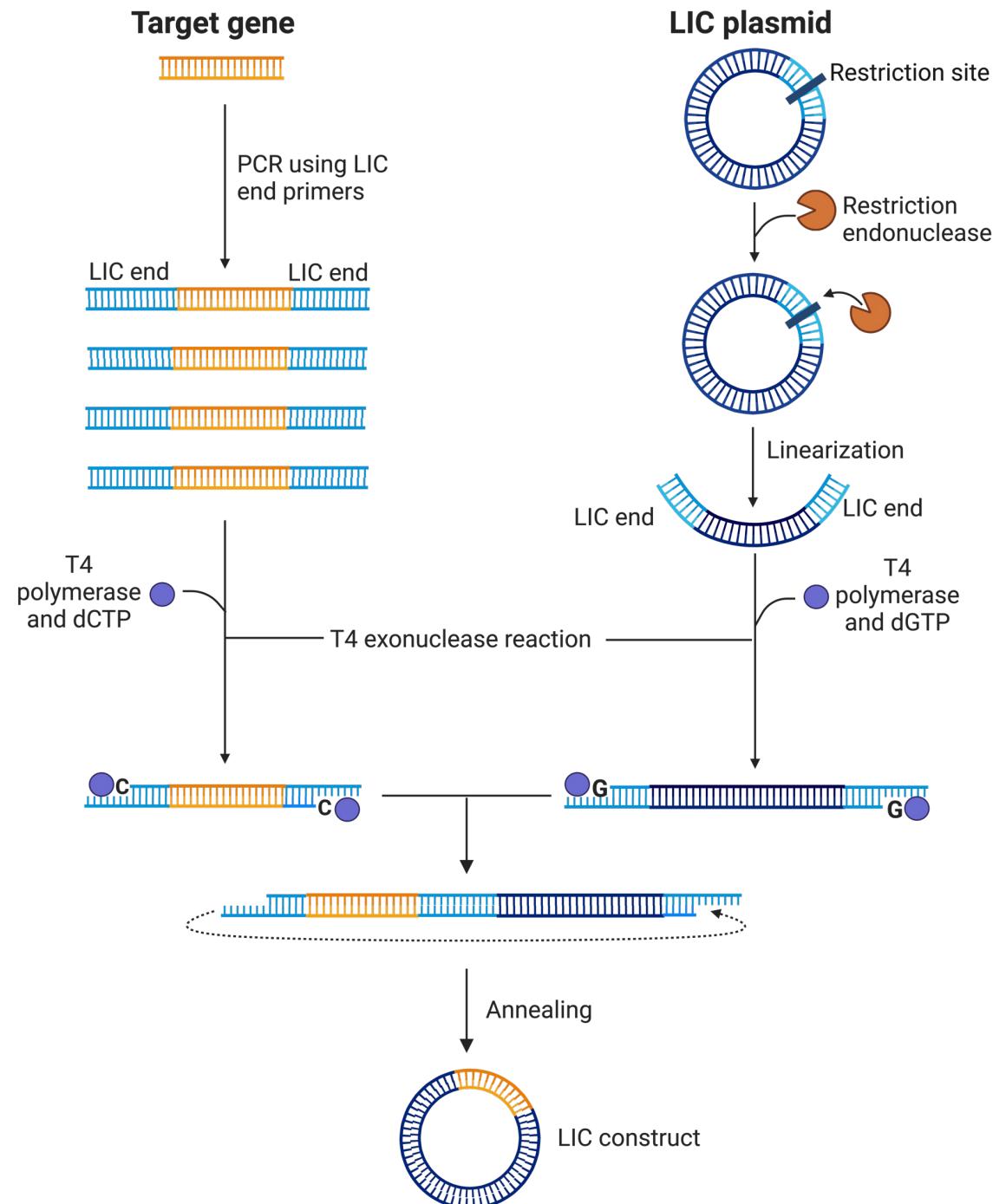
Overhangs are designed to be complementary in the PCR primers for the insert, based on the vector sequence and chosen restriction site.

In the presence of a single free dNTP, T4 polymerase continues as an exonuclease until a base complementary to the free nucleotide is exposed on the single-strand overhang.

T4 polymerase then resumes polymerase activity, adding the free base and becoming stuck at this point due to the absence of other free bases.

The resulting product, with annealed overhangs, can be directly transformed into *E. coli* cells. Nicks in the circular DNA will be repaired during the normal bacterial replication process.

# Cloning PCR Products (Ligation Independent Cloning)



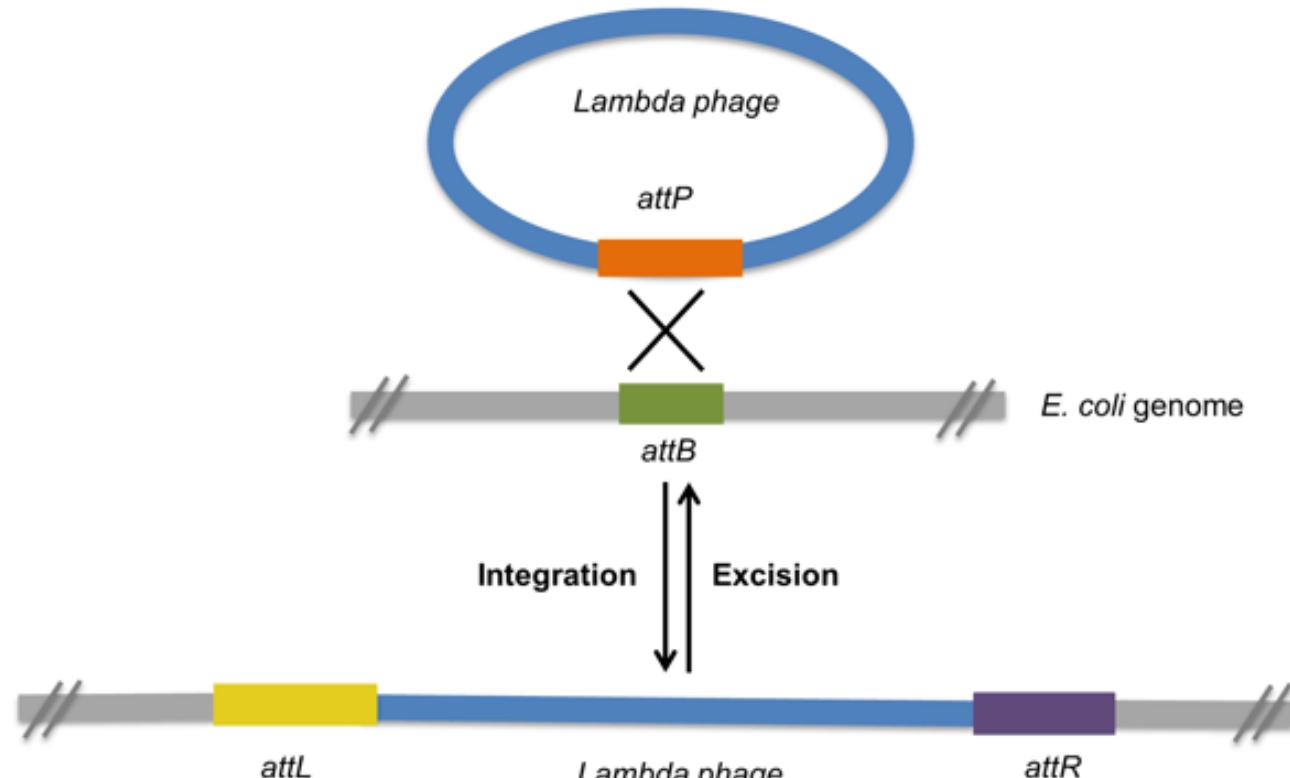
# Cloning PCR Products (Gateway Cloning)

In vitro version of integration and excision recombination reactions in lambda phage-bacteria interaction.

Recombination facilitated by *attP* (phage) and *attB* (bacteria) attachment sites.

Recombination results in integration of phage into bacterial genome flanked by *attL* and *attR* sites.

Gateway vectors have modified *att* sites for easy cloning of desired DNA sequences.



# Cloning PCR Products (Gateway Cloning)

## BP Reaction

Occurs between *attB* sites (insert) and *attP* sites (donor vector) and catalyzed by BP Clonase enzyme mix.

Generates entry clone with DNA of interest flanked by *attL* sites.

Excises *ccdB* gene from donor vector.

## LR Reaction

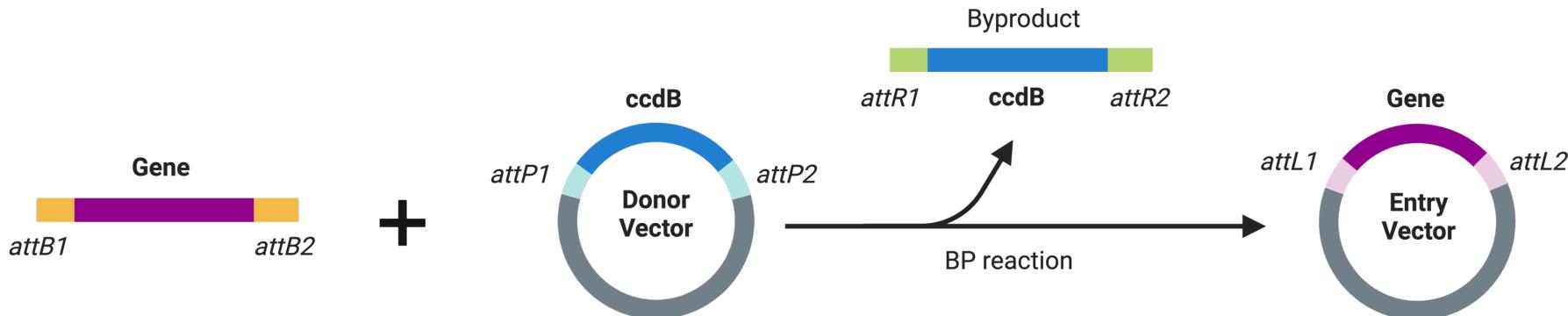
Occurs between *attL* sites (entry clone) and *attR* sites (destination vector) and catalyzed by LR Clonase enzyme mix.

Generates expression clone with DNA of interest flanked by *attB* sites.

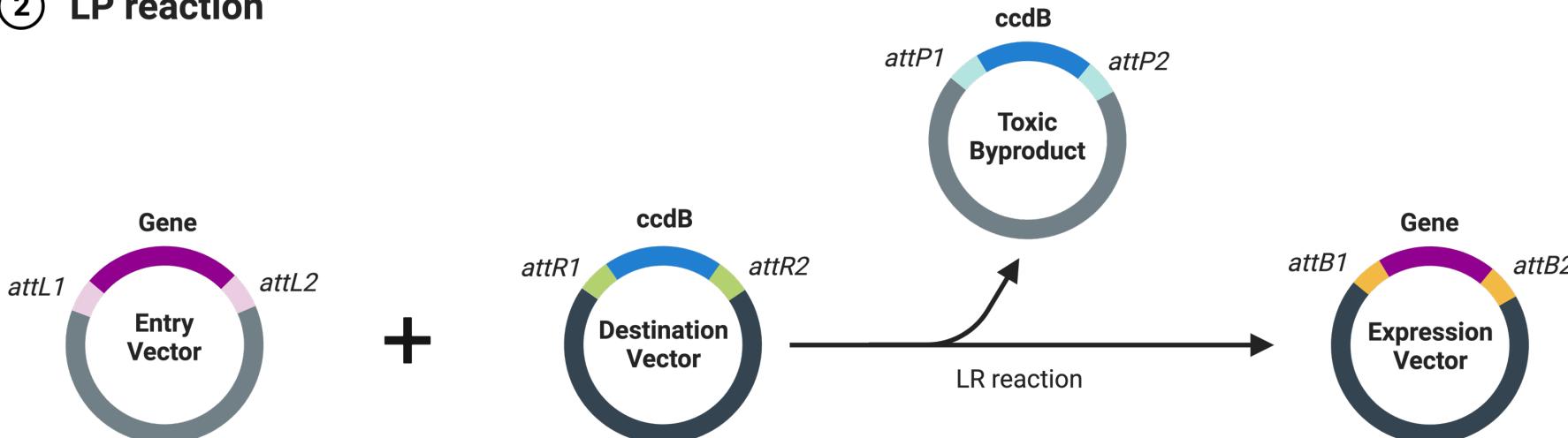
Excises DNA fragment containing *ccdB* gene from destination vector.

# Cloning PCR Products (Gateway Cloning)

## ① BP reaction



## ② LP reaction



# Cloning PCR Products (Golden Gate Cloning)

Relies on Type IIS restriction enzymes that can cleave outside their recognition sequence, creating four base flanking overhangs, which can be customized for directing DNA fragment assembly.

Recognition sites do not appear in the final construct, enabling scarless cloning.

## Cloning Scheme:

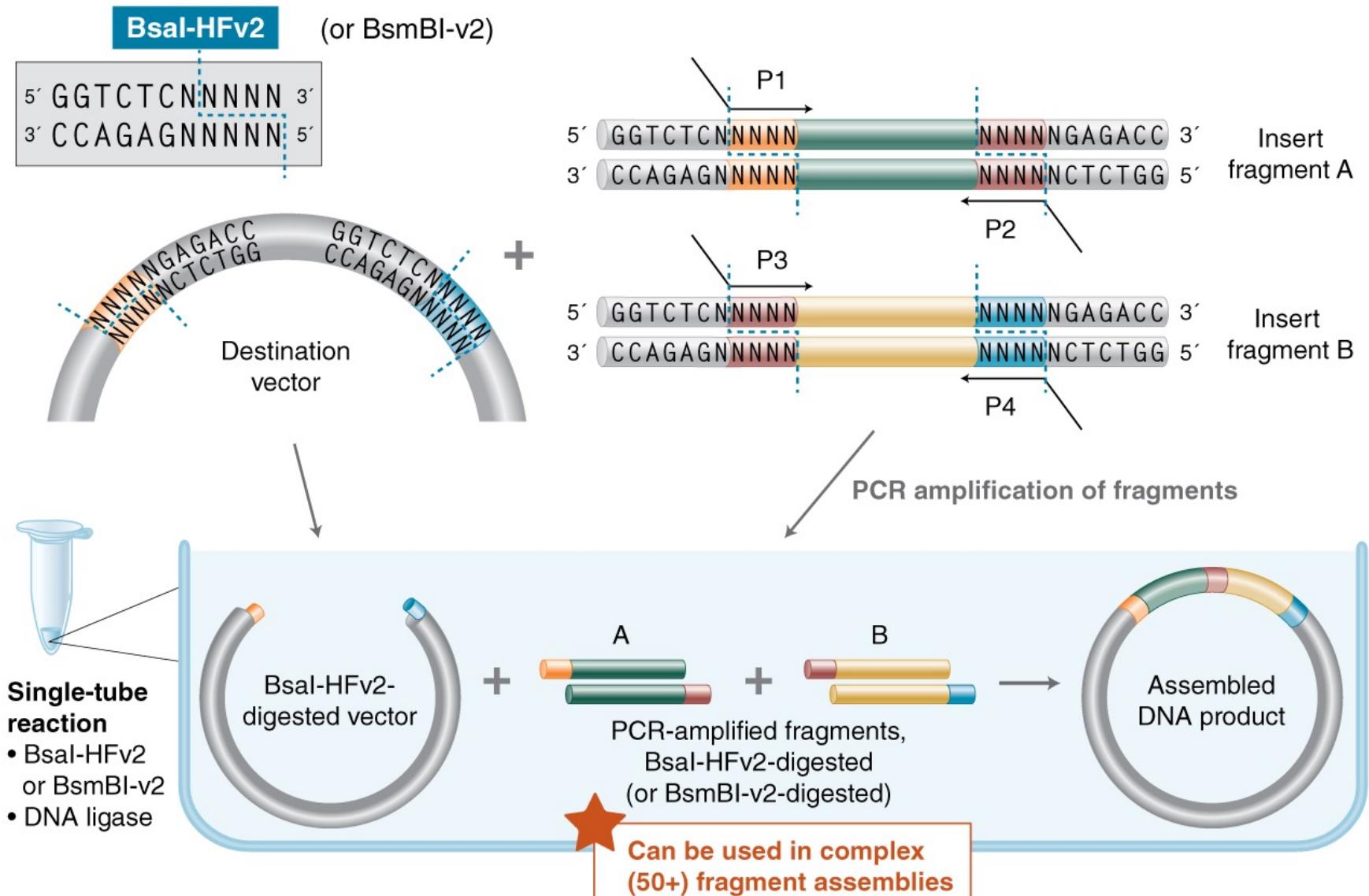
Gene of interest designed with Type IIS sites (e.g., Bsal or BbsI) located outside cleavage site.

These sites are eliminated during digestion/ligation, not present in final construct.

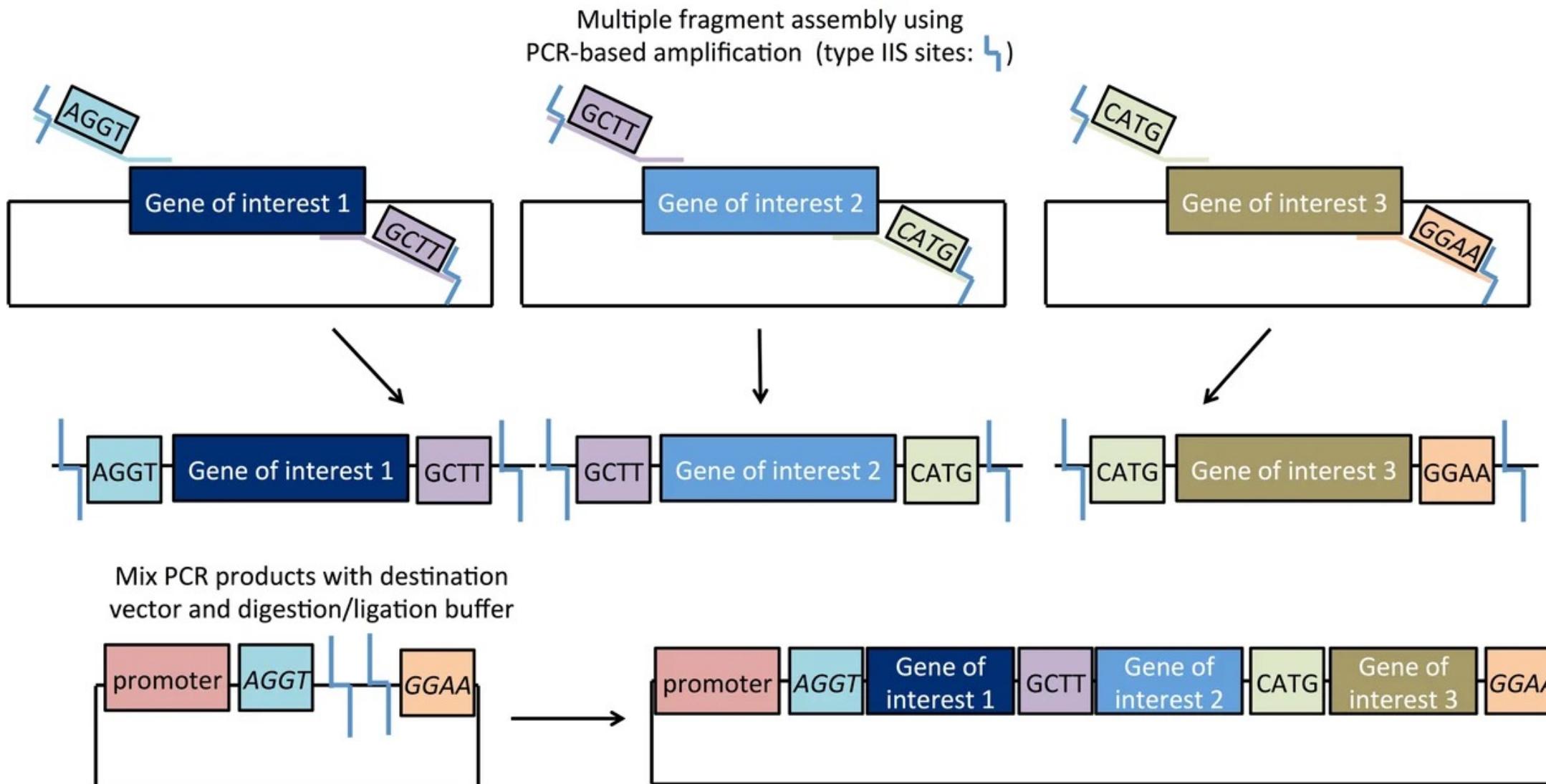
Destination vector has complementary overhangs guiding final ligation product assembly.

Fragment with 5' overhang TGGA and 3' overhang TCCG ligated into vector with matching overhangs.

# Cloning PCR Products (Golden Gate Cloning)



# Cloning PCR Products (Golden Gate Cloning)



# The Gibson Assembly

The Gibson Assembly method is a cloning procedure that allows the cloning of two or more fragments without the need for restriction enzyme digestion or compatible restriction sites

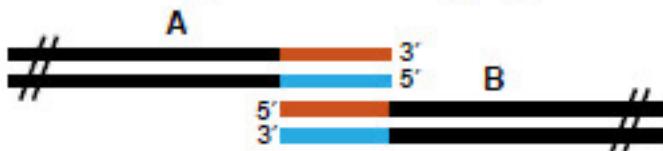
Gibson Assembly efficiently joins multiple overlapping DNA fragments in a single-tube isothermal reaction

The Gibson Assembly Mix includes three different enzymatic activities that perform in a single buffer:

1. The exonuclease creates single-stranded 3' overhangs that facilitate the annealing of fragments that share complementarity at one end (overlap region).
2. The DNA polymerase fills in gaps within each annealed fragment.
3. The DNA ligase seals nicks in the assembled DNA.

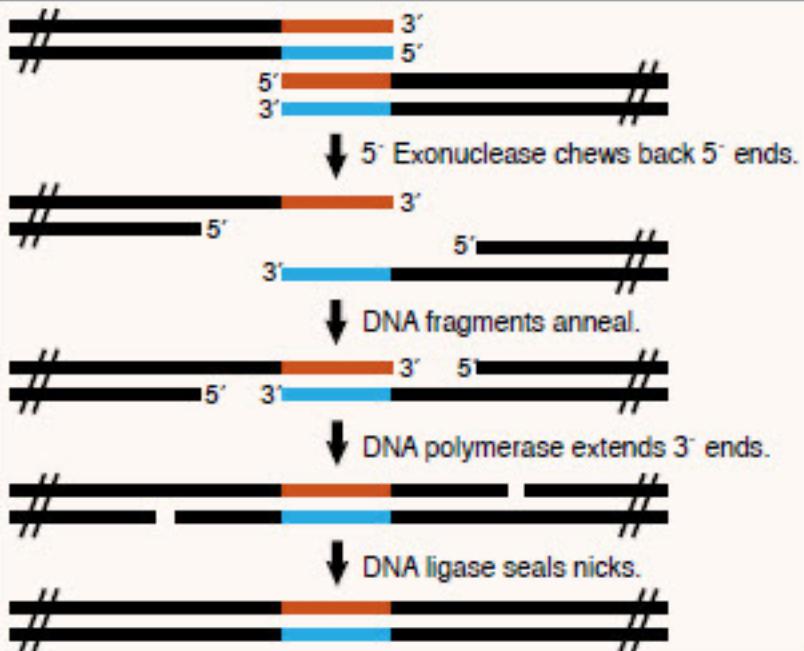
# The Gibson Assembly

dsDNA fragments with overlapping ends.



Add fragments to  
Gibson Assembly  
Master Mix.

## Gibson Assembly

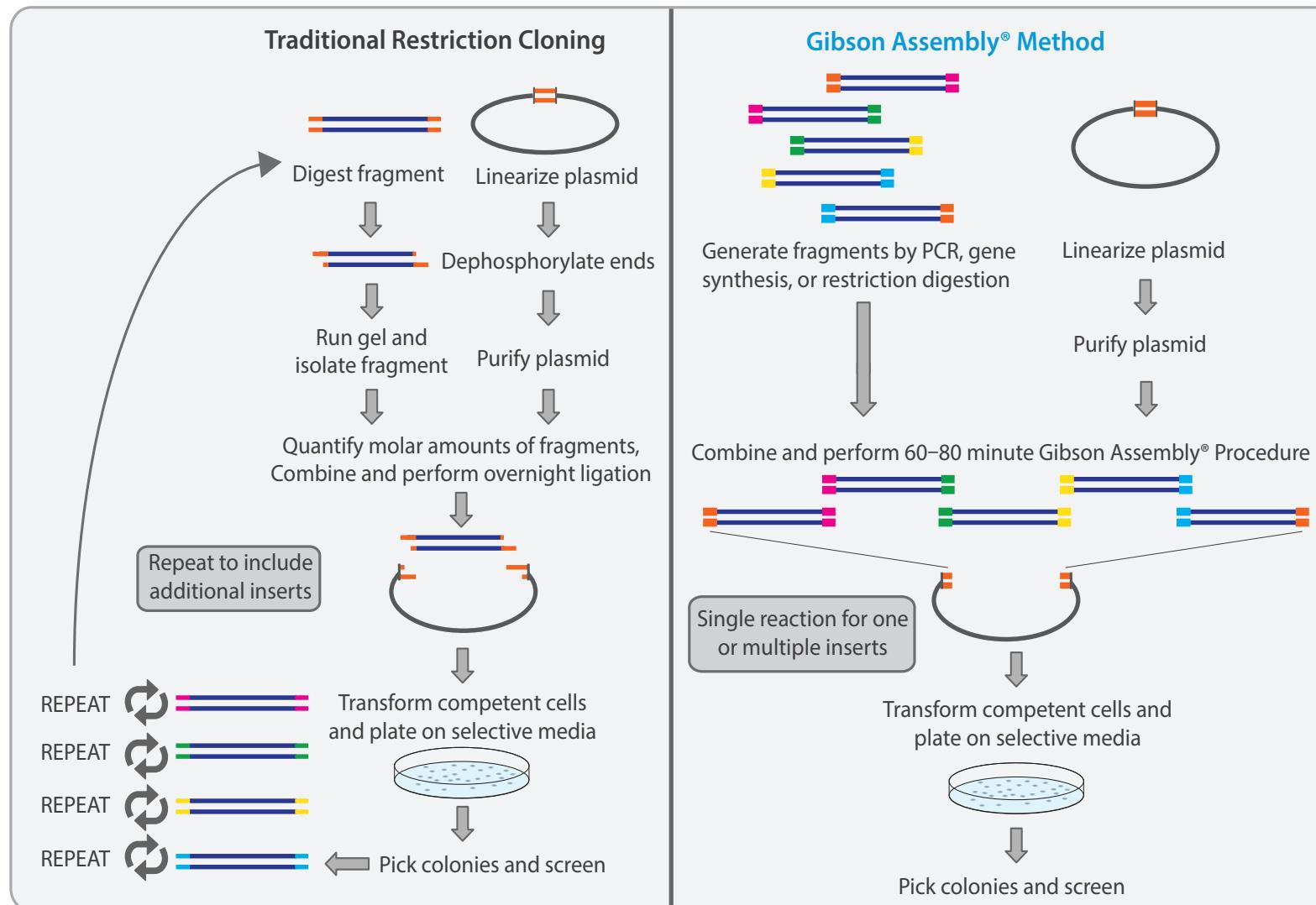


Incubate at 50°C  
for 15–60 minutes.

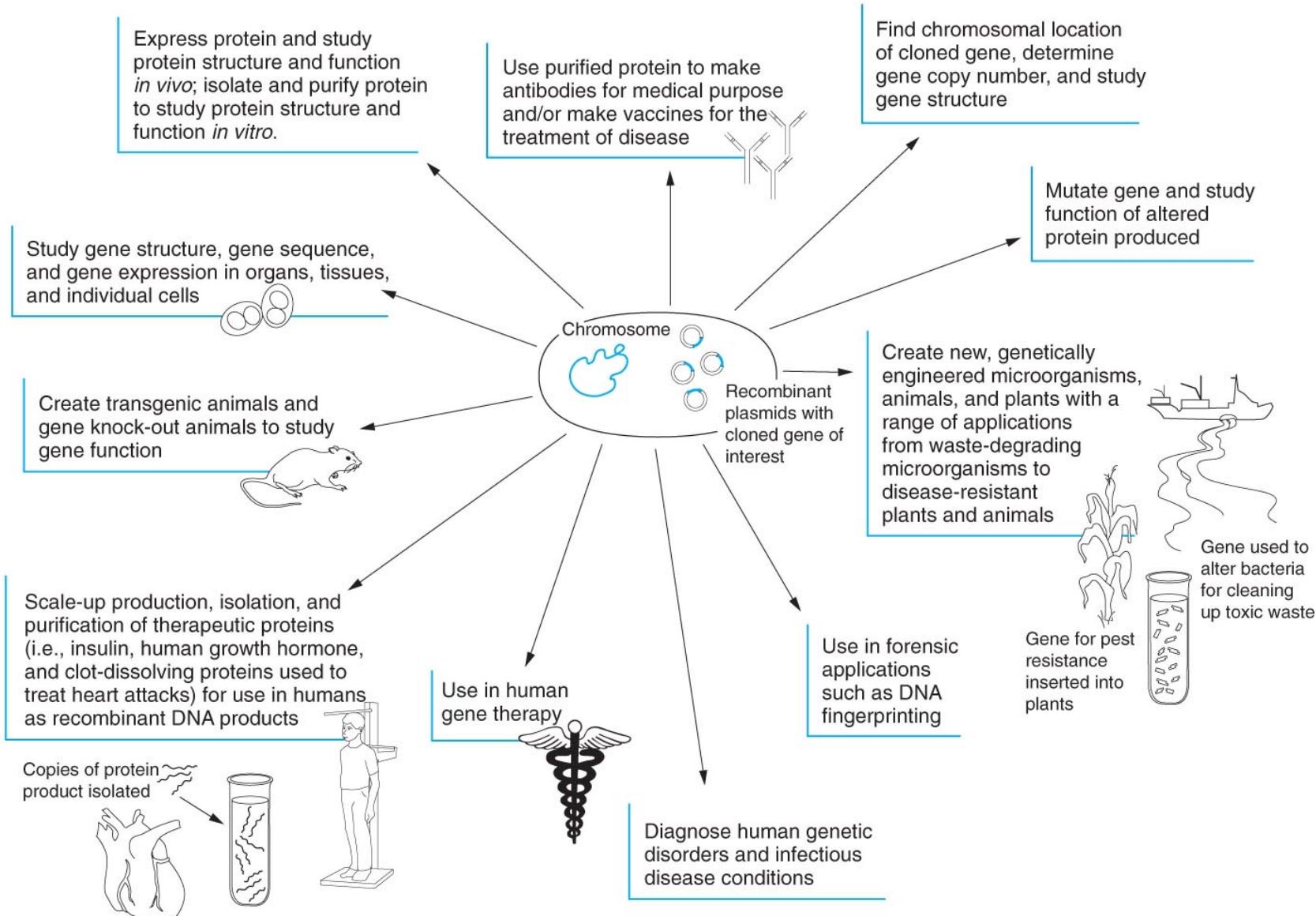


Fully Assembled DNA

# The Gibson Assembly



# Applications of Recombinant DNA Technology



## 3.4 Laboratory Techniques

### *Agarose Gel Electrophoresis*

Separate and visualize DNA fragments based on size

Agarose is isolated from seaweed and when melted in a buffer solution and poured into a horizontal tray and as it cools it will form a semisolid gel containing small pores through which DNA will travel

The percentage of agarose used to make the gel determines the ability of the gel to separate DNA fragments of different sizes

High % gel (2%) allows to resolve smaller size fragments

Low % (0.5%) resolves larger size fragments

## Laboratory Techniques

### *Agarose Gel Electrophoresis*

To run a gel, it is submerged in a buffer solution that conducts electricity

DNA is loaded into small depressions called wells at the top of the gel

Electric current is applied through electrodes at opposite ends of the gel

DNA migrates according to its charge and size

## Laboratory Techniques

### *Agarose Gel Electrophoresis*

Rate of migration through the gel depends on the size of the DNA because the sugar phosphate backbone makes it always negatively charged

DNA migrates toward positive pole and is repelled by negative pole

Migration distance is inversely proportional to size of DNA fragment

Large fragments migrate slowly; smaller fragments migrate faster

## Laboratory Techniques

### *Agarose Gel Electrophoresis*

Tracking dye is added to the samples to monitor DNA migration during electrophoresis

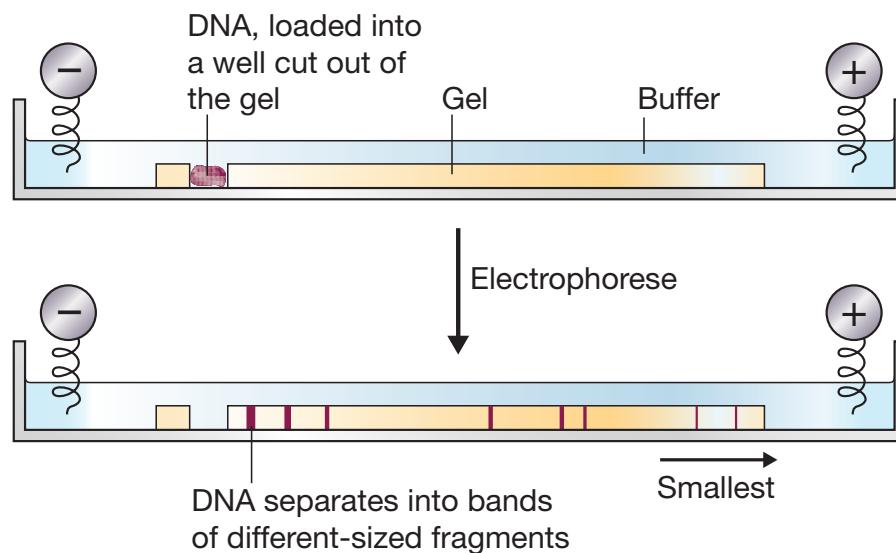
DNA can be visualized after electrophoresis by the addition of DNA staining dyes

**Ethidium bromide:** intercalate between DNA base pairs and it fluoresces under ultraviolet light

Then a picture can be taken to document the gel results

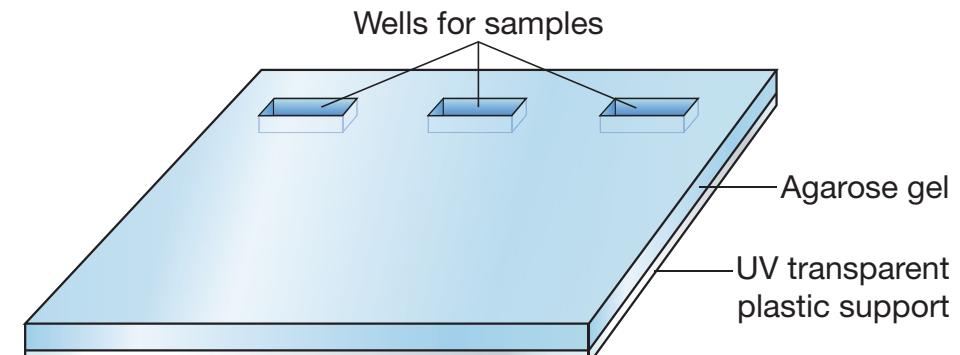
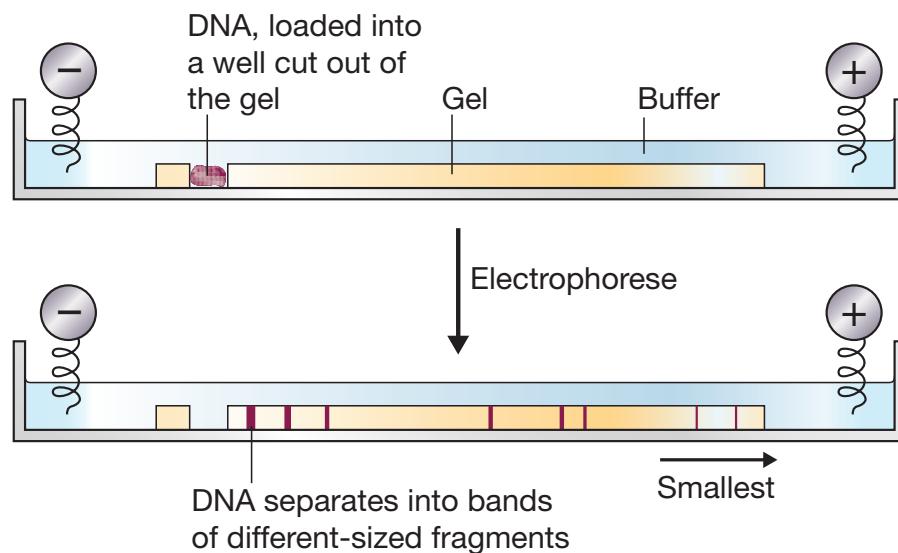
# Laboratory Techniques

## Agarose Gel Electrophoresis



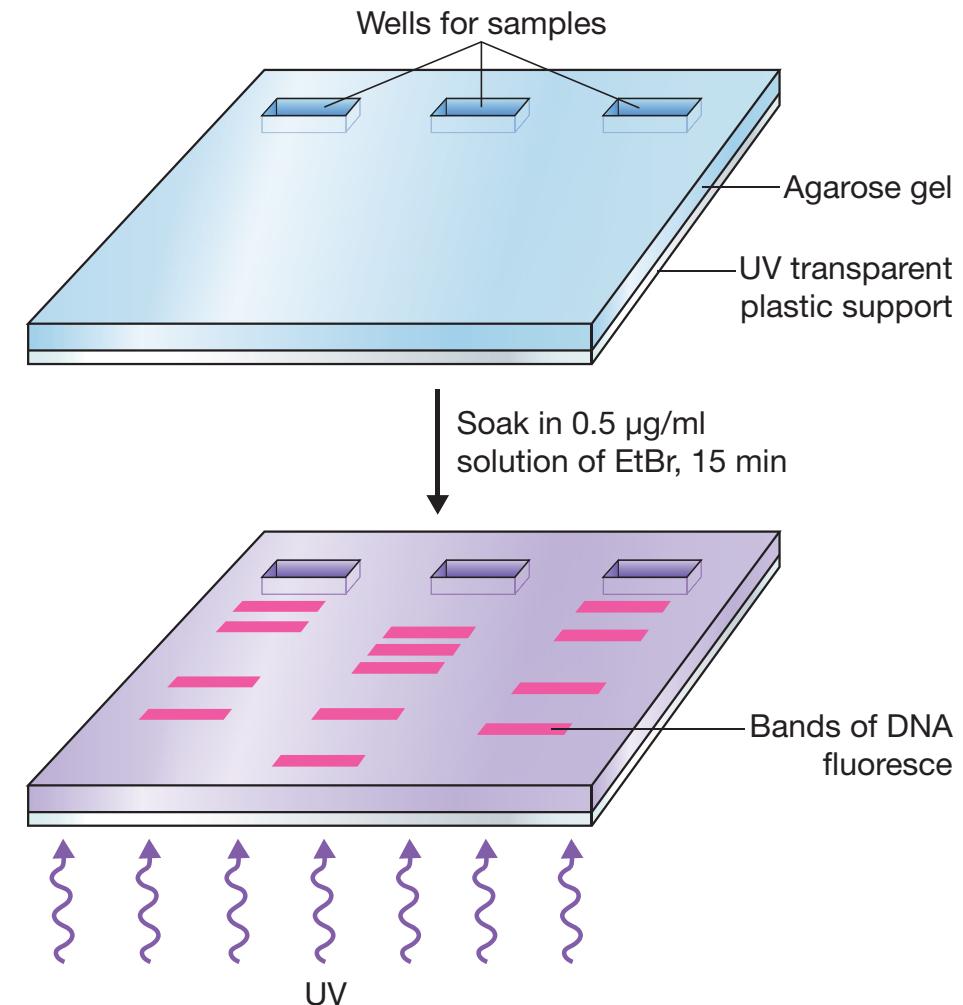
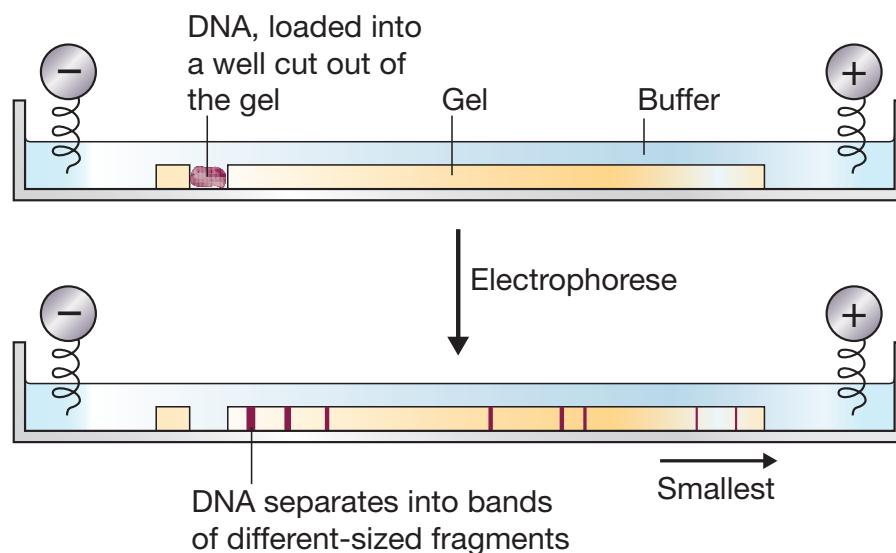
# Laboratory Techniques

## Agarose Gel Electrophoresis



# Laboratory Techniques

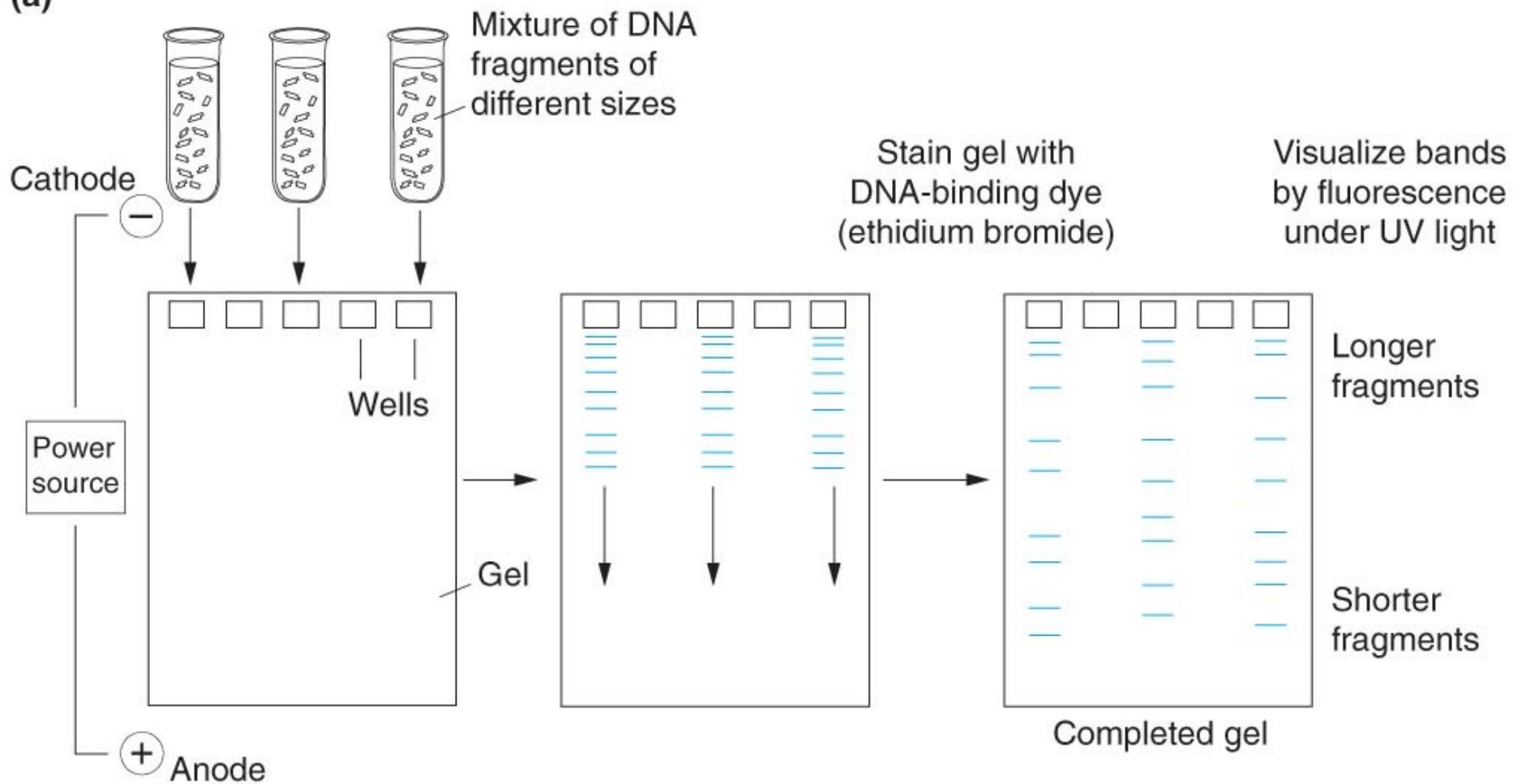
## Agarose Gel Electrophoresis



# Laboratory Techniques

## Agarose Gel Electrophoresis

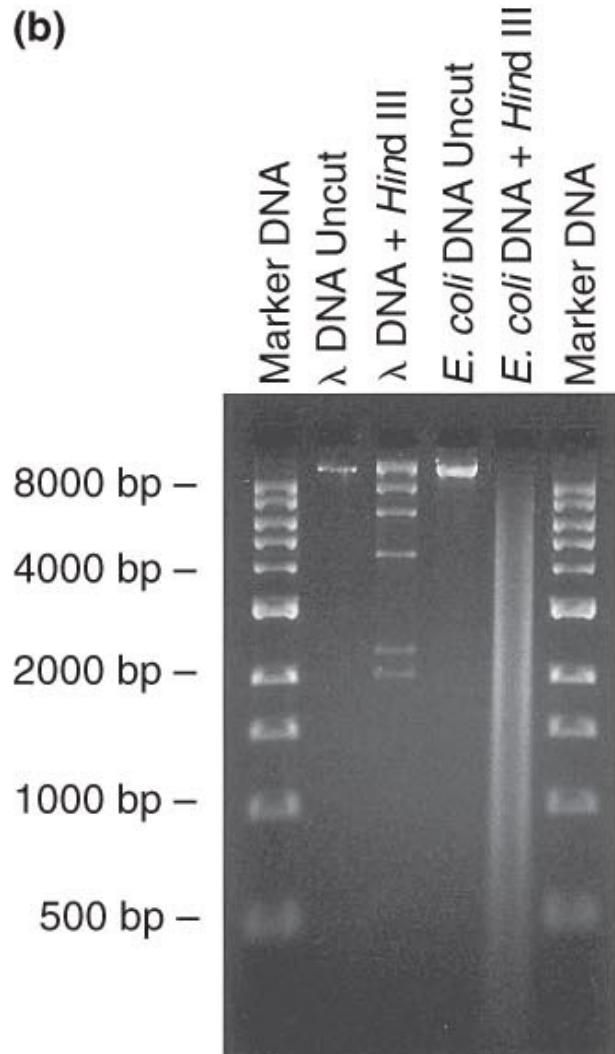
(a)



# Laboratory Techniques

## Agarose Gel Electrophoresis

(b)



# Laboratory Techniques

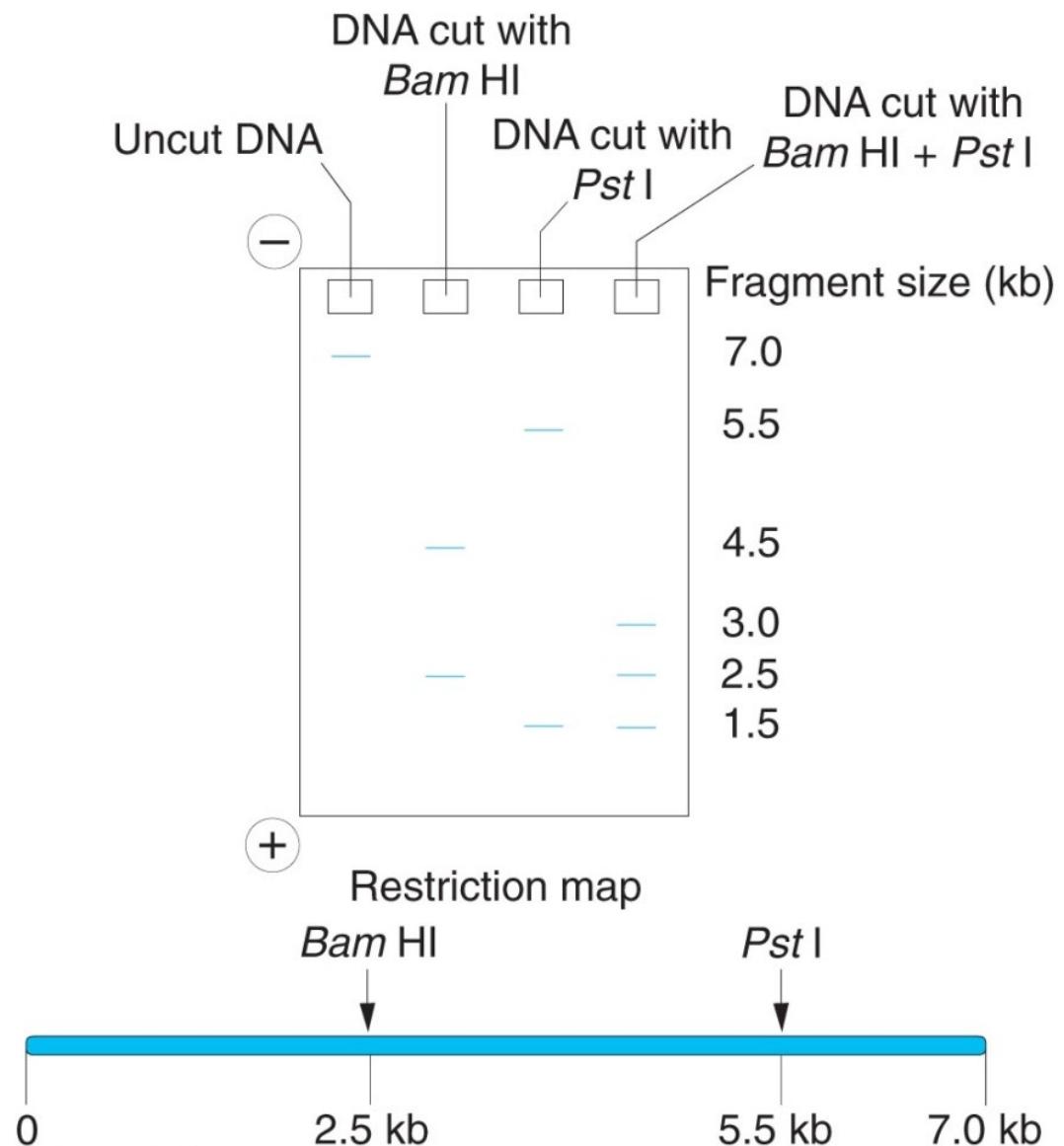
## *Restriction mapping*

Cut cloned gene with restriction enzymes to pinpoint location of the cutting sites

Knowing restriction map is useful for making clones of small pieces of the DNA which is called **subcloning**

These small pieces of DNA can then be sequenced

# Restriction mapping



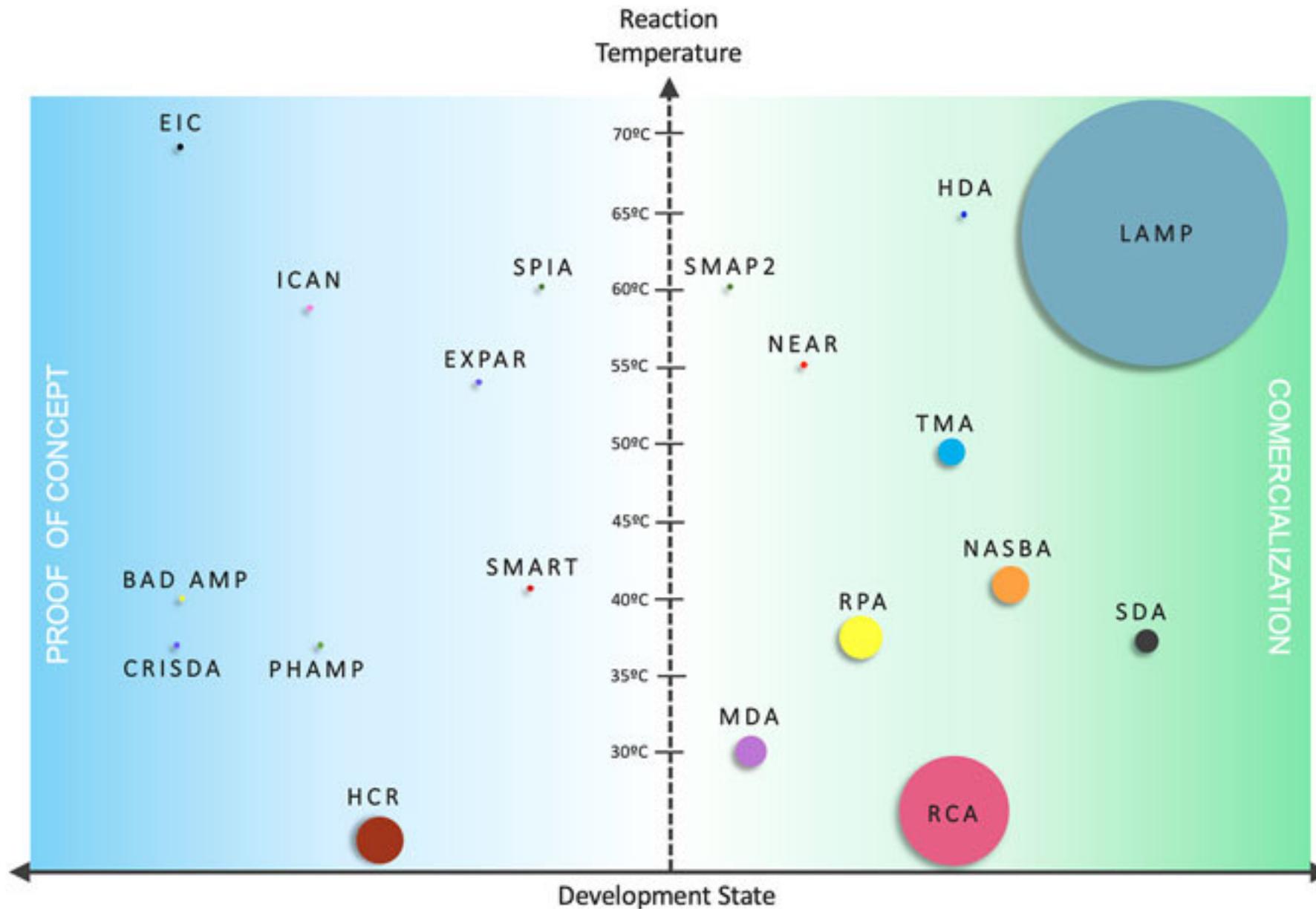
## Isothermal Amplification of Nucleic acids

- Amplification of DNA at constant temperature (25–65°C)
- Cheaper, faster, more sensitive, more resistant to inhibitors and doesn't require sophisticated and expensive equipment for detection of amplification
- Employ unique DNA polymerases for separating duplex DNA (high strand displacement activity), such as Klenow exo-, *Bsu* large fragment, and phi29 for moderate temperature reactions (25–40°C) and the large fragment of *Bst* DNA polymerase for higher temperature (50–65°C) reactions

# Isothermal Amplification of Nucleic acids

- Mainly being used for rapid diagnosis of infectious diseases
- Limitations may occur if precise target quantifications are requested or if amplification of several genes in a single tube
- Examples:
  1. Nucleic acid sequence-based amplification (NASBA)
  2. Strand displacement amplification (SDA)
  3. Rolling circle amplification (RCA)
  4. The loop-mediated isothermal amplification (LAMP)
  5. Helicase-dependent amplification (HDA)
  6. Recombinase polymerase amplification (RPA)

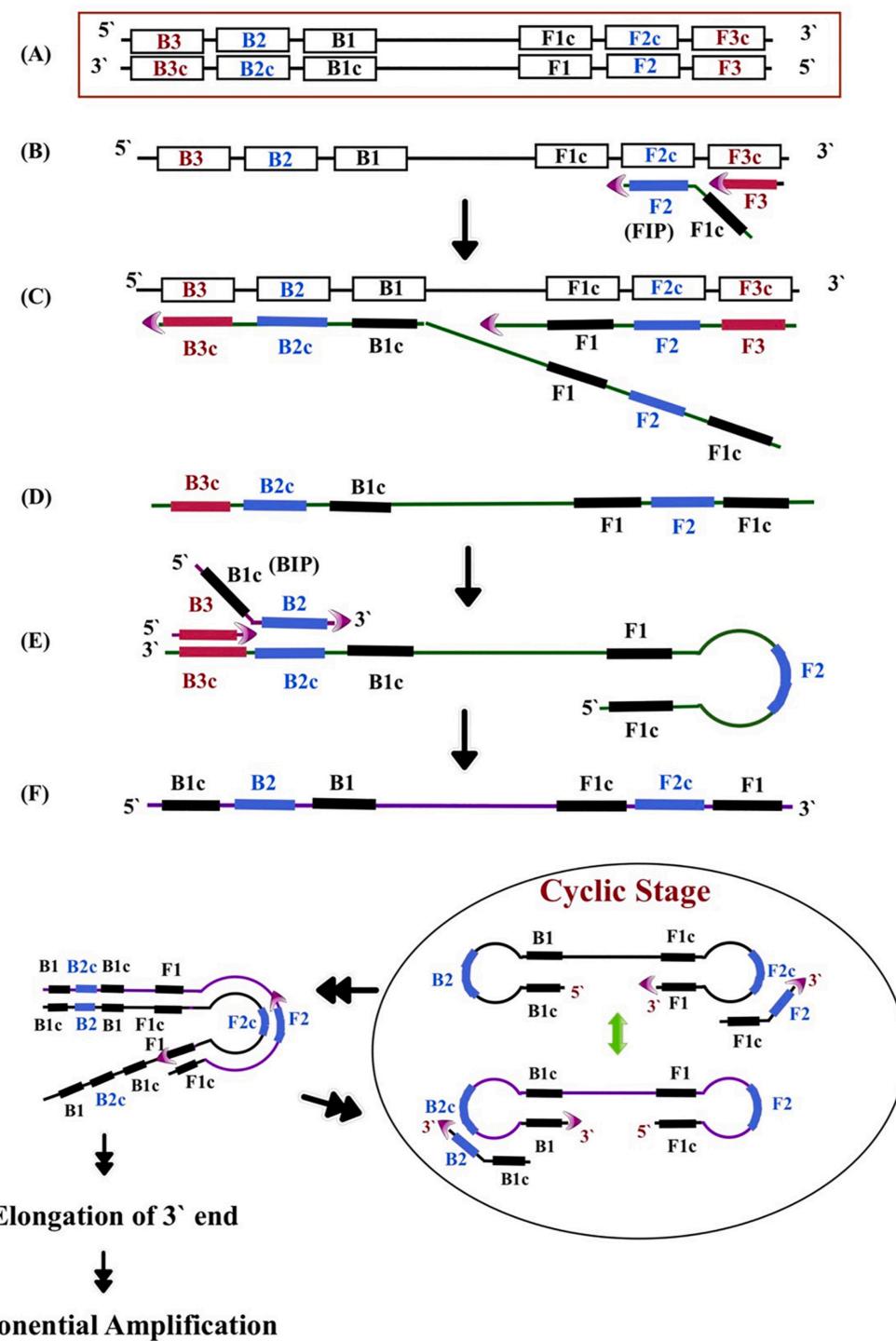
# Isothermal Amplification of Nucleic acids



# Loop-Mediated Isothermal Amplification (LAMP)

- Reaction temperature: 65°C
- Amplicon size: <250 bp
- DNA product: long, branched
- Uses 4-6 primers recognizing 6-8 distinct regions of target DNA.
- Amplification is so extensive that the magnesium pyrophosphate produced during the reaction can be seen by eye, making LAMP well-suited for field diagnostics.
- LAMP is compatible with fluorescent and colorimetric detection

- A. Priming sites on both strands of dsDNA,
- B. The amplification process initiates by the annealing of FIP primer annealing to the plus strand. Upon the elongation of FIP from the 3' end of the F2 region by *Bst* polymerase, the outer primer hybridizes at the upstream position.
- C. Elongation of F3 along with the template displaces the downstream FIP-extended strand.
- D. while the complementary regions at the 5' end of the separated strand form a loop, the 3' end serves as a template for reverse primer annealing.
- E. Formation of dumbbell-like intermediate as a template for the cyclic stage at the end of the non-cyclic stage.



# Recombinase polymerase amplification (RPA)

- Highly sensitive and selective isothermal amplification technique, operating at 37–42°C
- Amplicon size: <1,000 bp
- DNA product: short, discrete
- Requires minimal sample preparation and capable of amplifying as low as 1–10 DNA target copies in less than 20 min
- RPA is compatible with lateral flow strips and fluorescent detection

## Steps:

A. Recombinase proteins form complexes with each primer

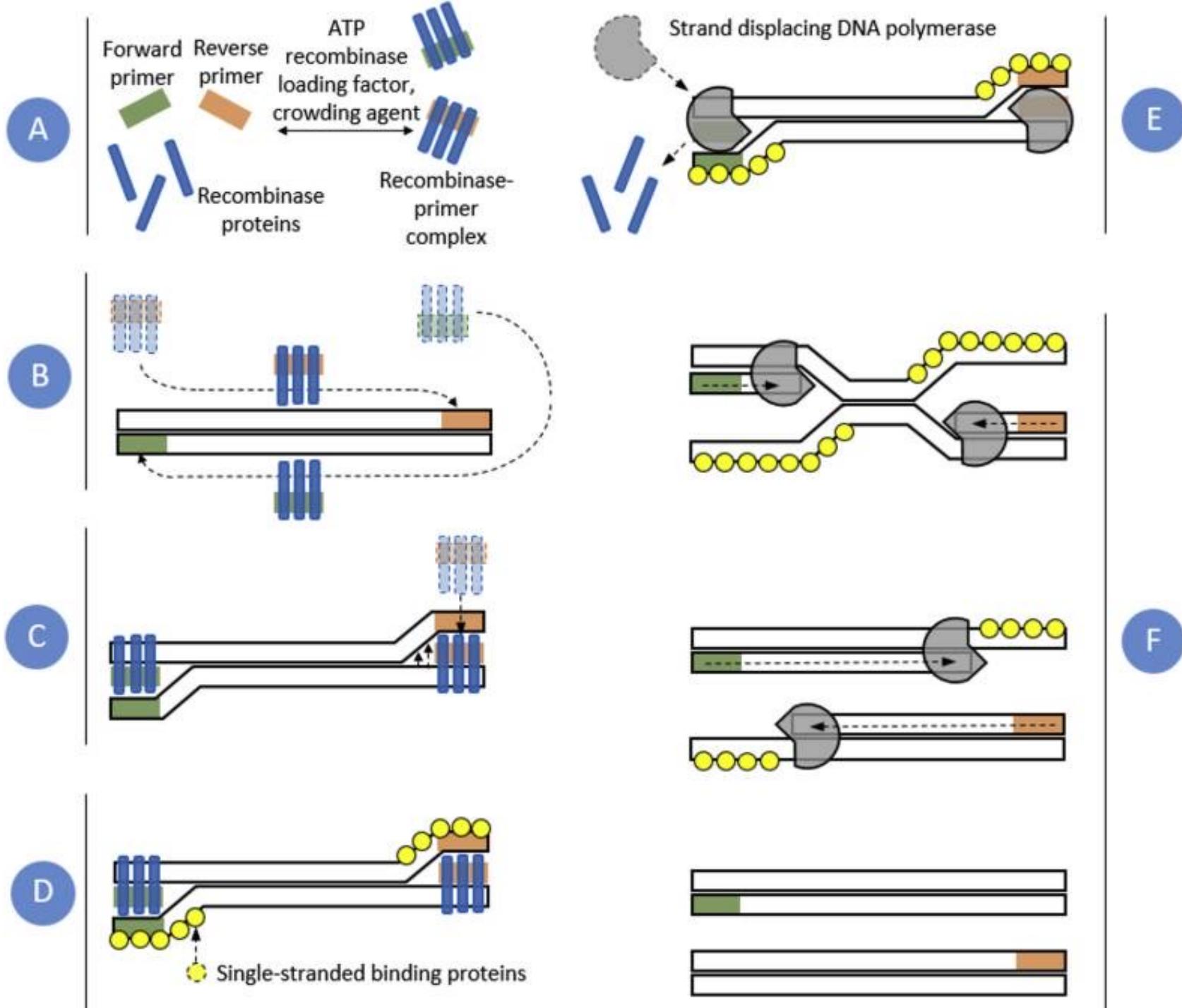
B. Recombinase scans DNA for homologous sequences

C. The primers are inserted at the cognate site by the strand-displacement activity of the recombinase

D. Single stranded binding proteins stabilize the displaced DNA chain

E. The recombinase then disassembles leaving the 3'-end of the primers accessible to a strand displacing DNA polymerase

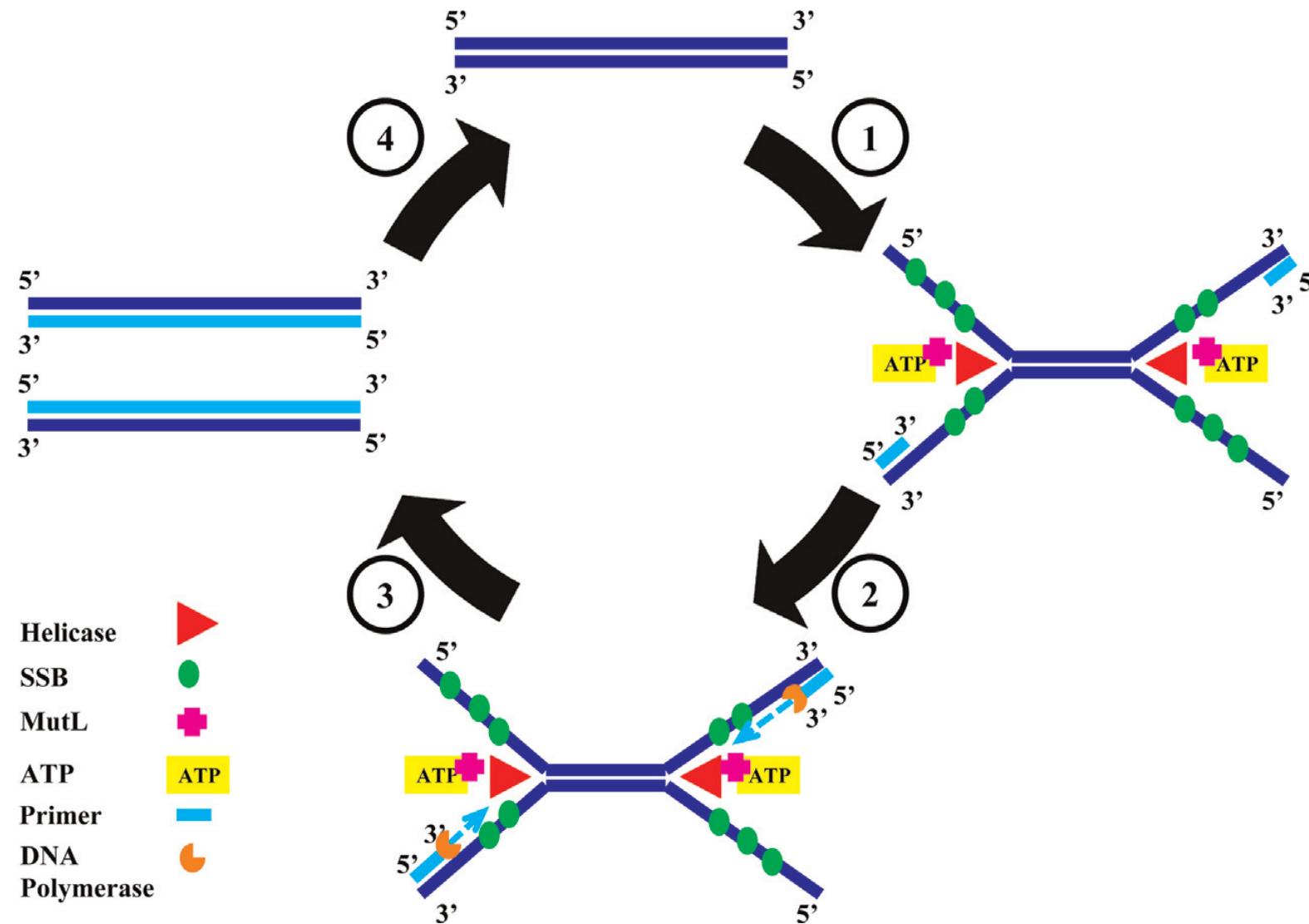
F. Elongation by the DNA polymerase



# Helicase-dependent amplification (HDA)

- HAD mimics the replication fork scheme created during DNA replication in vivo, where a helicase is used to separate the double-stranded DNA.
- Single strand binding proteins (SSB) prevents the unwound DNA from re-annealing and enabling the two primers to bind. An exo- Klenow fragment of DNA polymerase I then extended the template
- Amplicon size: Up to a whole genome
- DNA product: long, discrete
- Highly sensitive and selective isothermal amplification technique, operating at 37–42°C
- HDA is compatible with lateral flow strips and fluorescent detection

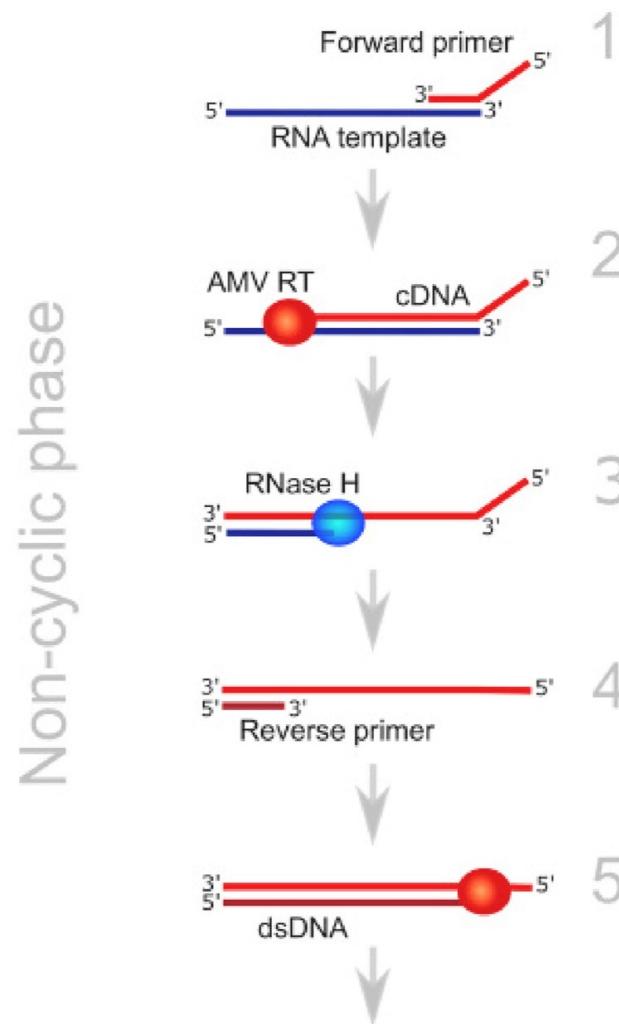
# Helicase-dependent amplification (HDA)



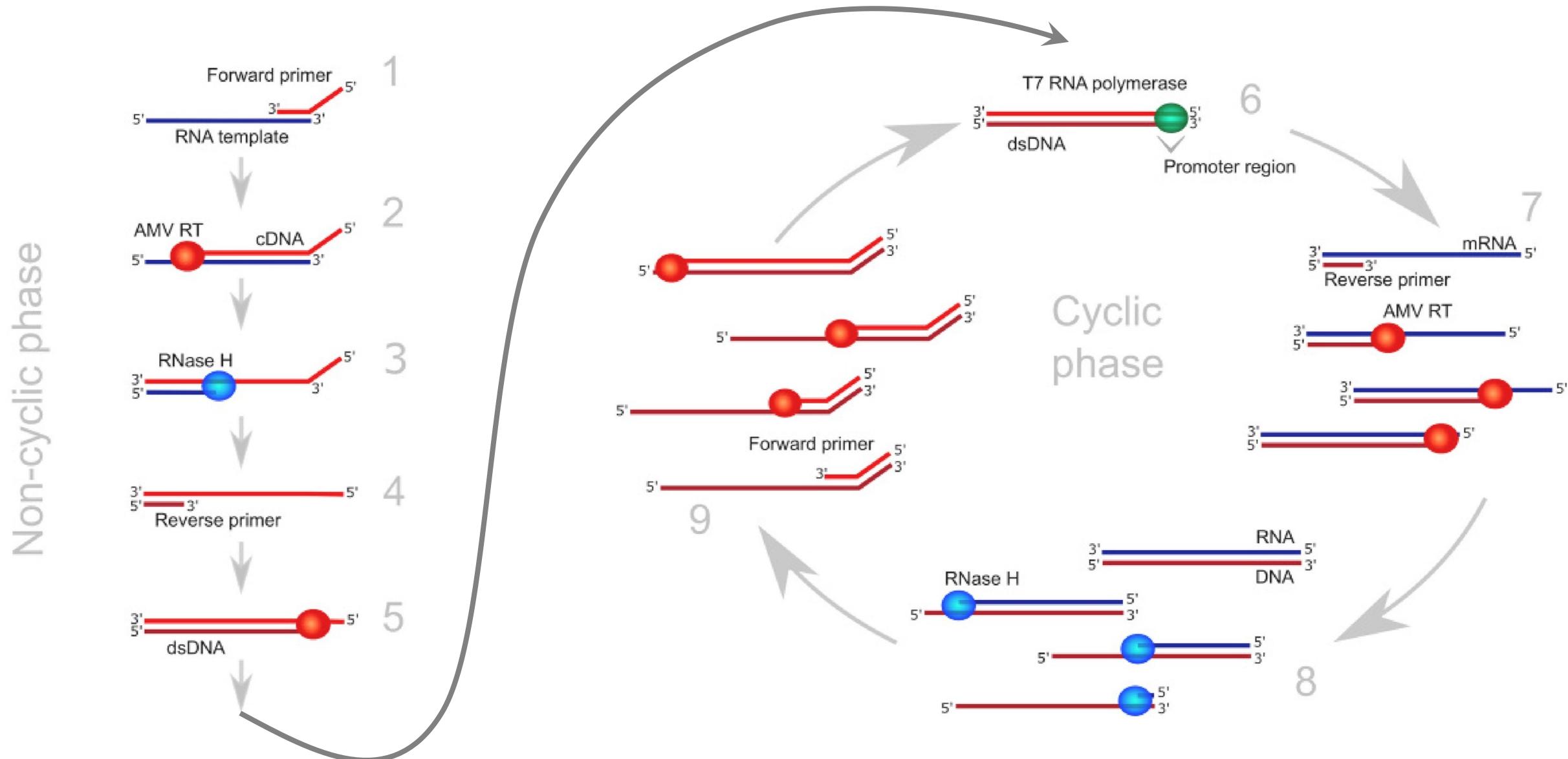
# Nucleic acid sequence-based amplification (NASBA)

- The method can selectively amplify messenger RNA (mRNA) even in the presence of genomic DNA at 41 °C by utilizing two primers and three enzymes
- **Enzymes:** avian myeloblastosis virus (AMV) reverse transcriptase, RNase H, and T7 RNA polymerase
- **Primers:**
  1. Forward anti-sense with a non-complementary promoter sequence for the corresponding T7 RNA polymerase at 5'end
  2. Shorter reverse one designed to hybridize with cDNA generating from P1.
- Amplicon size: 100-150 bp
- DNA product: short, discrete

# Nucleic acid sequence-based amplification (NASBA)



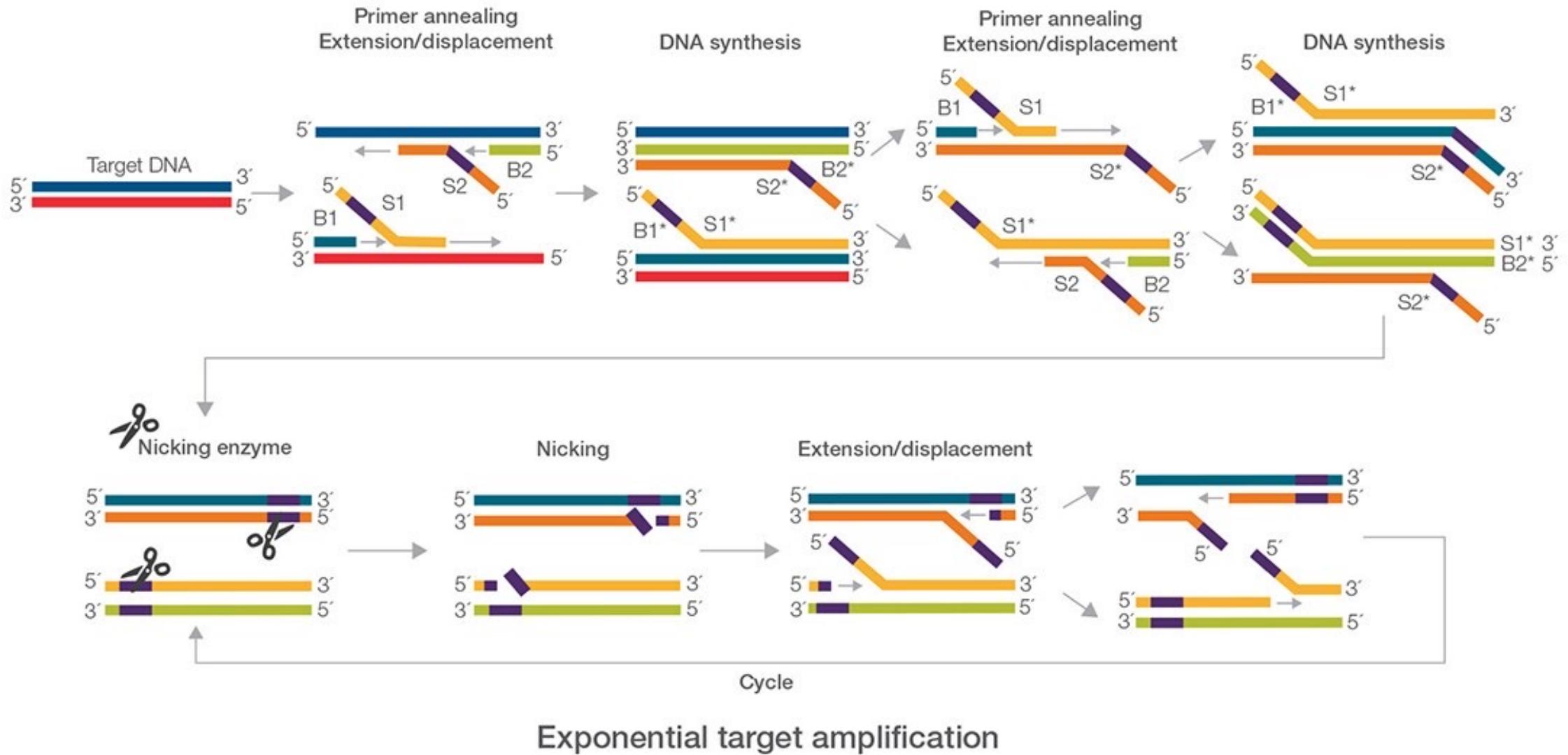
# Nucleic acid sequence-based amplification (NASBA)



# Strand Displacement Amplification (SDA)

- SDA uses
  1. A restriction endonuclease to nick the unmodified strand of the DNA target and
  2. An exonuclease-deficient DNA polymerase exo-Klenow polymerase to displace the downstream DNA strand at the nick sites
- The SDA reaction contains two parts:
  1. The generation of DNA templates containing restriction sites and the cyclic amplification of target DNA.
    - In the first part, primers S1 and S2, which are designed to have Hinc II recognition sites at the 5' end and target sequences for directed hybridization at the other end, specifically introduce a Hinc II restriction site into the extended strand by binding with target sequences.
    - Bumper primers B1 and B2, which are adjacent to primers S1 and S2, are extended by DNA polymerase with strand replacement activity to release the single extension strand of primer S1 and S2.
    - The backward primers hybridize to the single strand containing a Hinc II restriction site, and the bumper primers are also extended to synthesize new dsDNA
  2. The next step is a cycle process.
    - The restriction endonuclease Hinc II can cleave the restriction site at one strand separated from the dsDNA
    - Subsequently, a nick is formed and the free 3'-end is extended by the polymerase.

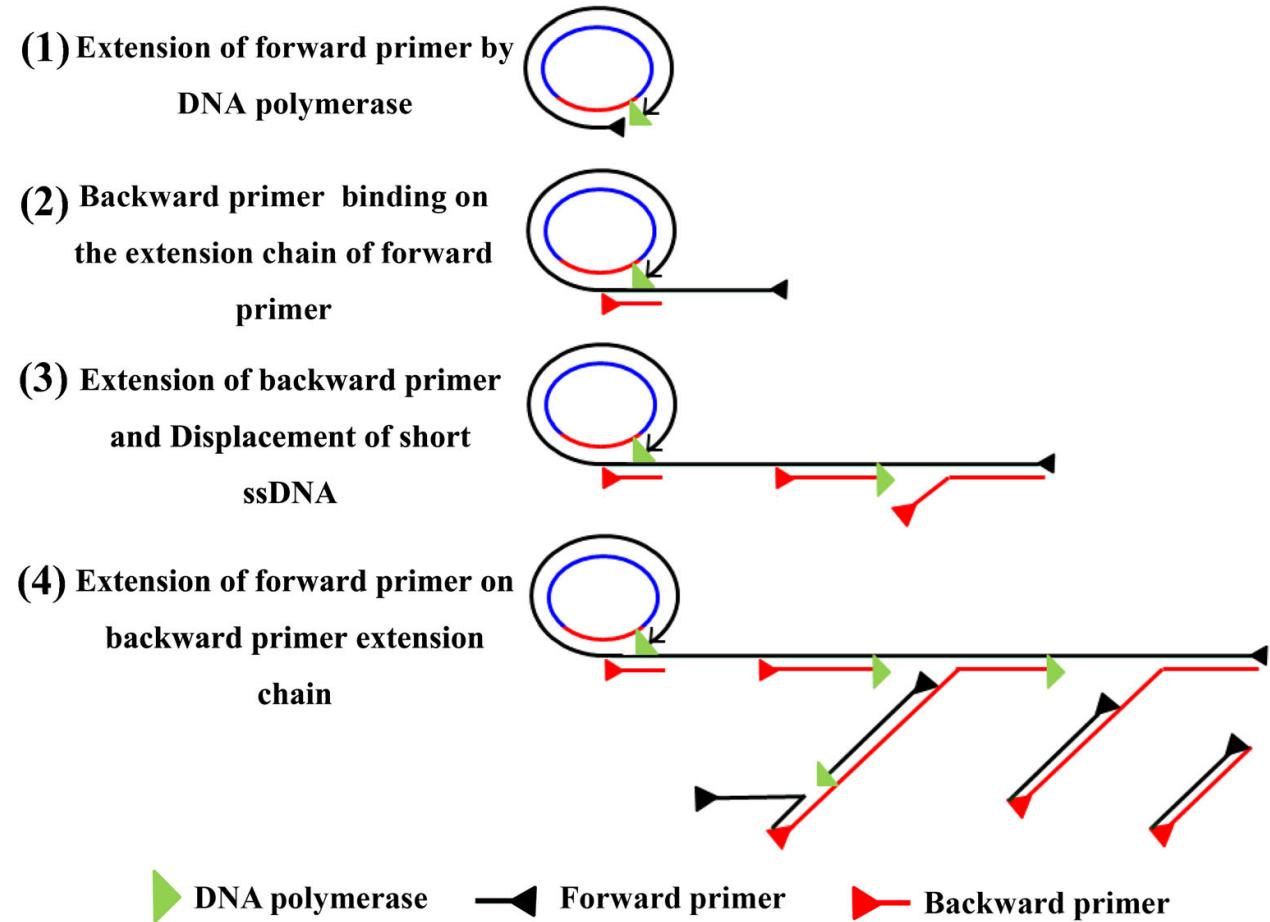
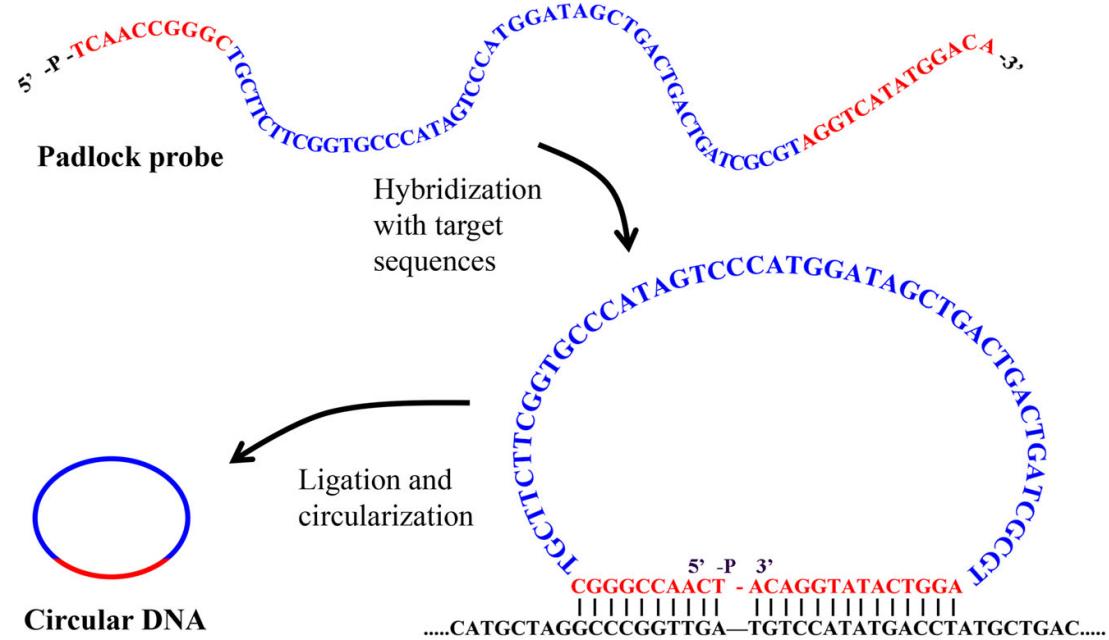
# Strand Displacement Amplification (SDA)



# Rolling Circle Amplification (RCA)

- RCA relies on the high polymerase processivity and strand displacement activity of Phi29 bacteriophage DNA polymerase ( $\varphi$ 29DNAP) to synthesize tandem repeats of DNAs
- The resulting amplicons are the multiple repeats of the circular template, which can subsequently be digested by enzymes into each individual short oligonucleotides (Liu et al., 1996).
- The benefits of RCA are two-fold:
  1. The reaction mechanism is simple, only requiring a single primer and a productive enzyme; and the reaction is isothermal, with an incubation temperature as low as 23 °C.
  2. The circular DNA is the predominant template for amplification even in the presence of linear DNA, and the yield is very high.

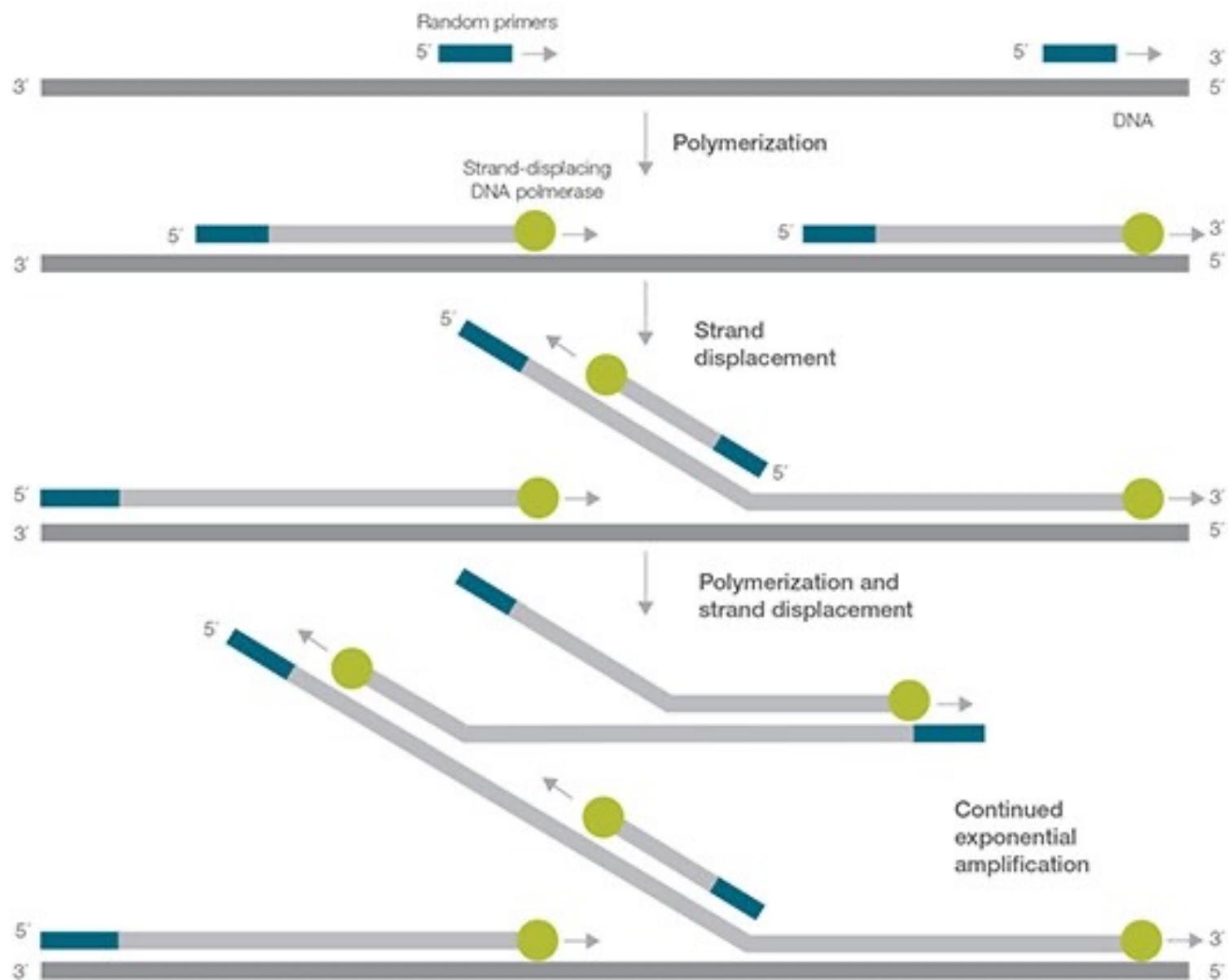
# Rolling Circle Amplification (RCA)



# Whole Genome Amplification (WGA)

- Two sets of non-specific primers designed to annealing with random regions on each strand of target DNA are used
- These random primers are phosphorothioate-modified hexamers to resist 5' to 3' exonuclease activity of DNA polymerase
- Following the hybridization of primers, strand-displacement DNA polymerase initiates the amplification from the 3'ends of each primer.
- Synthesis of the new strand proceeds until the polymerase reaches the downstream primer on the same strand.
- Polymerase displaces the intervening primer and keeps going through the template DNA
- Each time a strand goes under displacement, priming occurs on its separated single-stranded section.
- Therefore, in a short time, following the exponential amplification, there would be hyper-branched intermediates and dsDNA amplicons.

## Whole genome amplification (WGA)



Technology	Reaction temperature	Reaction time	Primers	Amplicon size	Target	Sensitivity	Detection method
LAMP	60–65 °C	15–60 mins	4–6 primers	>20 kb	dsDNA (RNA)	Single copy	Fluorescence, colorimetric, turbidity, lateral flow
WGA	30–40 °C	60–180 mins	Random hexamers	Unlimited	dsDNA		Fluorescence, colorimetric
RPA	37 °C	30–60 mins	2 primers	<1 kb	dsDNA	Single copy	Fluorescence, lateral flow, Gel electrophoresis
HDA	65 °C	~90 mins	2 primers	~150 nt	dsDNA	Single copy	Fluorescence, colorimetric, lateral flow
NASBA	40–50 °C	~60 mins	2 primers	~150 nt	ssRNA (DNA)	Single copy	Fluorescence, ELISA, Gel electrophoresis
RCA	30–65 °C	60–90 mins	1 primer	~150 nt	Circular DNA (RNA)	10 copies	Fluorescence, colorimetric, turbidity
SDA	30–55 °C	~120 mins	4 primers	~100 nt	ssDNA (RNA)	10 copies	Fluorescence, Gel electrophoresis

# Fluorescence *in situ* hybridization (FISH)

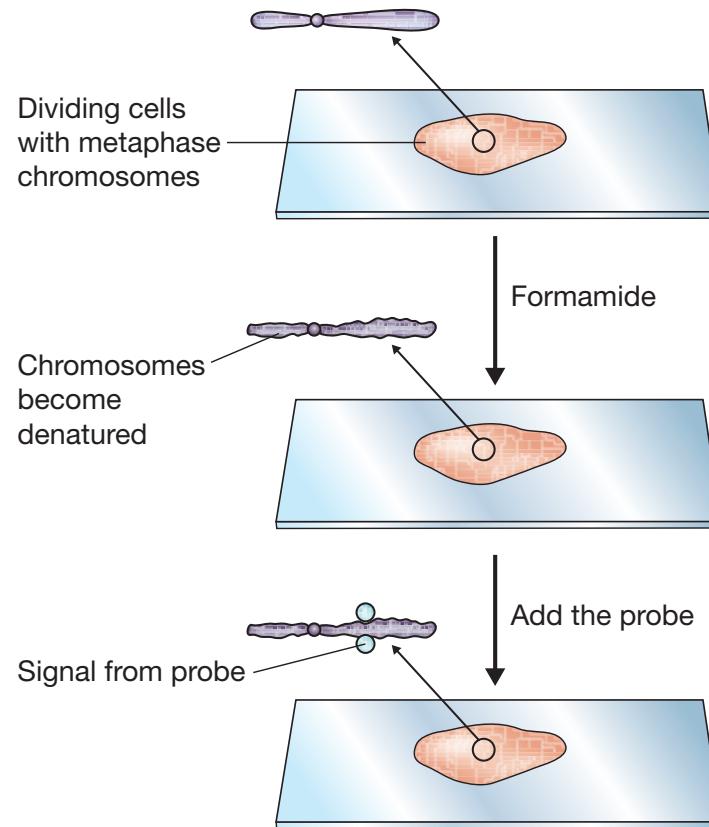
Identify which chromosome contains a gene of interest

DNA or RNA probe for gene of interest is labeled with fluorescent nucleotides

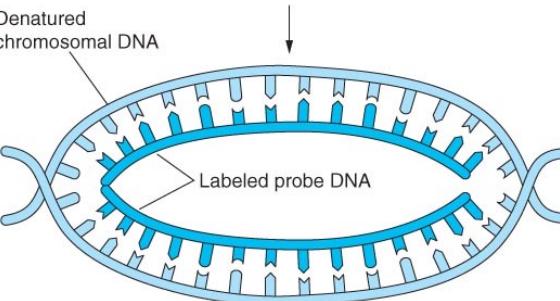
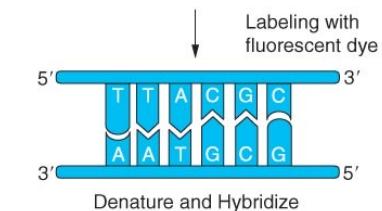
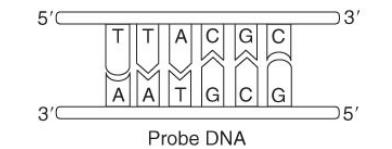
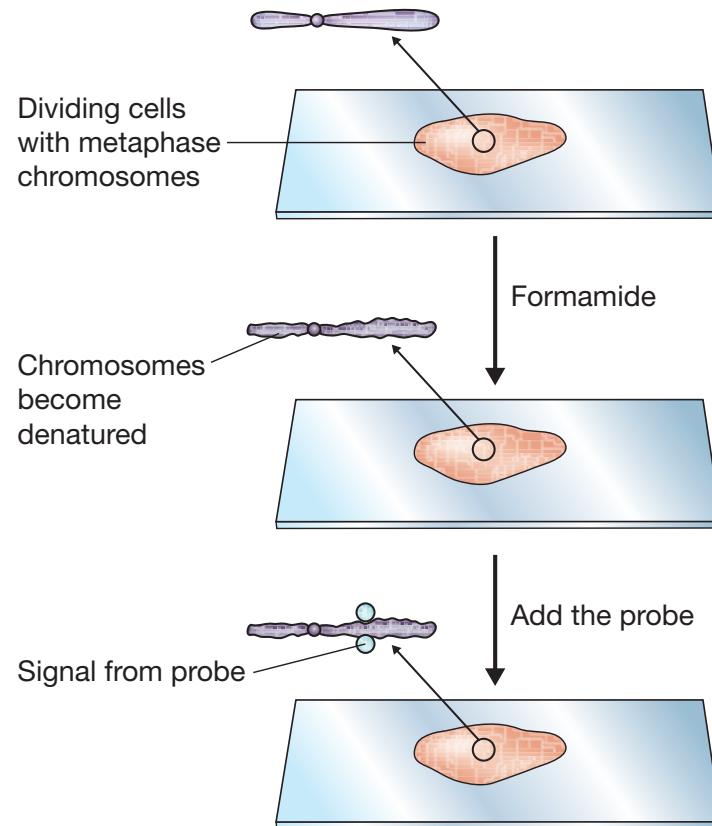
FISH used to analyze genetic disorders

FISH used to determine which cells in a particular organ are expressing the particular mRNA

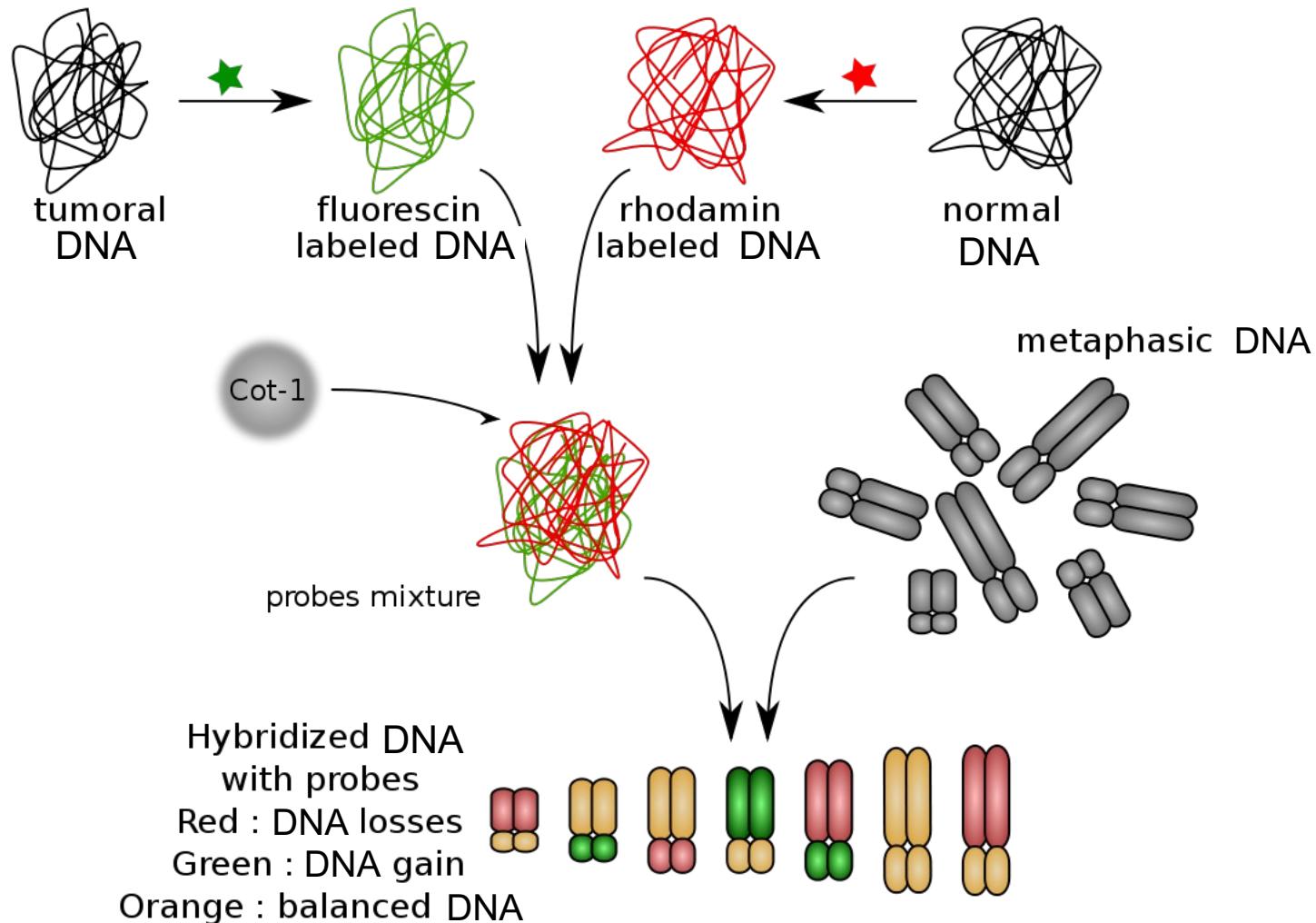
# Fluorescence in situ hybridization (FISH)



# Fluorescence in situ hybridization (FISH)



# Comparative Genome Hybridization (CGH)



# **Southern Blotting**

Used to determine:

1. Gene copy number
2. Gene mapping
3. Gene mutation detection
4. PCR product confirmation
5. DNA fingerprinting

# Southern Blotting

DNA is digested with restriction enzymes and fragments are separated by agarose gel electrophoresis

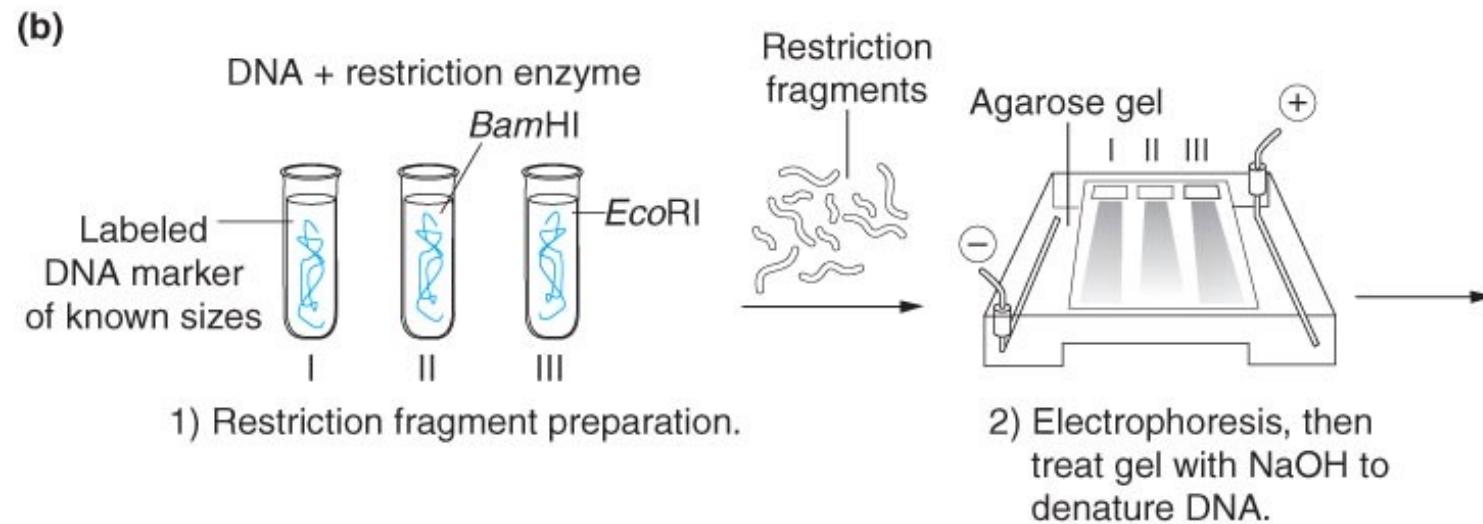
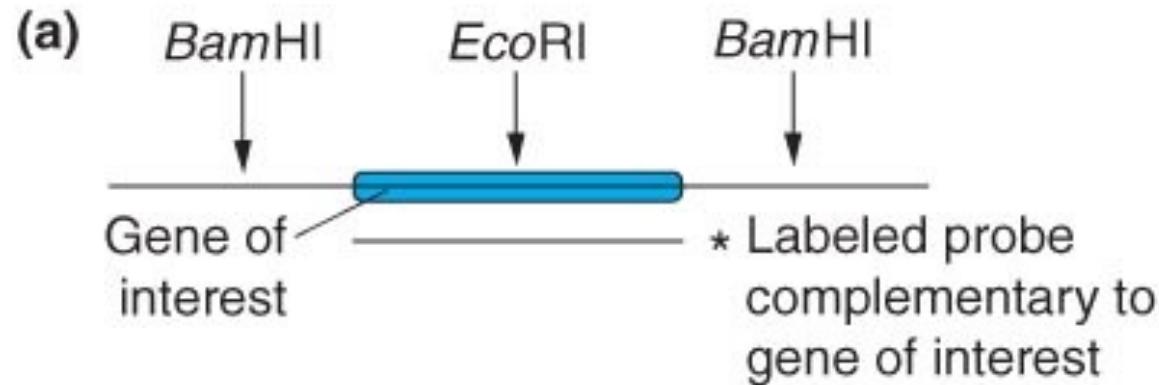
Gel is treated with alkaline solution to denature the DNA

Fragments are transferred onto a nylon or nitrocellulose filter (called blotting)

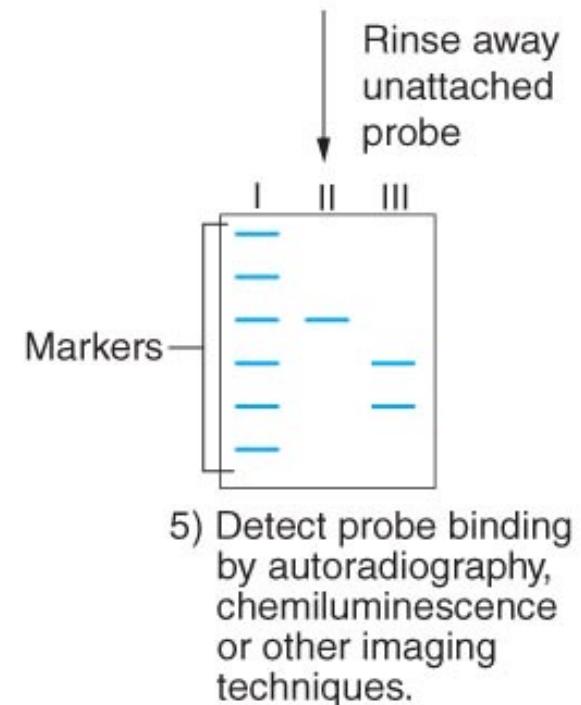
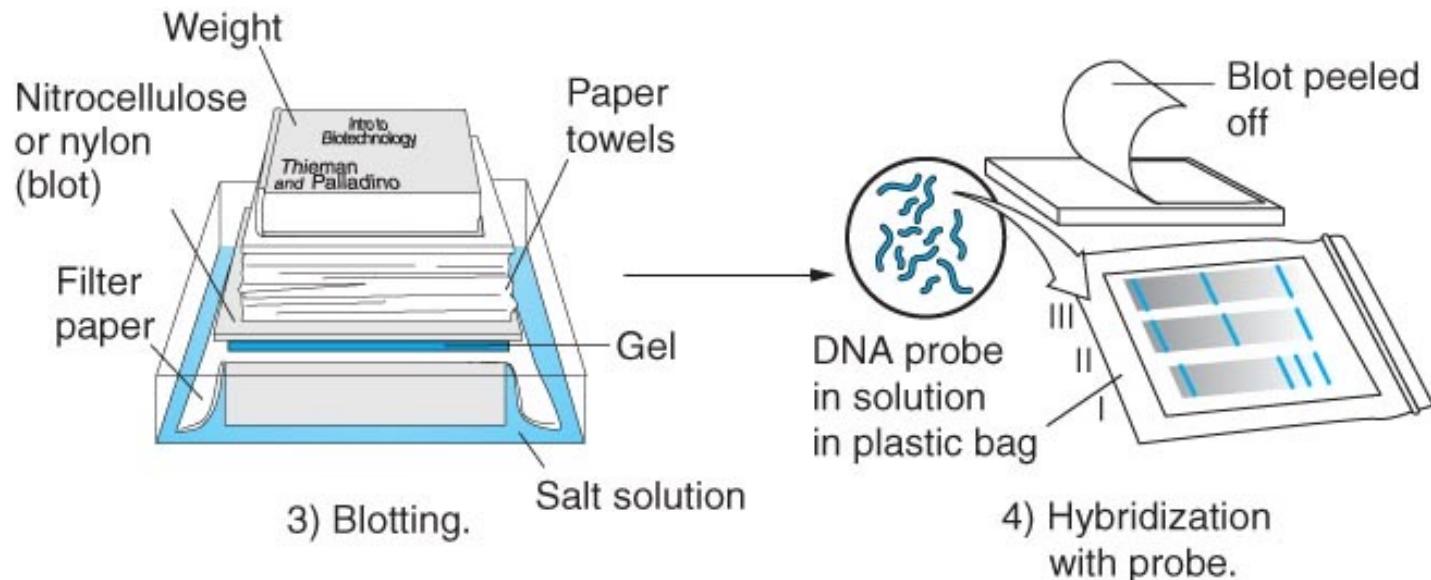
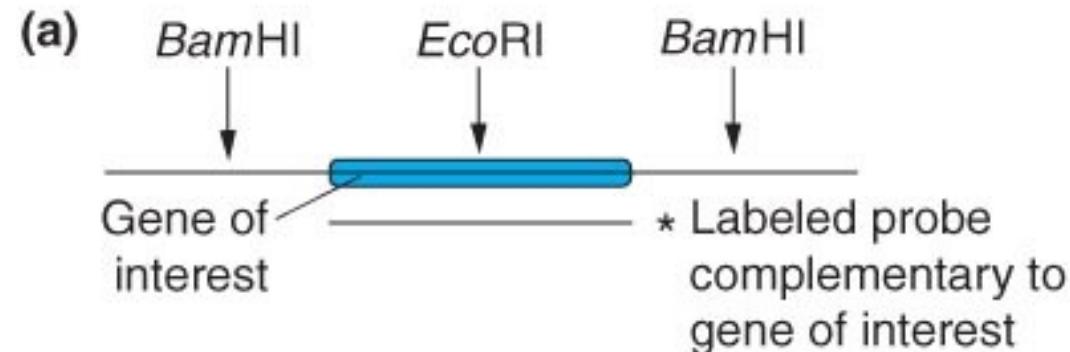
Filter (blot) is baked or exposed to UV light to permanently attach the DNA

Filter (blot) is incubated with a labeled probe and exposed to film by autoradiography

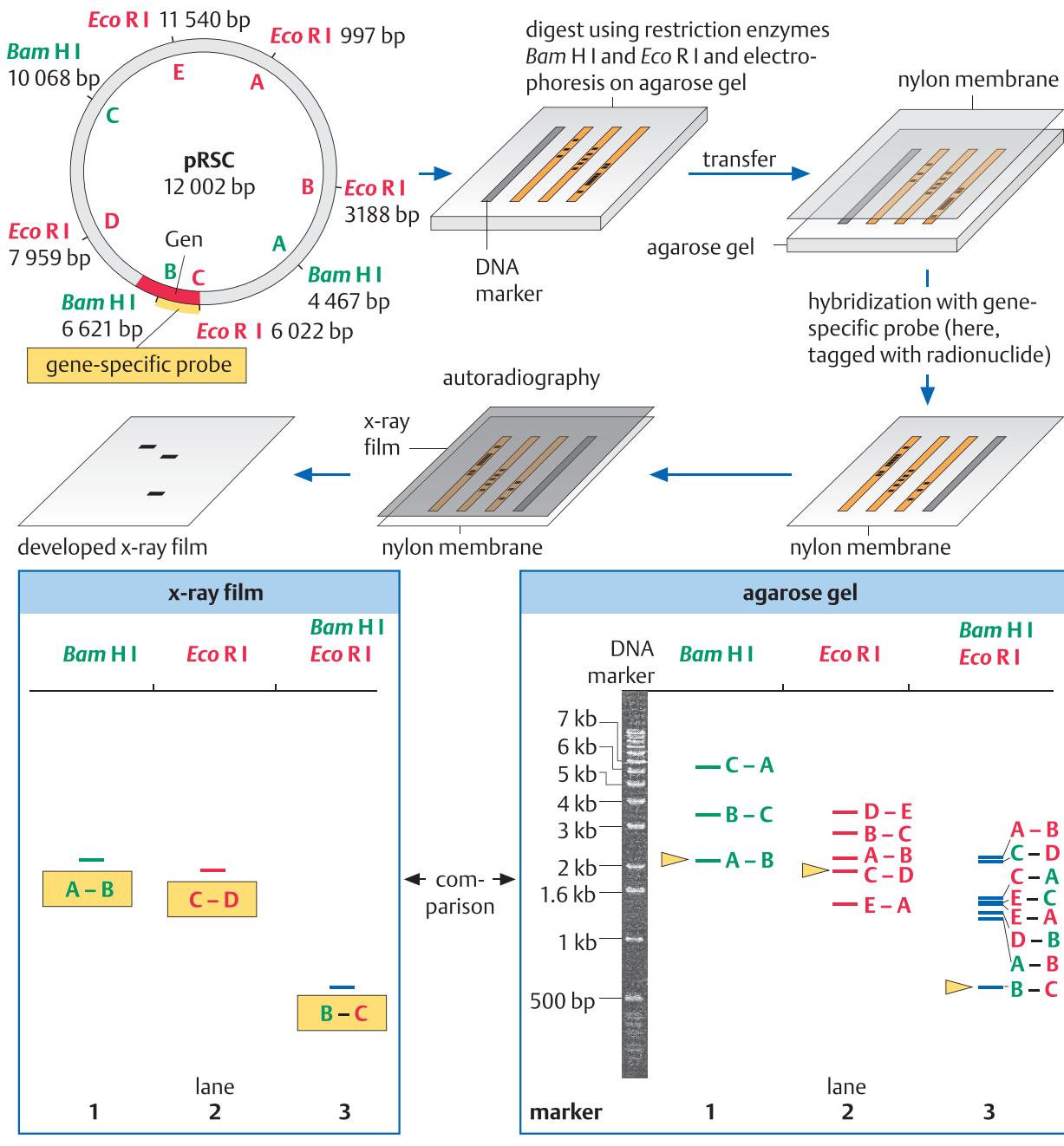
# Southern Blotting



# Southern Blotting



### Southern blot



## **Other types of Blotting**

**Northern blotting**

(the separation and blotting of RNA molecules,

**Western blotting**

(the separation and blotting of proteins)

