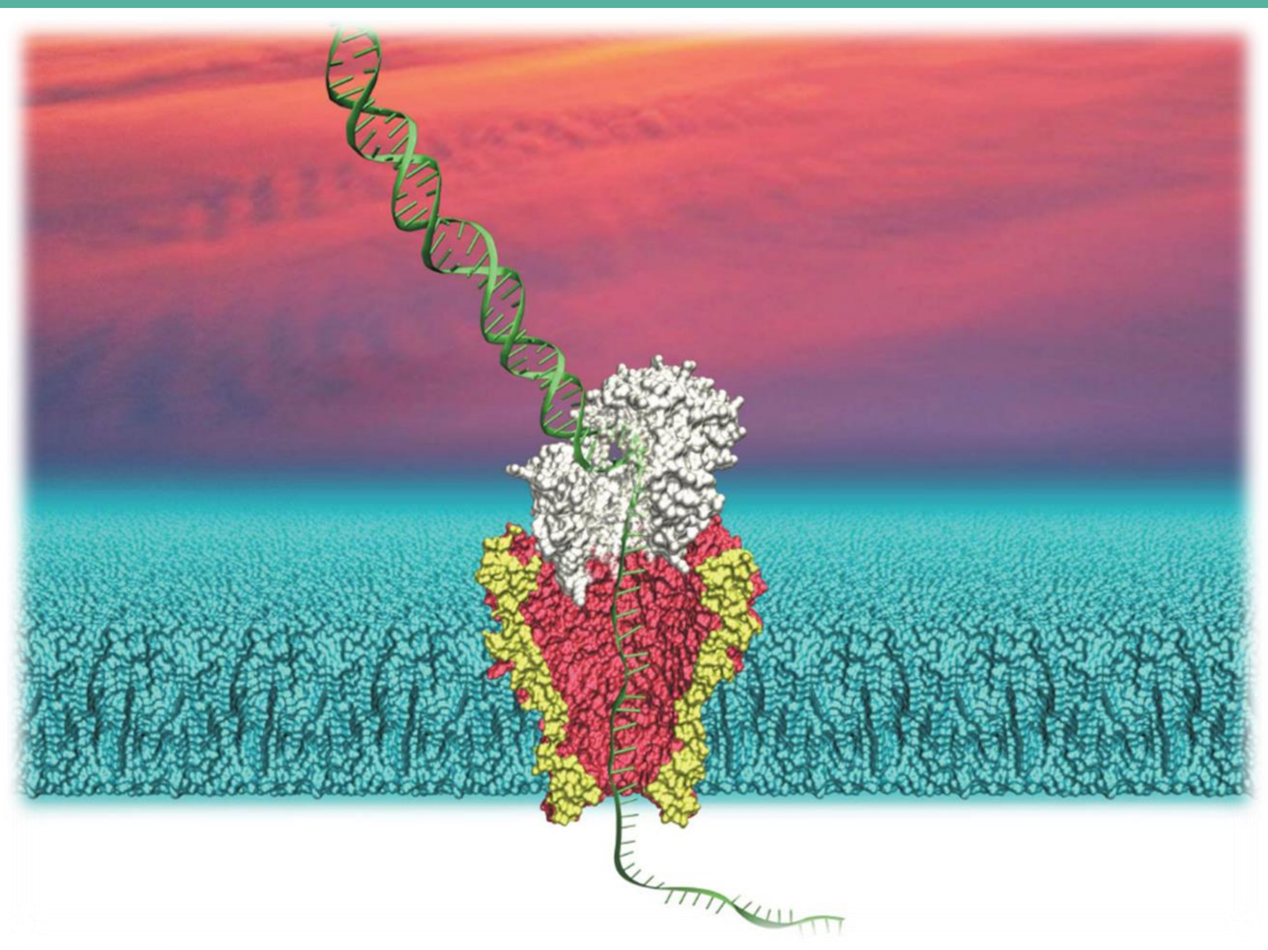
# Chapter 19

# DNA Technology



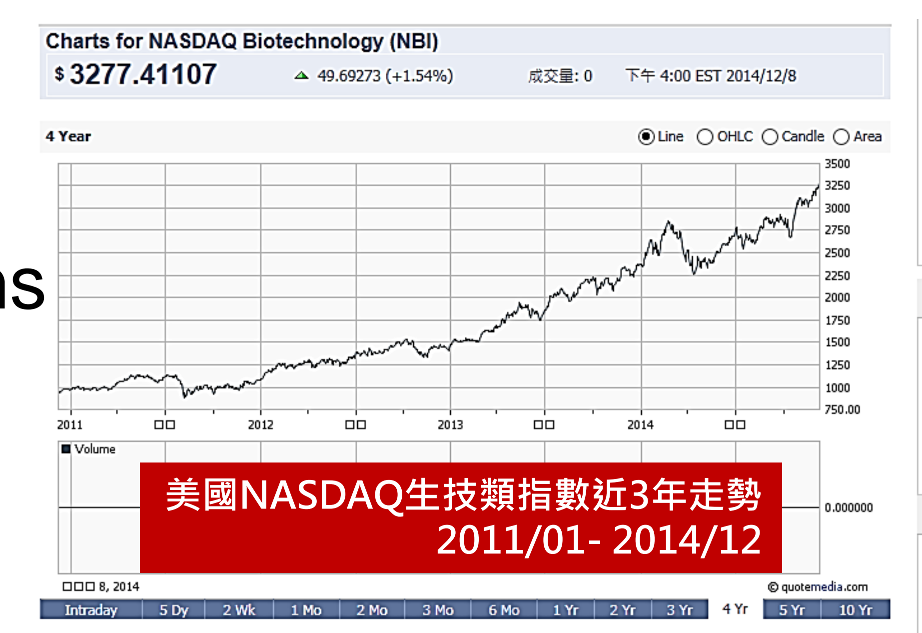
Modified by YJ Chuang at NTHU-MS

# Overview: The DNA Toolbox

- **One of the greatest achievements of modern science has been the sequencing of the human genome (2003)**--- depended on advances in **recombinant DNA technology**
- In **recombinant DNA**, nucleotide sequences from two different sources, often two species, are combined *in vitro* into the same DNA molecule
  - Large-scale sequencing of the genomes of various species was under way since then

# DNA technology & Biotechnology

- **Biotechnology** is the manipulation of organisms or their components to make useful products




The applications of **DNA technology** affect everything from agriculture, to criminal law, to medical research. Thus, DNA technology has revolutionized biotechnology

# Milestones in DNA Technology

2006 Induced pluripotent stem cell

1997 Reproductive cloning of mammals (Dolly)

1989 Gene therapy (first success)

1972-1990s cDNA library

2000 Reverse Transcription-PCR

1977 DNA sequencing (Sanger)

1983-85 Polymerase chain reaction/PCR

1952 Electrophoresis

1993 Real-time qPCR

1969–1980s *in situ* Hybridization

1990-2004 Next-generation sequencing

1950 1960 1970 1980 1990 2000 2010

1995 Microarray

2010s Genome Editing (CRISPR/Cas System)

1970 1st DNA sequencing

1970 Discovery of reverse transcriptase

1970 Discovery of Restriction enzymes

1972 Recombinant protein expression

1972 Gene/Molecular cloning

1975 Animal cloning (Frog)

# Concept 19.1: DNA sequencing and DNA cloning are valuable tools for genetic engineering and biological inquiry

- A gene's complete nucleotide sequence can be determined using a process called **DNA sequencing** 定序
- The first automated procedure was based on a technique called dideoxy or chain termination sequencing, developed by Frederick Sanger
- In the last 15 years, "next-generation sequencing" techniques have been developed that are much faster

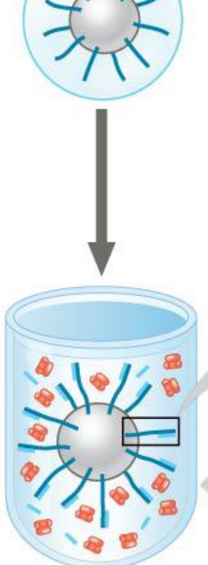
# Next-Generation Sequencing (NGS) 次世代定序

- "Next-generation sequencing" techniques use a single template strand that is immobilized and amplified to produce an enormous number of identical fragments
- Thousands or hundreds of thousands of fragments (400–1,000 nucleotides long) are sequenced in parallel
- This is considered as a type of high-throughput technology

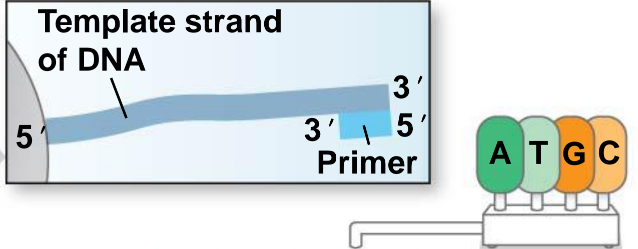
  高通量

Technique

# Next-Generation Sequencing (NGS)
次世代定序



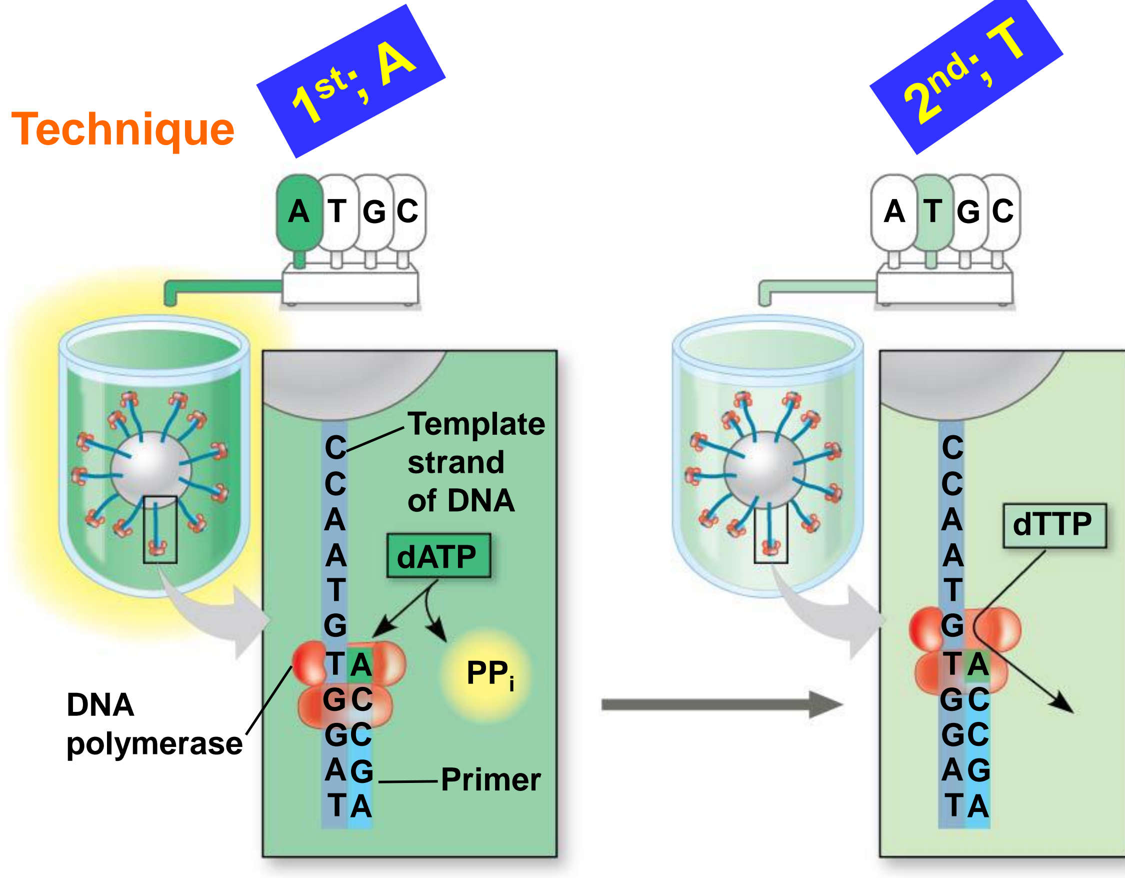
1. Genomic DNA is fragmented.
2. Each fragment is isolated with a bead.
3. Using PCR, $10^6$ copies of each fragment are made, each attached to the bead by 5′ end.
4. The bead is placed into a well with DNA polymerases and primers.




一次反應只用一種 去氧核苷酸
dATP, dGTP, dTTP, dCTP (**dNTP**)
沖洗後，才換下一種; 4個輪換

5. A solution of each of the four nucleotides is added to all wells and then washed off. The entire process is then repeated.

# Next-Generation Sequencing (NGS)
次世代定序

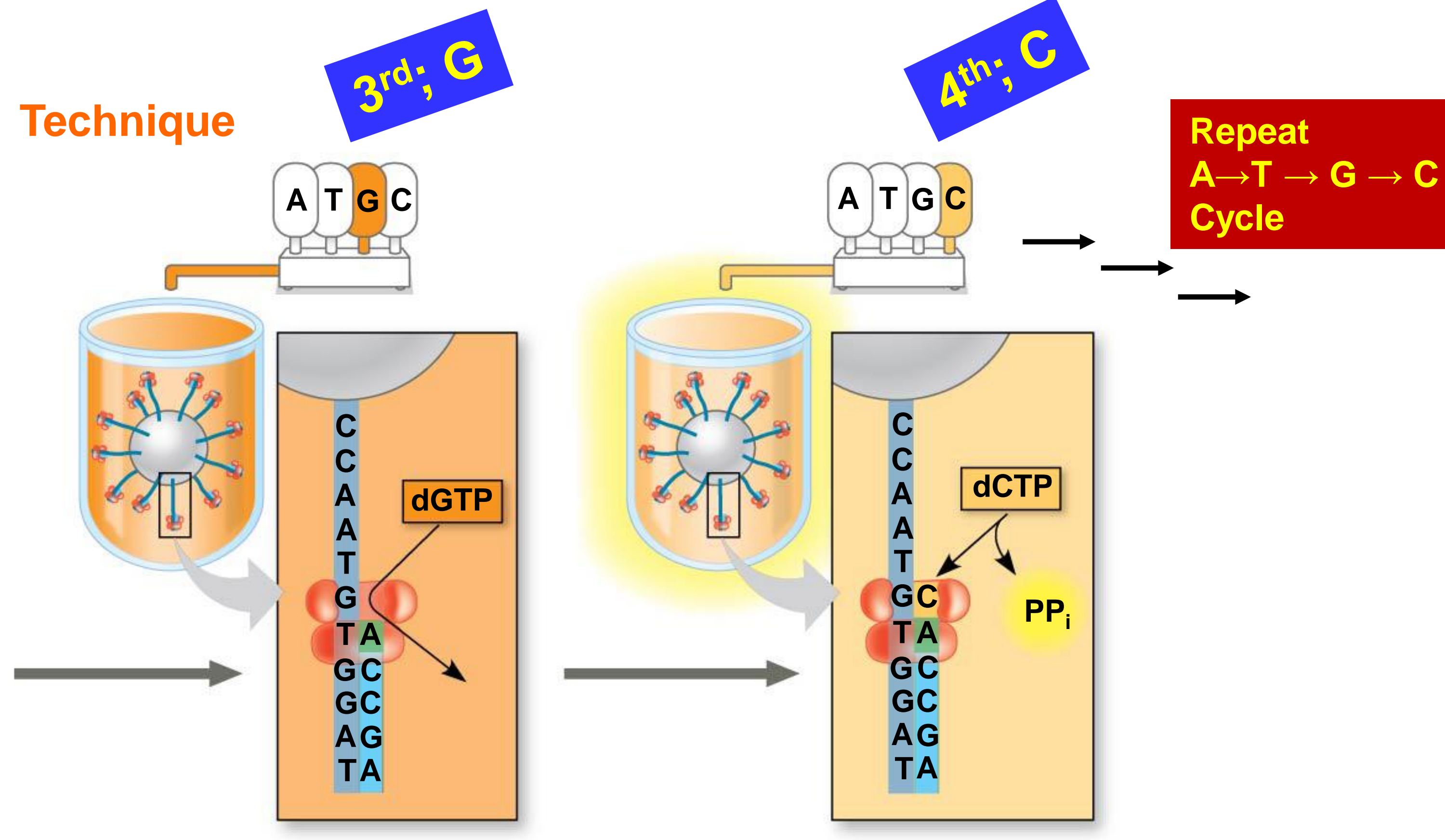



6 If a nucleotide is joined to a growing strand, **PP$_i$ is released, causing a flash of light that is recorded.**

7 If a nucleotide is not complementary to the next template base, no PP$_i$ is released, and no flash of light is recorded.

*No match (no DNA elongation), No signal*

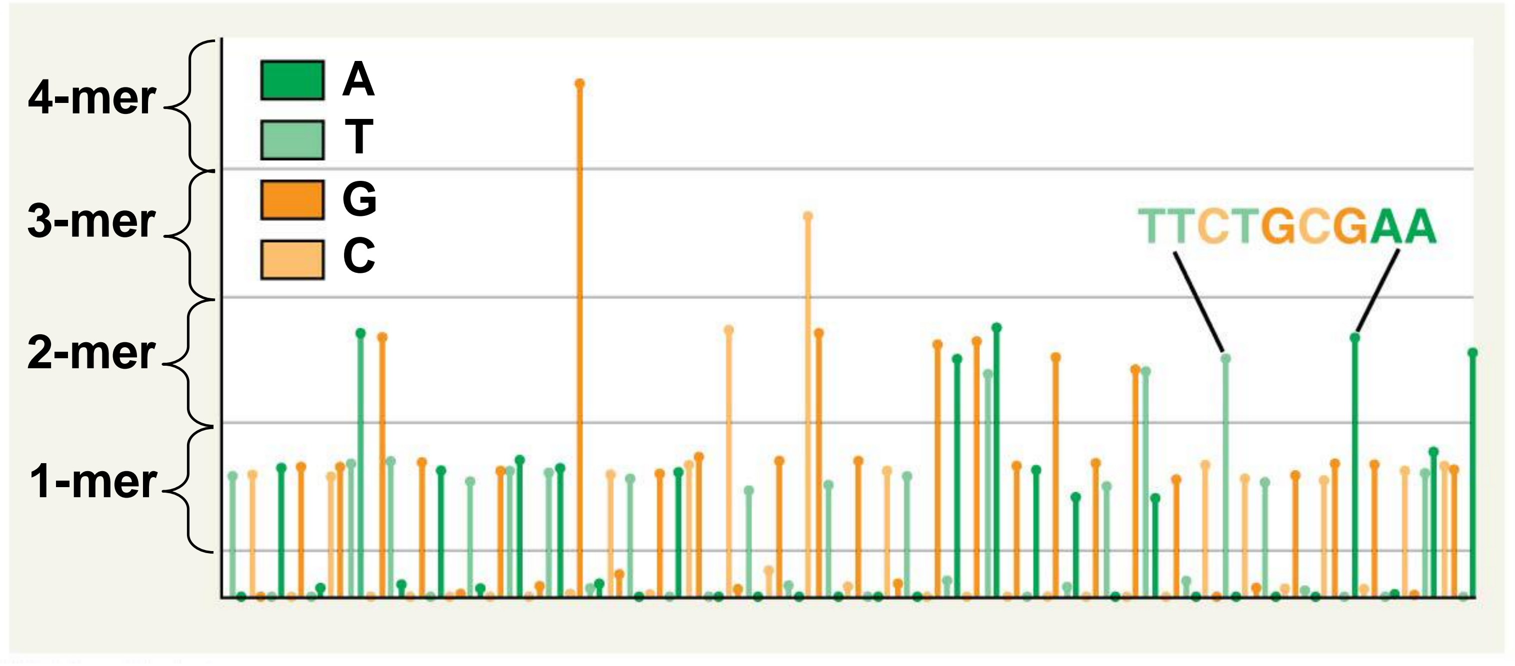
# Next-Generation Sequencing (NGS)
# 次世代定序




8 The process is repeated until every fragment has a complete complementary strand. The pattern of flashes reveals the sequence.

Figure 19.4d

**Next-Generation Sequencing (NGS)**
**次世代定序**

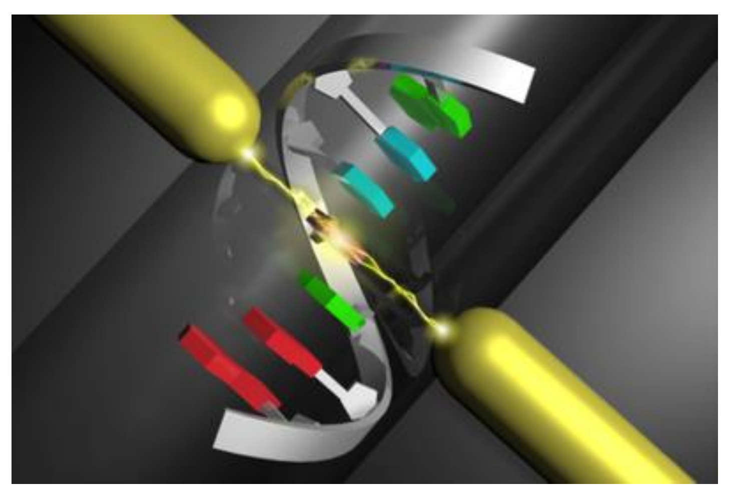



dATP, dGTP, dTTP, dCTP 4個依設定輪換，有反應 (complementary base pairing) ，即記錄藉以完成定序

# And the sequence technology keeps advancing….

- In “**third- and fourth-generation sequencing**,” the techniques used are even faster and less expensive than the previous, while providing spatial information

請自主學習



Real-time sequencing of single DNA (or RNA) molecules


**Reference**:
•Schadt EE et al. **A window into third-generation sequencing.** Hum. Mol. Genet. (2010)
•19(R2):R227
•Liang J et al. **Single-cell sequencing technologies: current and future.** J Genet Genomics. (2014)

# DNA Cloning

- **How to do DNA cloning:**
  - (1) 目標：**DNA of interest**
  - (2) 載體：**Plasmids (cloning vector):** **replication origin, multiple cloning sites, selection marker (for example: ampicillin resistance gene; LacZ gene : β-galactosidae** digests **X-gal** into blue product**)**
  - (3) 宿主：**Host**: i.e. bacteria (E. Coli), yeast
- Cloned genes are useful for making copies of a particular gene and producing a protein product

Figure 19.5a

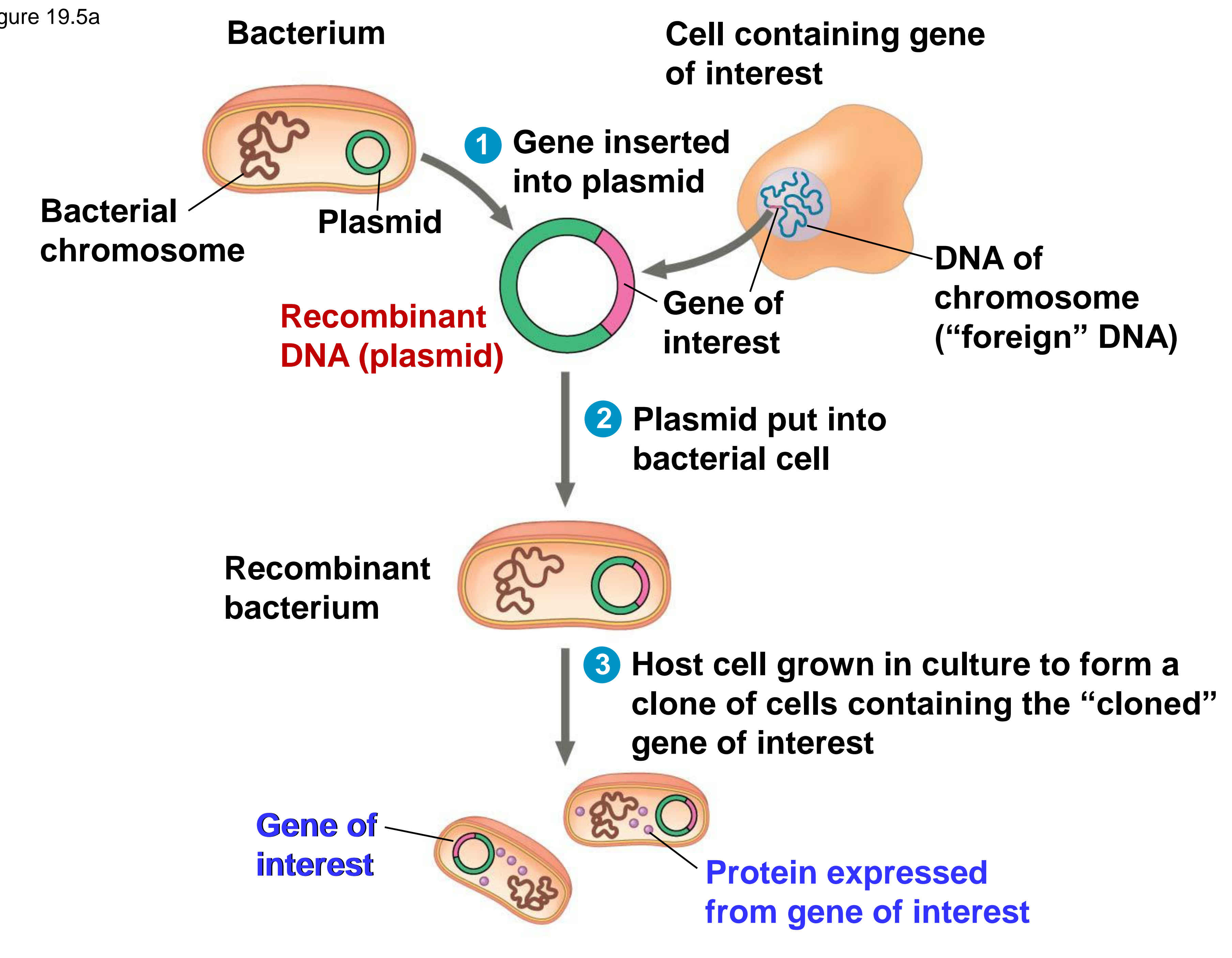
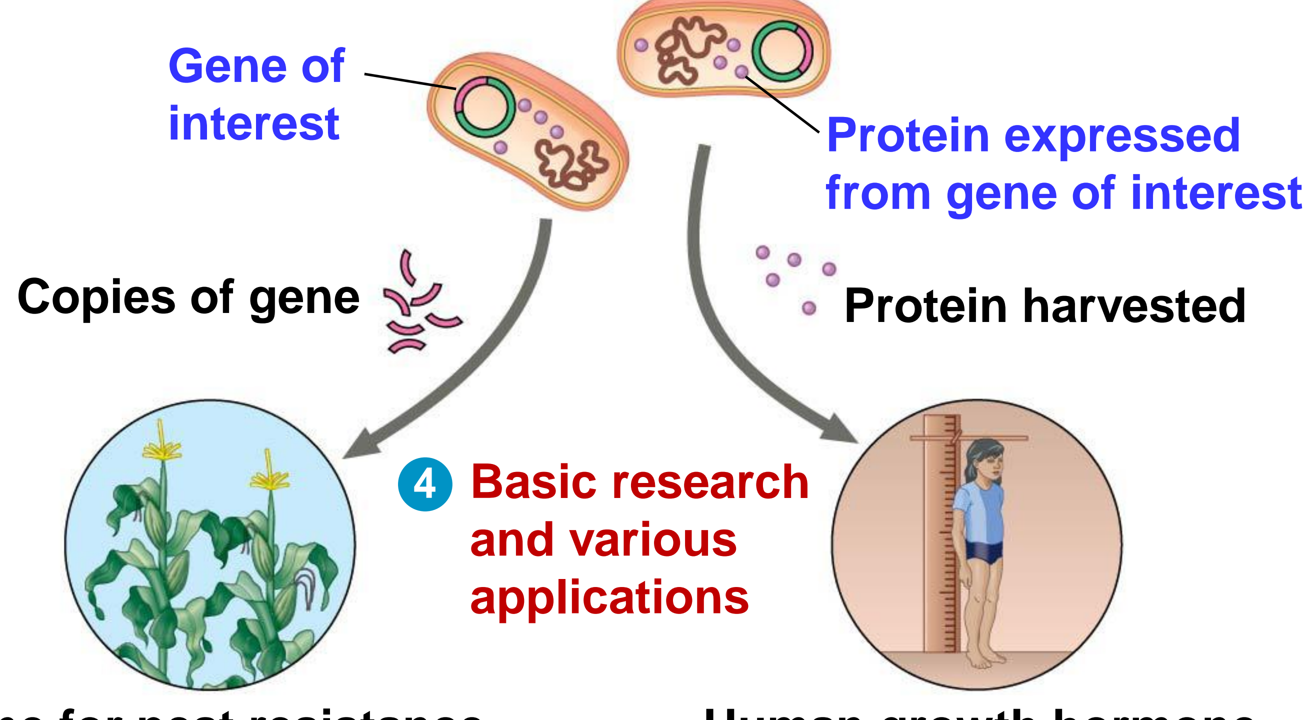
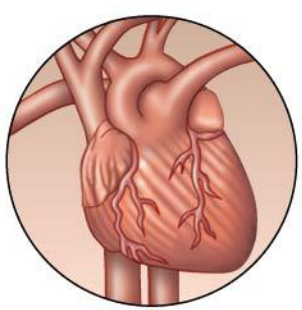


Figure 19.5b



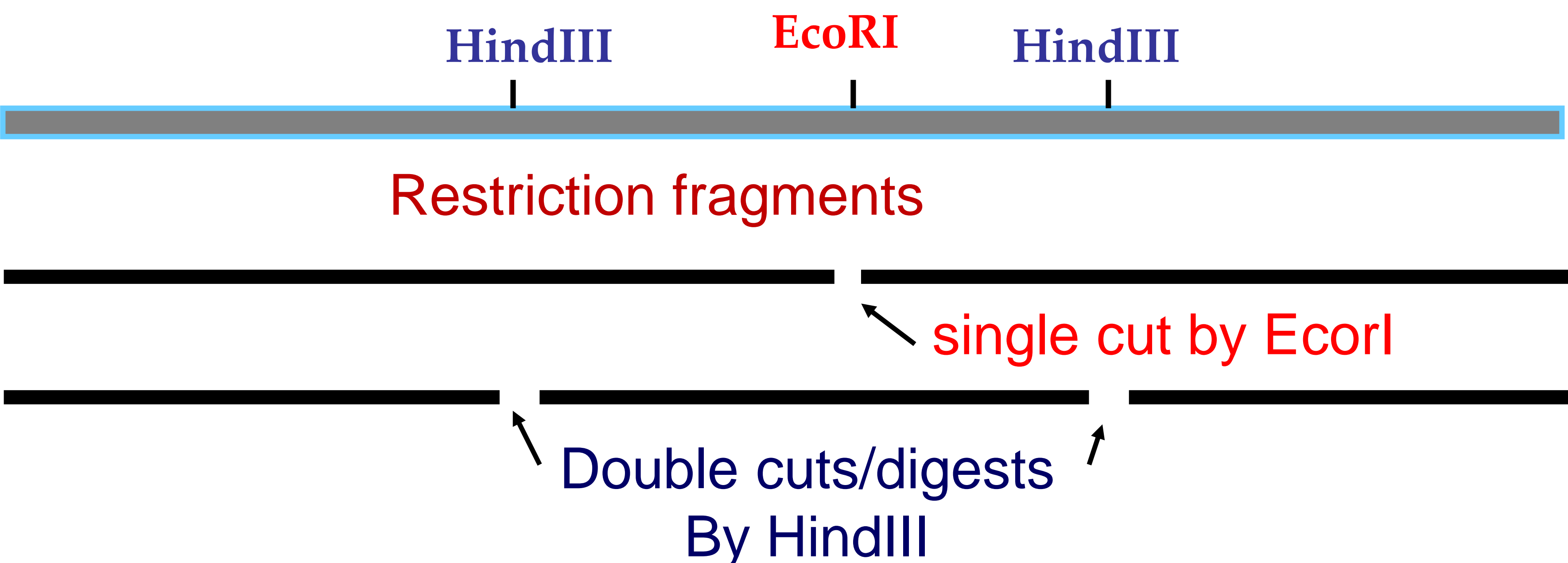
Gene used to alter bacteria for cleaning up toxic waste



Protein dissolves blood clots in heart attack therapy

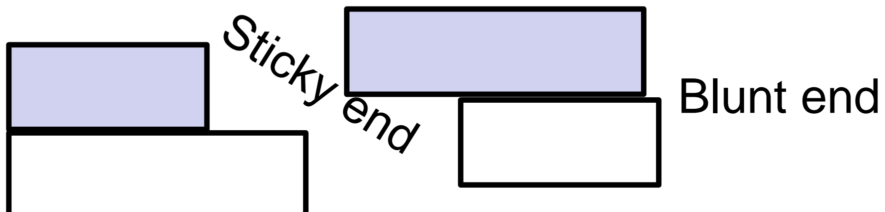
# How to Make Recombinant DNA: Restriction Enzymes

(1) Bacterial **restriction enzymes** -- cut DNA molecules at a limited number of specific DNA sequences (4-8 nucleotides), called **restriction sites** – G|AATTC (EcoRI),



# Using Restriction Enzymes to Make Recombinant DNA

- The most useful restriction enzymes cut DNA in a staggered way, producing fragments with "**sticky ends**" that bond with complementary sticky ends of other fragments




PLAY Animation: Restriction Enzymes

# DNA ligase

- **DNA ligase** is an enzyme that seals the bonds between restriction fragments

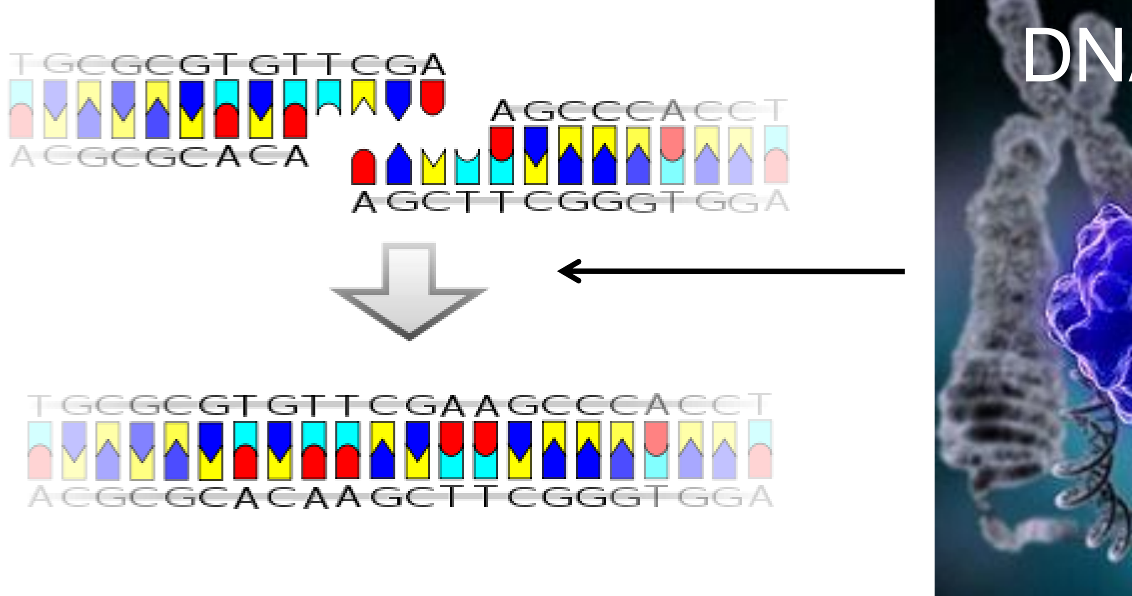


Figure 19.6a

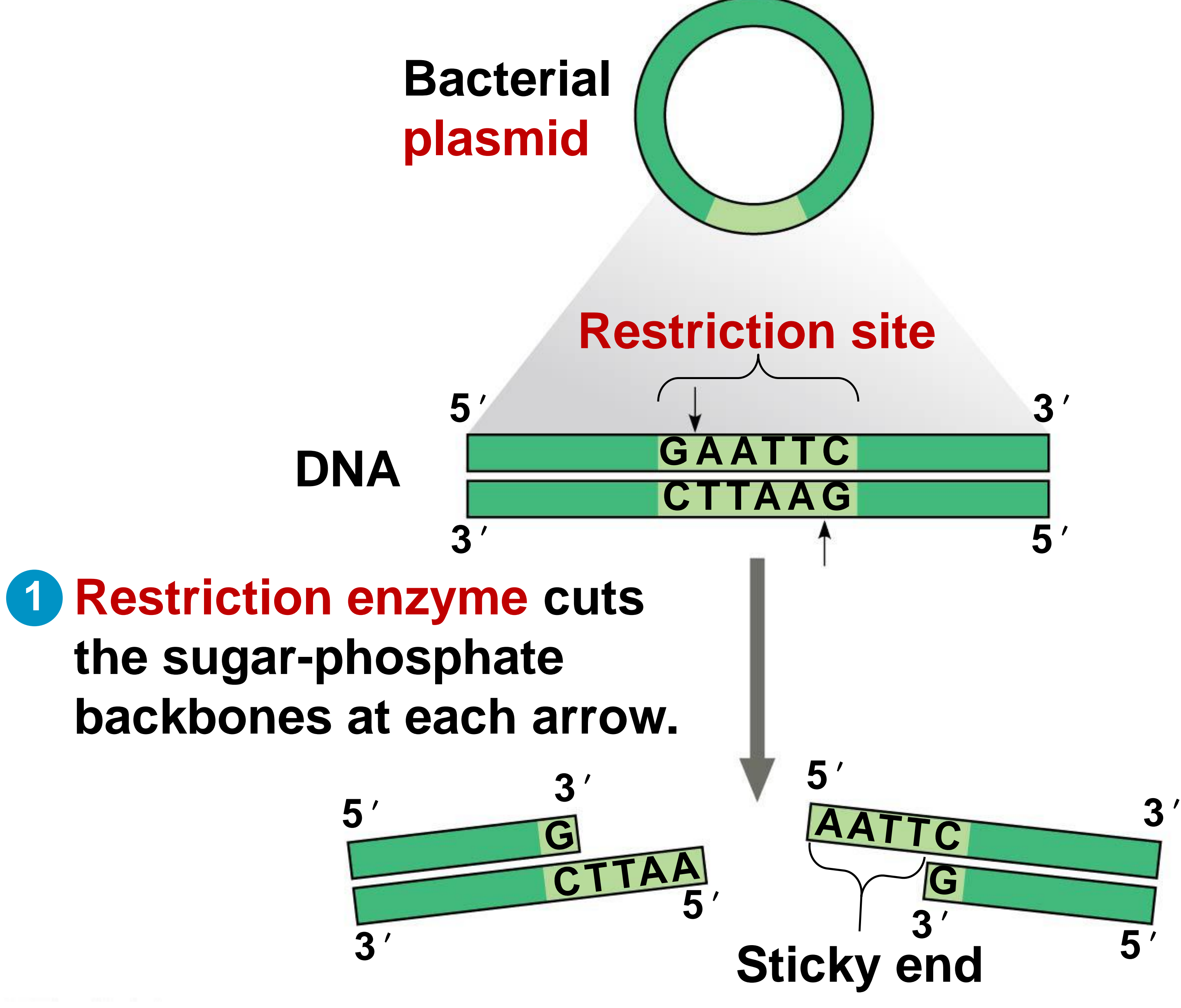


Figure 19.6b

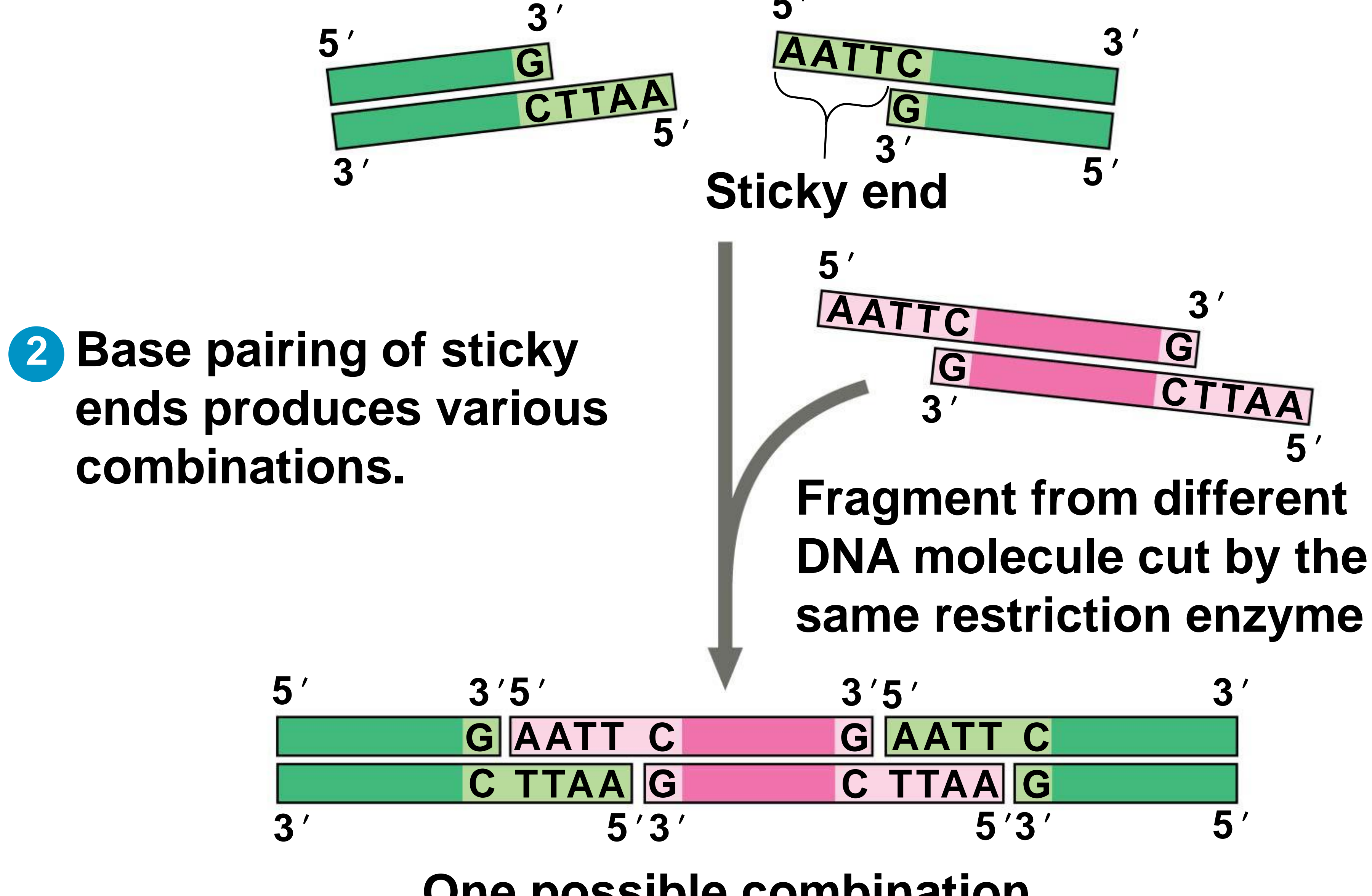
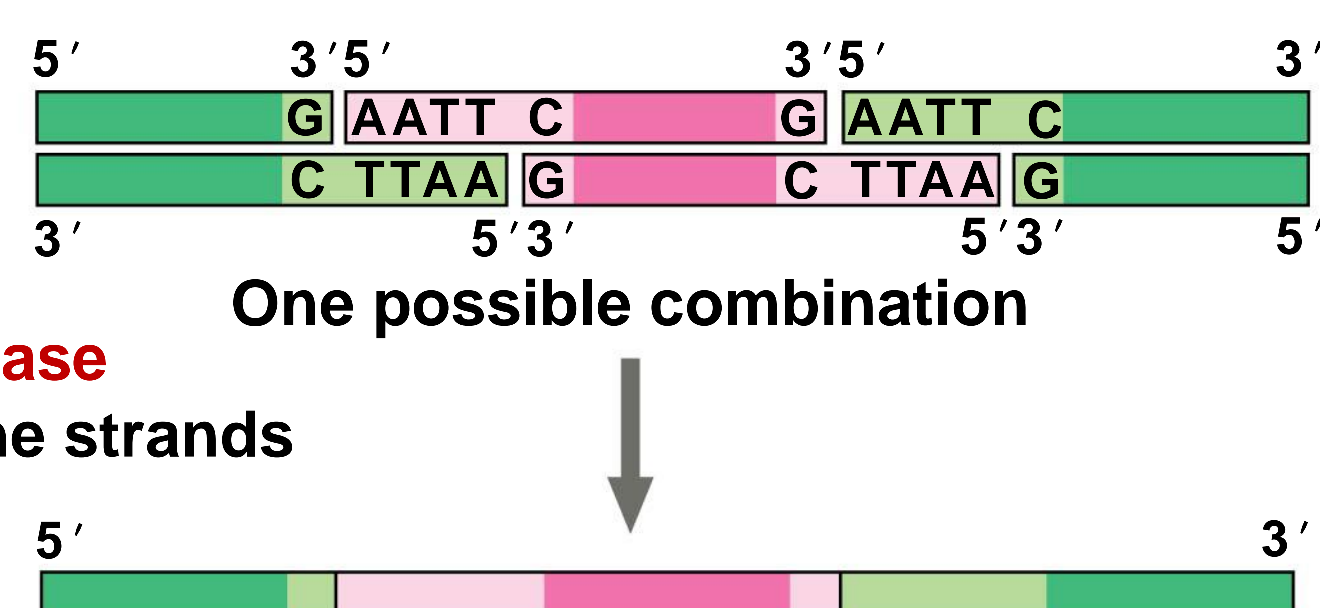
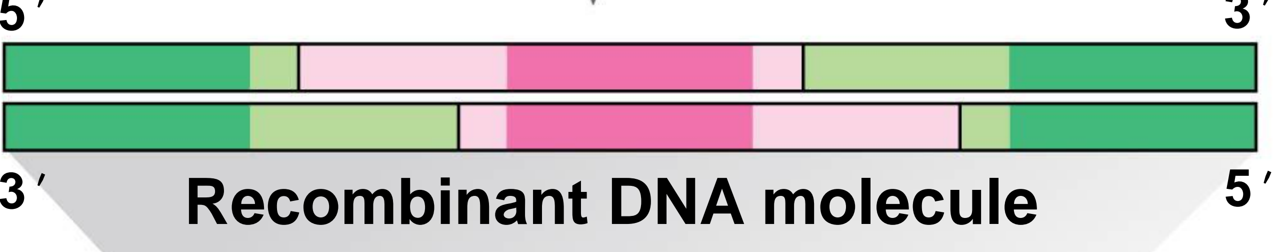
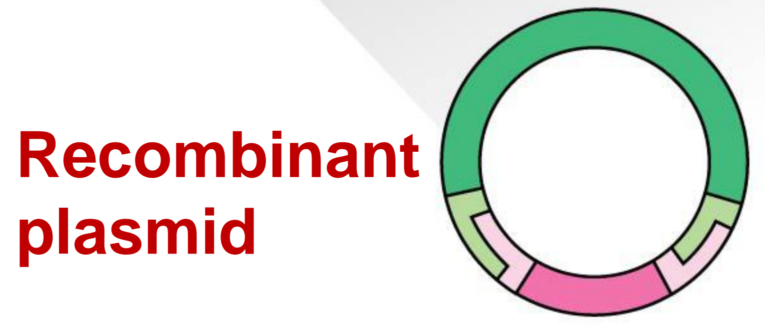


Figure 19.6c




3 **DNA ligase** seals the strands



# DNA assay: DNA Gel Electrophoresis 膠體電泳

- One indirect method of rapidly analyzing and comparing DNA molecules is **gel electrophoresis**
  - This technique uses a gel as a molecular sieve (分子篩) to separate nucleic acids or proteins by size
  - A electrical current is applied that causes charged molecules to move through the gel
  - Molecules are sorted into **"bands" by their size**

Fig. 20-9a

**TECHNIQUE**

# Gel electrophoresis

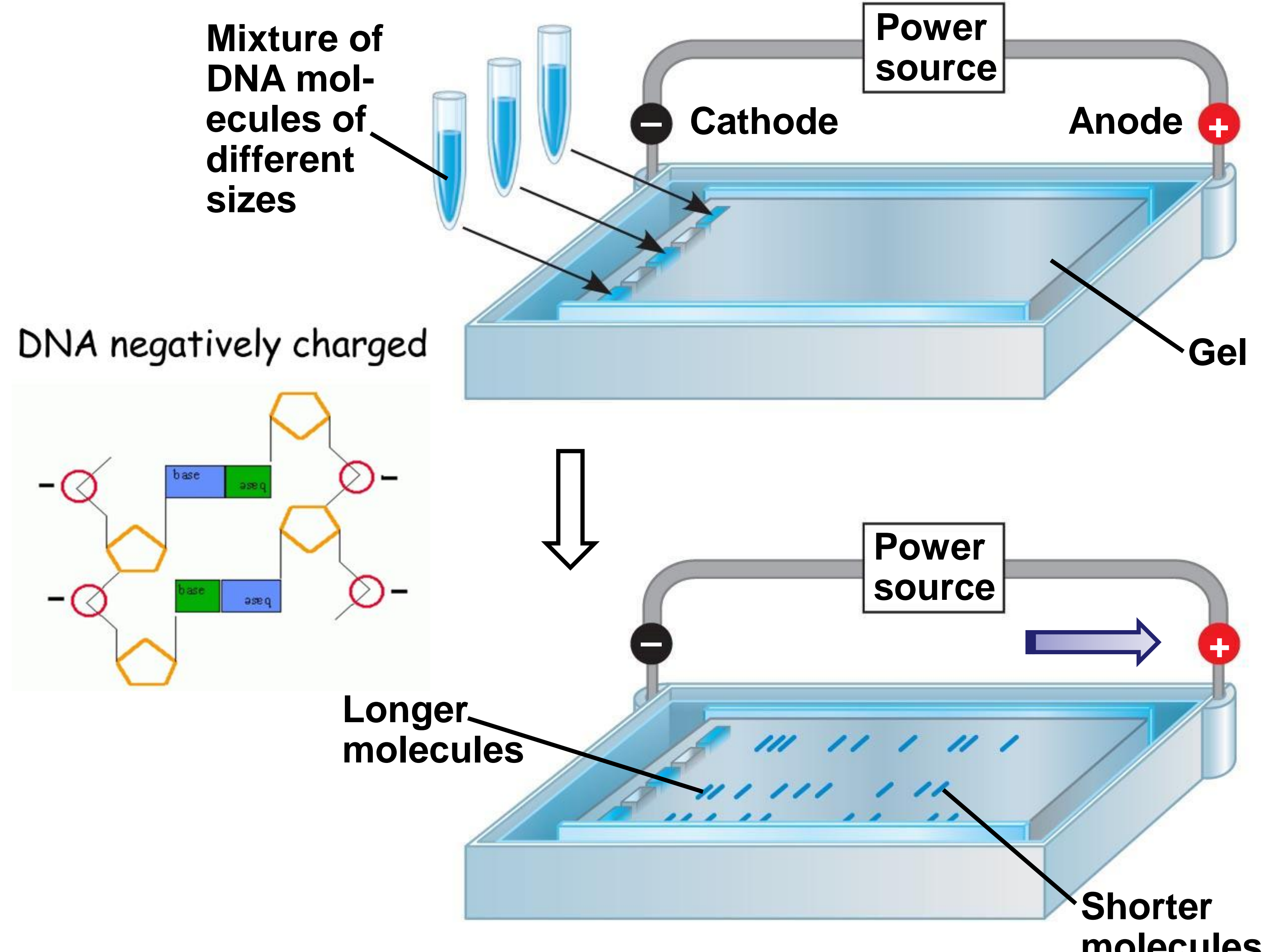
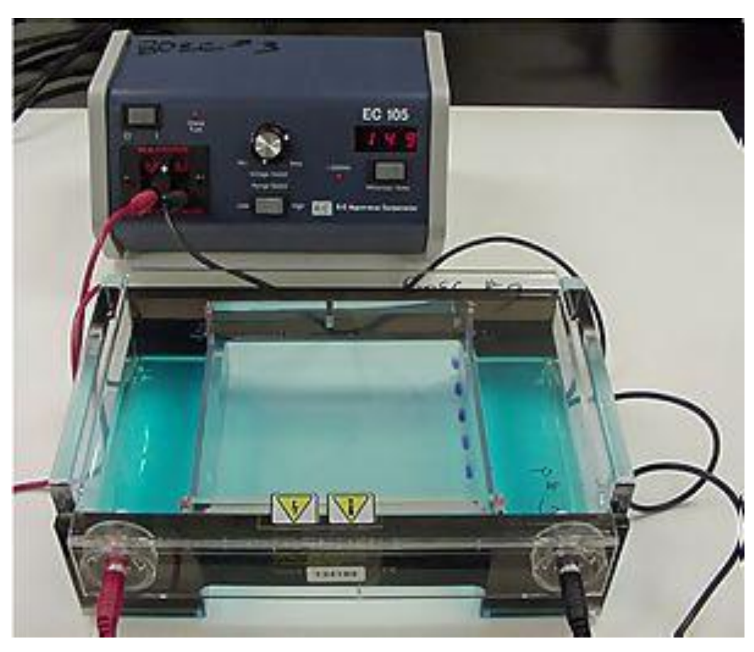
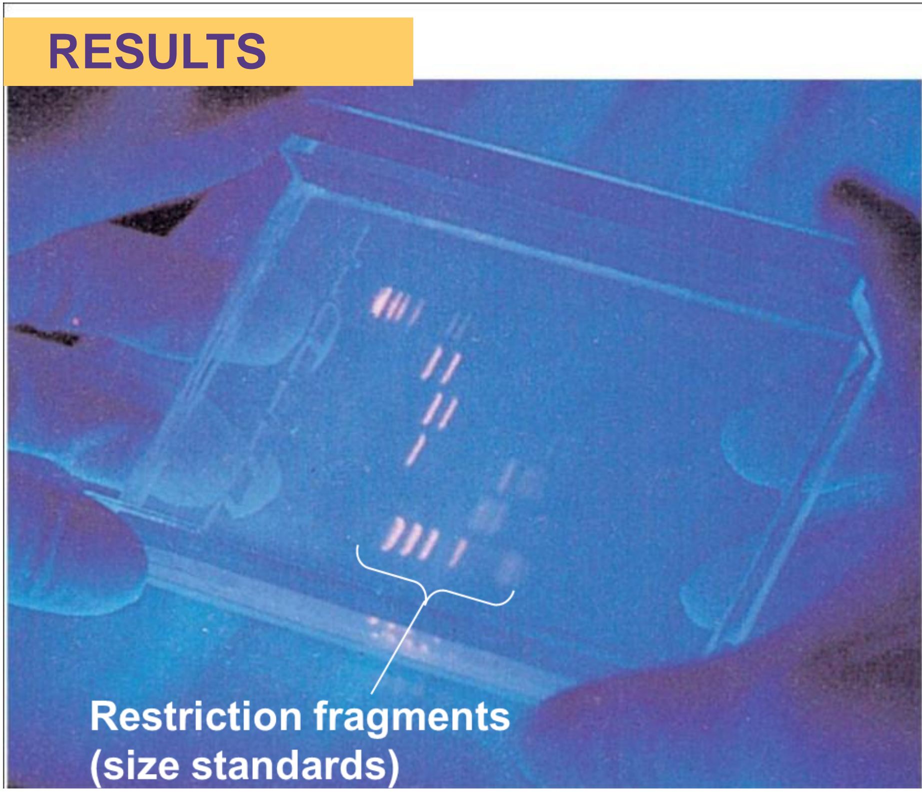
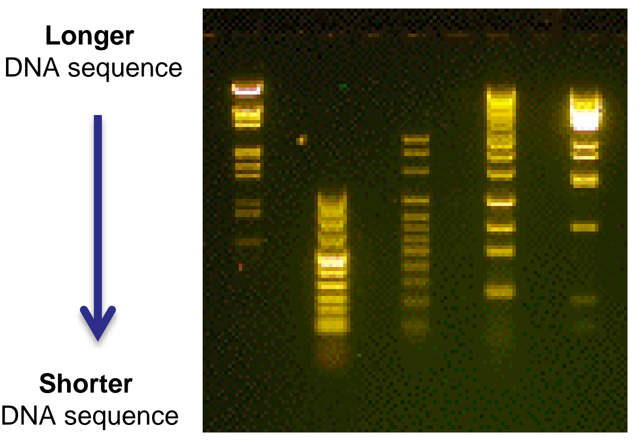


Fig. 20-9b

# Gel electrophoresis




Visualization by **EtBr staining (old method)**

**Ethidium Bromide** is now be replaced by safer fluorescent staining reagents, such SYBR gold.

# Amplifying DNA *in Vitro*: The Polymerase Chain Reaction (PCR)

- The **polymerase chain reaction, PCR**, can produce many copies of a specific target segment of DNA
- A three-step cycle — heating, cooling, and replication — brings about a chain reaction that produces an exponentially growing population of identical DNA molecules

# Significance of PCR in artificial gene replication

With **polymerase chain reaction (PCR)**, any specific segment of the target sequence within a DNA sample can be copied many times (amplified) completely *in vitro*.

**Key components in a PCR reaction:**

--Uses **primers** (with unique restriction sites to allow the product to be cloned into plasmid vectors) that bracket the desired sequence

--Uses a **heat-resistant DNA polymerase** (active at high temperature) isolated from prokaryotes living in hot spring: extend primers in 5’ $\longrightarrow$ 3’ direction

Fig.19-8

**TECHNIQUE**

Genomic DNA

5′ 3′

Target sequence

3′ 5′

Cycle 1 yields 2 molecules

1 Denaturation

5′ 3′

3′ 5′

2 Annealing

Primers

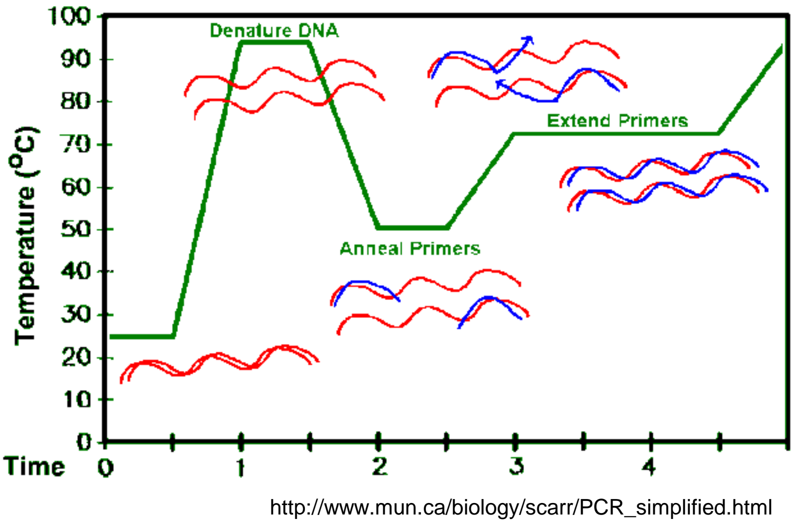
3 Extension

New nucleo- tides

Cycle 2 yields 4 molecules

Cycle 3 yields 8 molecules; 2 molecules (in white boxes) match target sequence

# Only tiny amount of DNA template (target sequence) is required



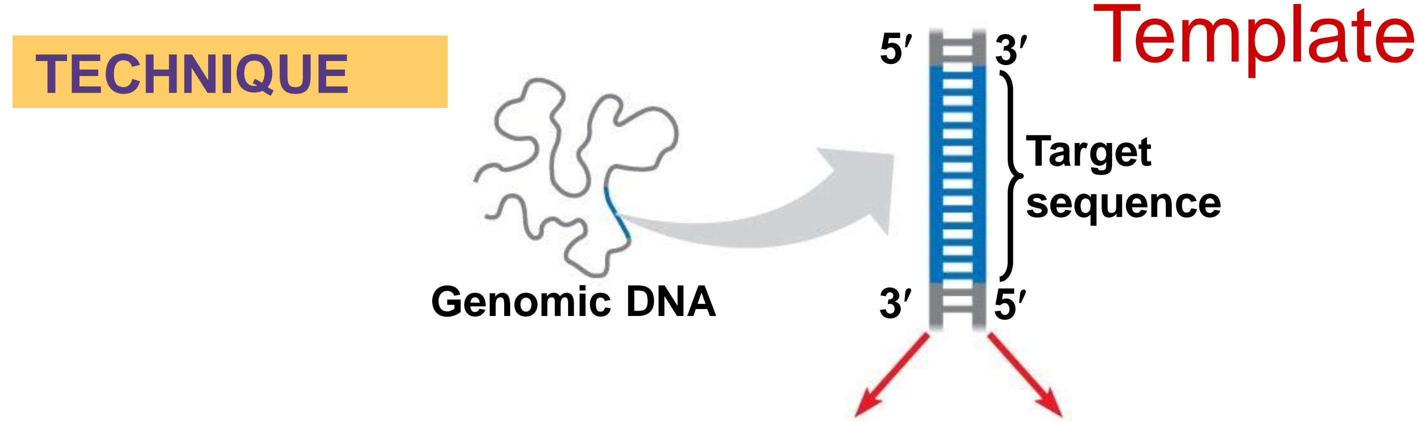

http://www.mun.ca/biology/scarr/PCR_simplified.html

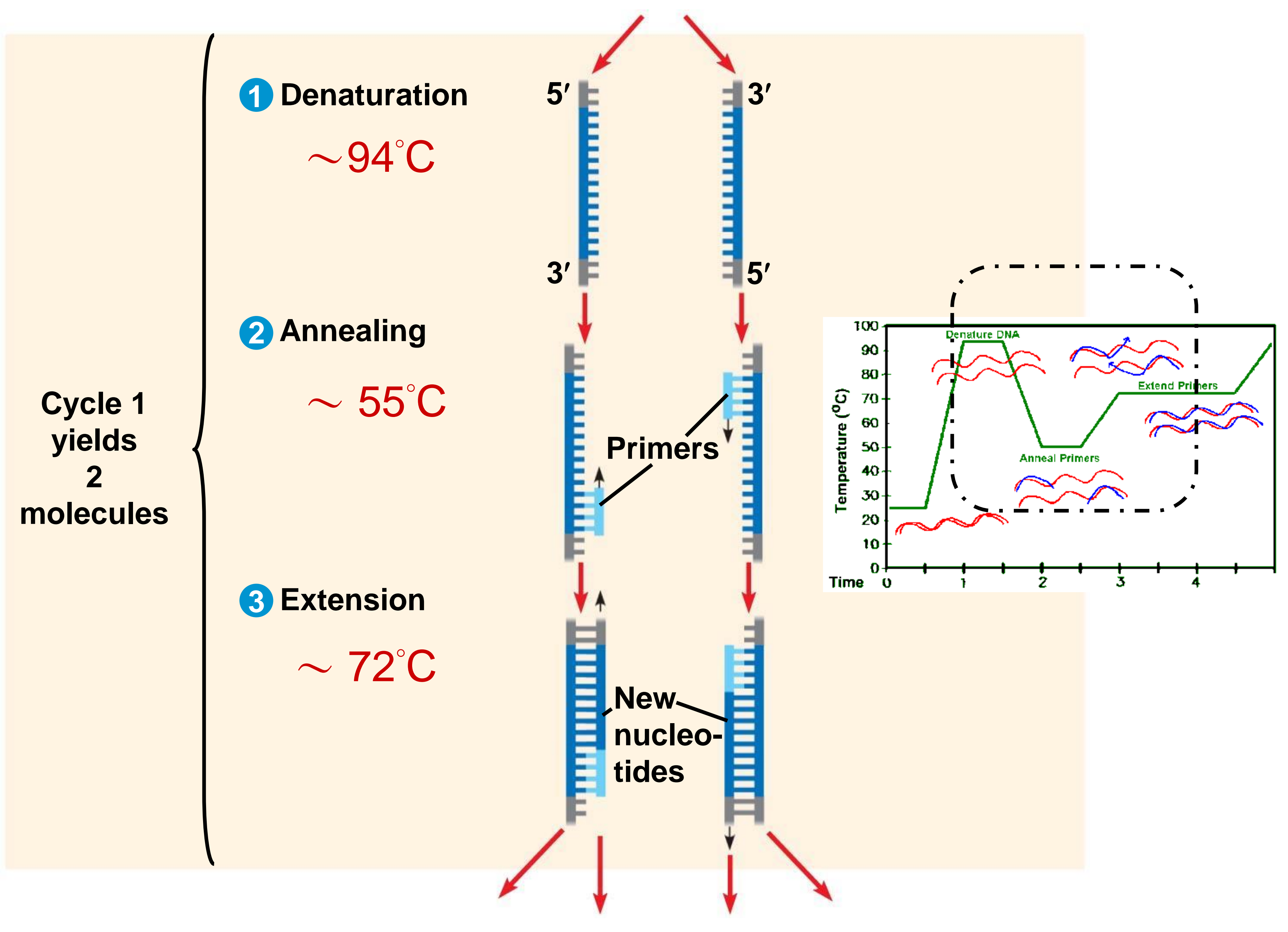
**Nobel prize in chemistry 1993**

Kary B. Mulis

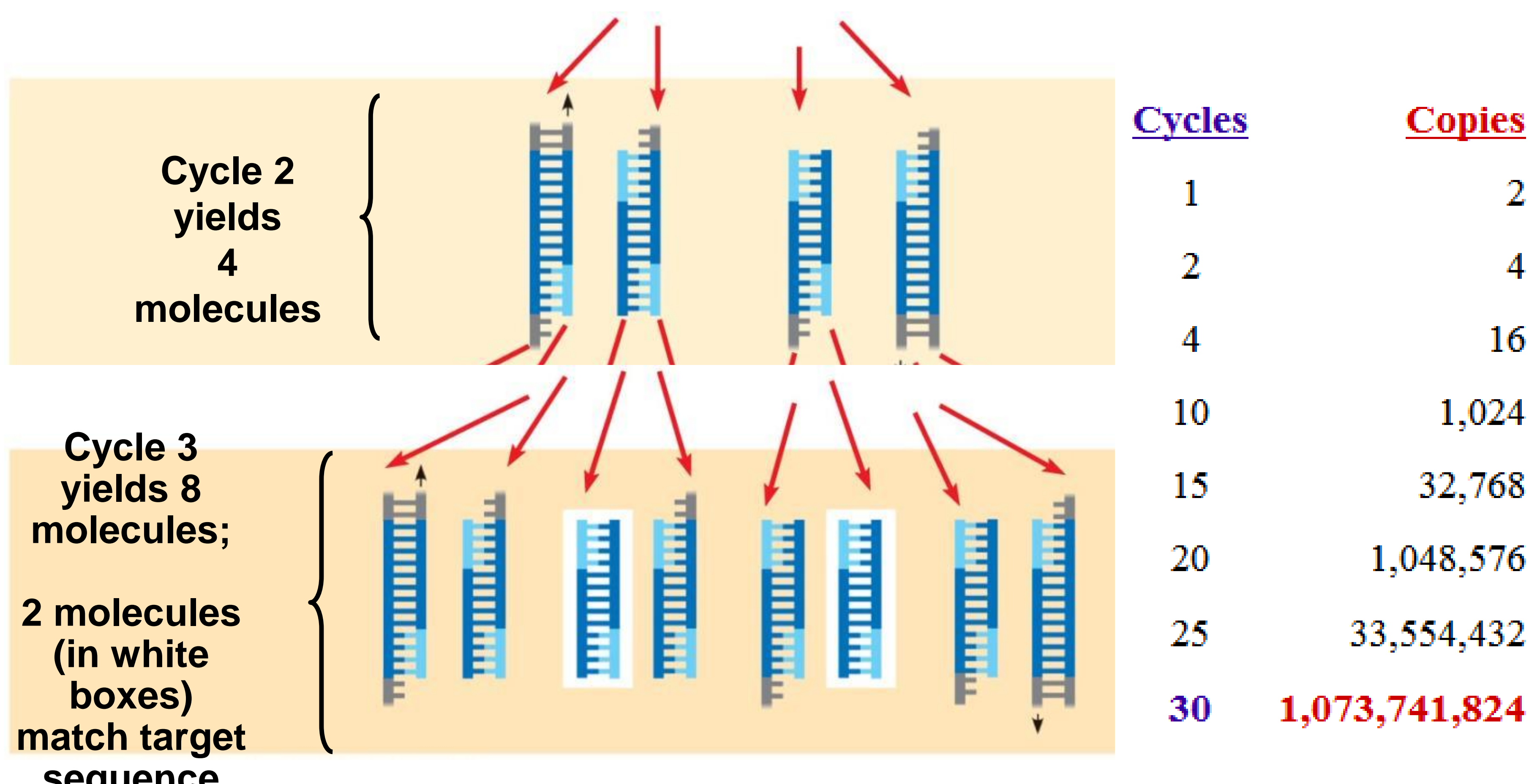
*“ for his invention of the polymerase chain reaction (PCR) method”*

# The polymerase chain reaction (PCR)





Cycle 1
yields
2
molecules
1 Denaturation
~94°C
5′
3′
3′
5′
2 Annealing
~ 55°C
Primers
3 Extension
~ 72°C
New
nucleo-
tides
100
90
80
70
60
50
40
30
20
10
0
Temperature (°C)
Time
0
1
2
3
4
Denature DNA
Extend Primers
Anneal Primers




# The polymerase chain reaction (PCR)

to generate a large number of copies of specific DNA sequence

# PCR through the ages

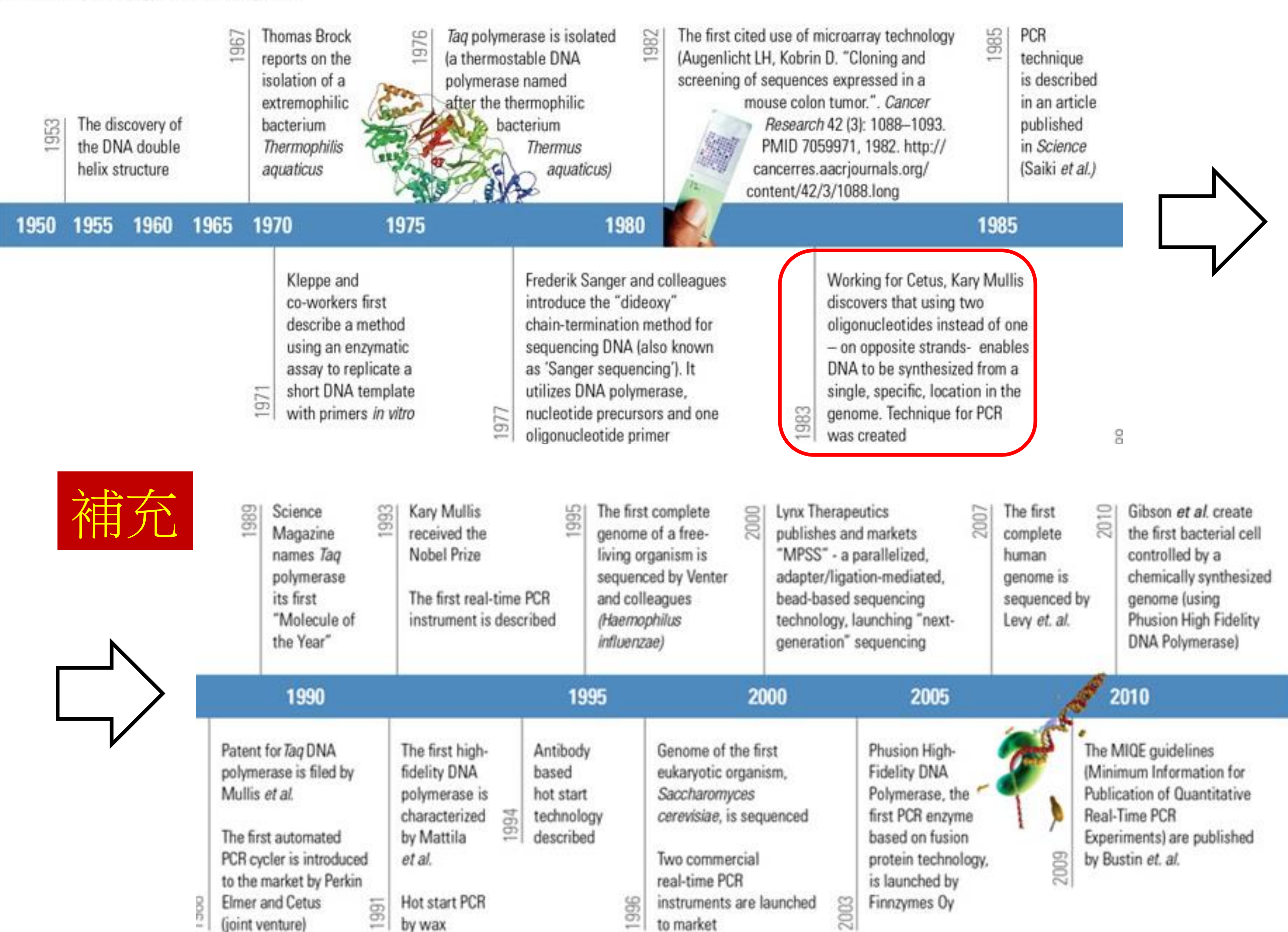


Figure 19.9

# Gene cloning

**DNA fragments obtained by PCR with restriction sites matching those in the cloning vector**

**Cut with same restriction enzyme used on cloning vector**

**A gene that makes bacterial cells resistant to an antibiotic is present on the plasmid.**

**Cloning vector (bacterial plasmid)**

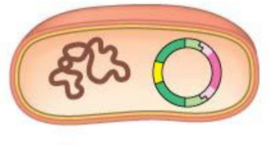
**Mix and ligate**

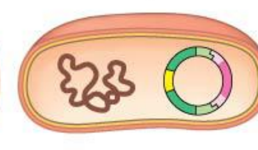
**Recombinant DNA plasmid**

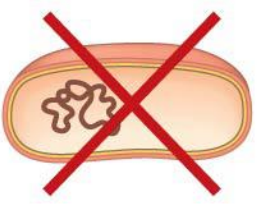
(with acquired resistance to antibiotic)

(selection by antibiotic)

**Only cells that take up a plasmid will survive**





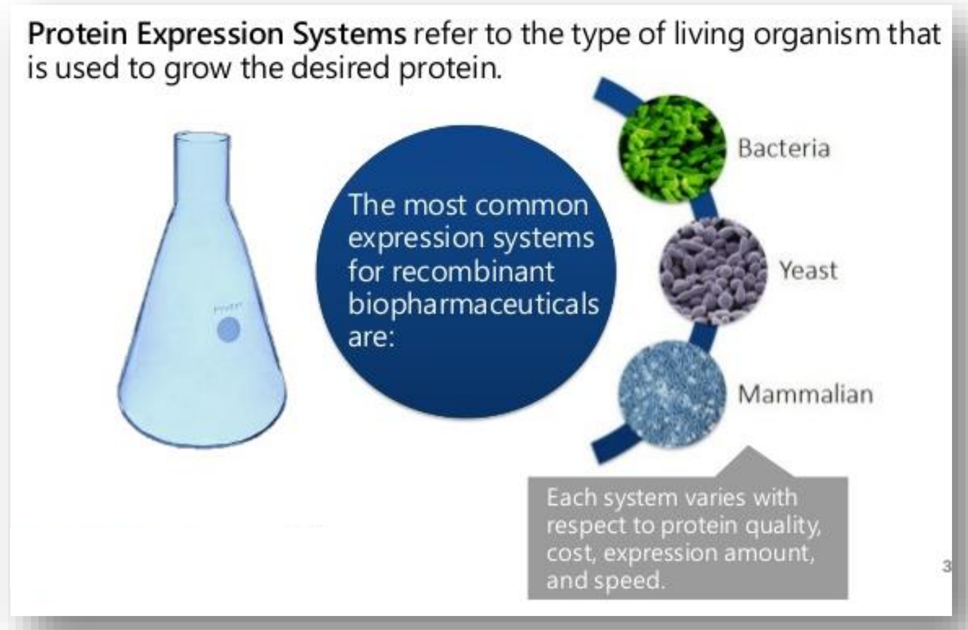


## DNA technology allows us to study the sequence, expression, and function of a gene

- DNA cloning allows researchers to
  - **Compare genes and alleles between individuals** (in term of sequence)
  - Locate **gene expression** in a body
  - Determine **the role of a gene** in an organism

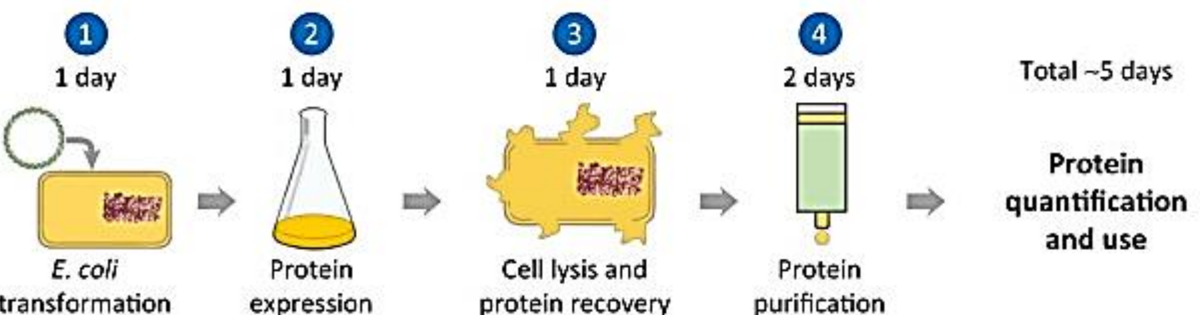
# Expressing Cloned Eukaryotic Genes

- After a gene has been cloned, its **protein product** can be produced in larger amounts for research
- Cloned genes can be expressed as protein in either bacterial or eukaryotic cells



# *Bacterial Expression Systems*

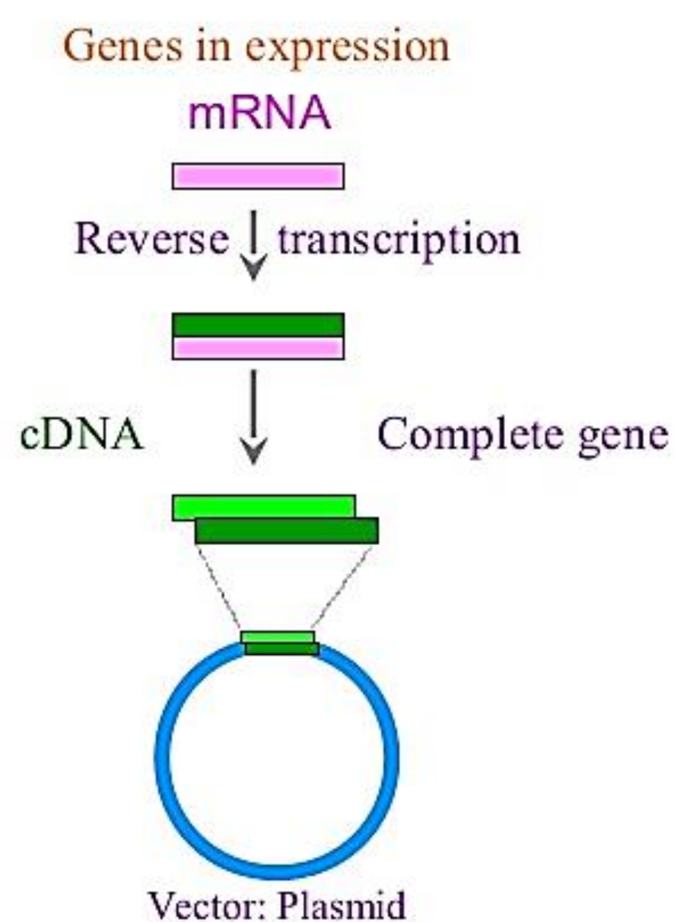
- Several technical difficulties hinder expression of cloned eukaryotic genes in bacterial host cells, such as no expression.
- To overcome differences in promoters and other DNA control sequences, scientists usually employ an **expression vector**, a cloning vector that contains a highly active bacterial promoter



# *Bacterial Expression Systems*

- Most popular methods to deliver plamids into *E.coli* – **heat shock** (42℃ for 45 seconds)
  - **Technical difficulties** hinder expression of cloned eukaryotic genes in bacterial host cells: no expression, **not soluble, not proper folded**, etc.

- Another difficulty with eukaryotic gene expression in bacteria is the presence of introns in most eukaryotic genes
- Researchers can avoid this problem by using **cDNA**, complementary to the mRNA, which contains only exons




*Read more on RT-PCR*

## *Eukaryotic DNA Cloning and Expression Systems*

- Molecular biologists can avoid eukaryote-bacterial incompatibility issues by using eukaryotic cells, such as yeasts, as hosts for cloning and expressing genes
- Even yeasts may not possess the proteins required to modify expressed mammalian proteins properly; which means **the post-translational modifications that many proteins require**, i.e. glyco-/lipo-proteins
- In such cases, cultured mammalian or insect cells may be used to express and study proteins

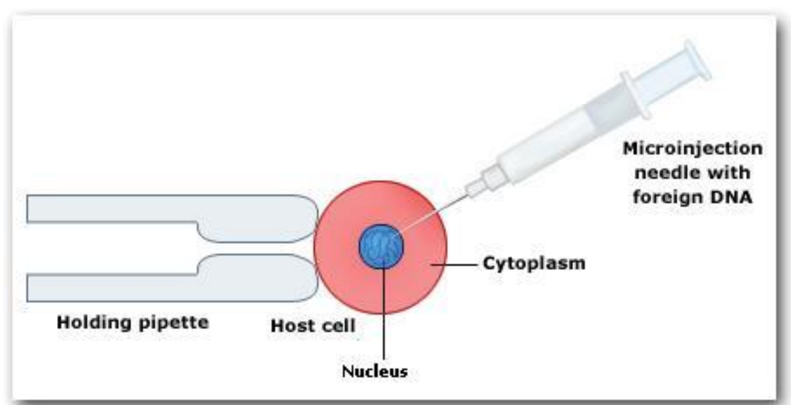
# Delivery gene/plasmid into the eukaryotic cells

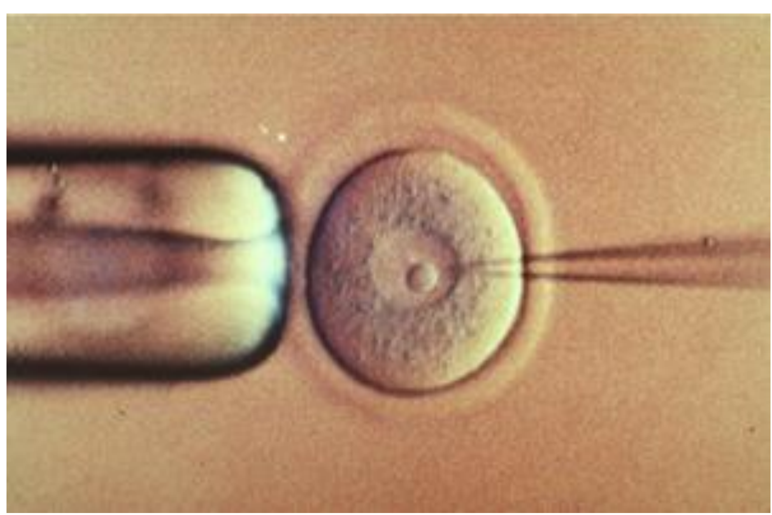
- One method of introducing recombinant DNA into eukaryotic cells is **electroporation**, applying a **brief electrical pulse** to create temporary holes in plasma membranes

- Alternatively, scientists can inject DNA into cells using microscopically thin needles (**microinjection**)

- Once inside the cell, the DNA is incorporated into the cell's DNA by natural genetic recombination

# Microinjection 顯微注射 for DNA Transfer



DNA microinjection

Microinjector

## *Cross-Species Gene Expression and Evolutionary Ancestry*

- The remarkable ability of bacteria to express some eukaryotic proteins underscores the shared evolutionary ancestry of living species
- For example, *Pax-6* is a gene that directs formation of a vertebrate eye; the same gene in flies directs the formation of an insect eye (which is quite different from the vertebrate eye)
- **The *Pax-6* genes in flies and vertebrates can substitute for each other**

# Analyzing Gene Expression

- ***In situ* hybridization** uses fluorescent dyes attached to probes to identify the location of specific mRNAs in place in the intact organism
  - *In situ* 在原位 hybridization (ISH) 偵測完整個體**上標的基因**的真實表現狀況

    Probes can be used to identify **where or when** a gene is transcribed in an organism

Figure 19.10a

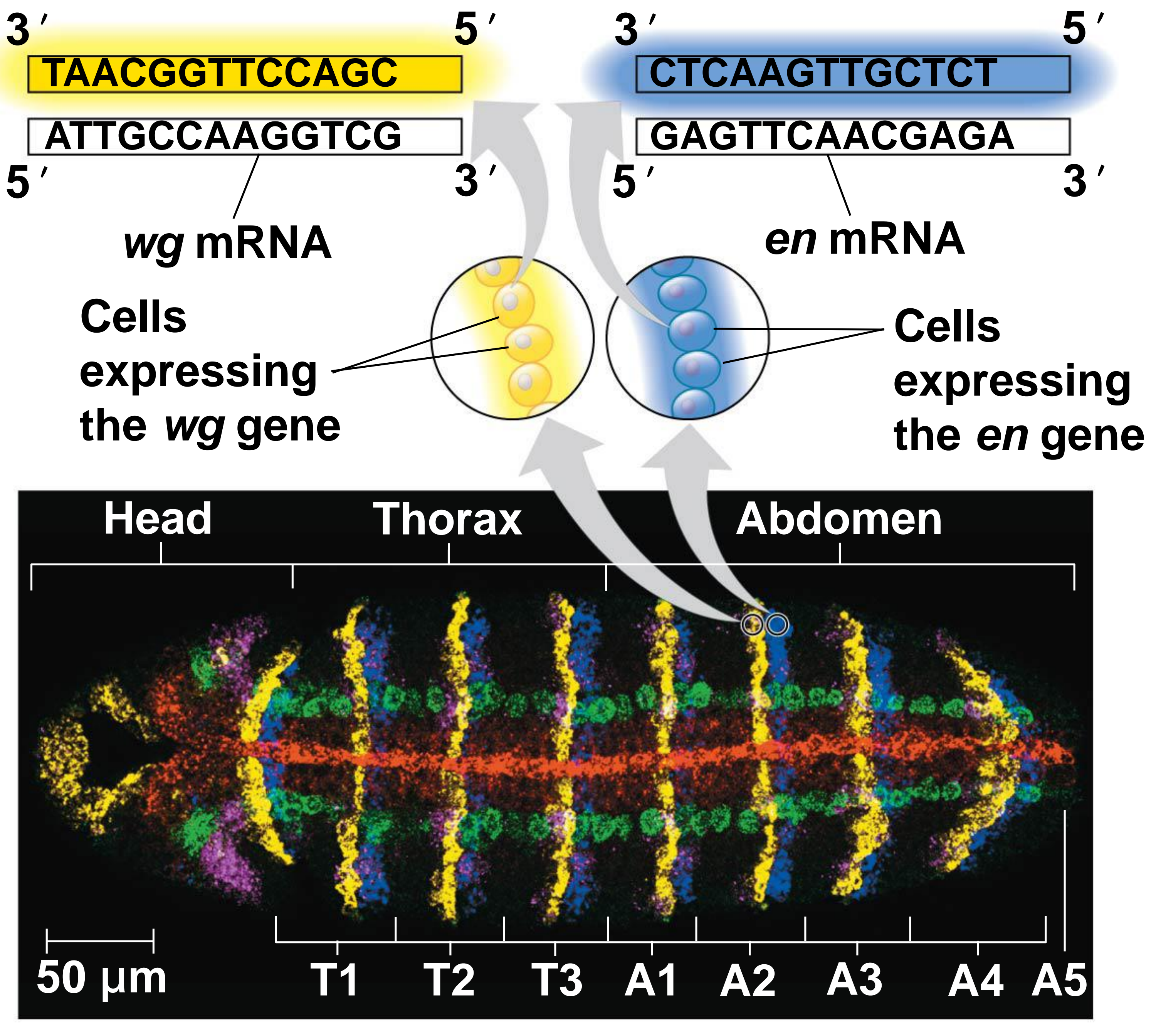
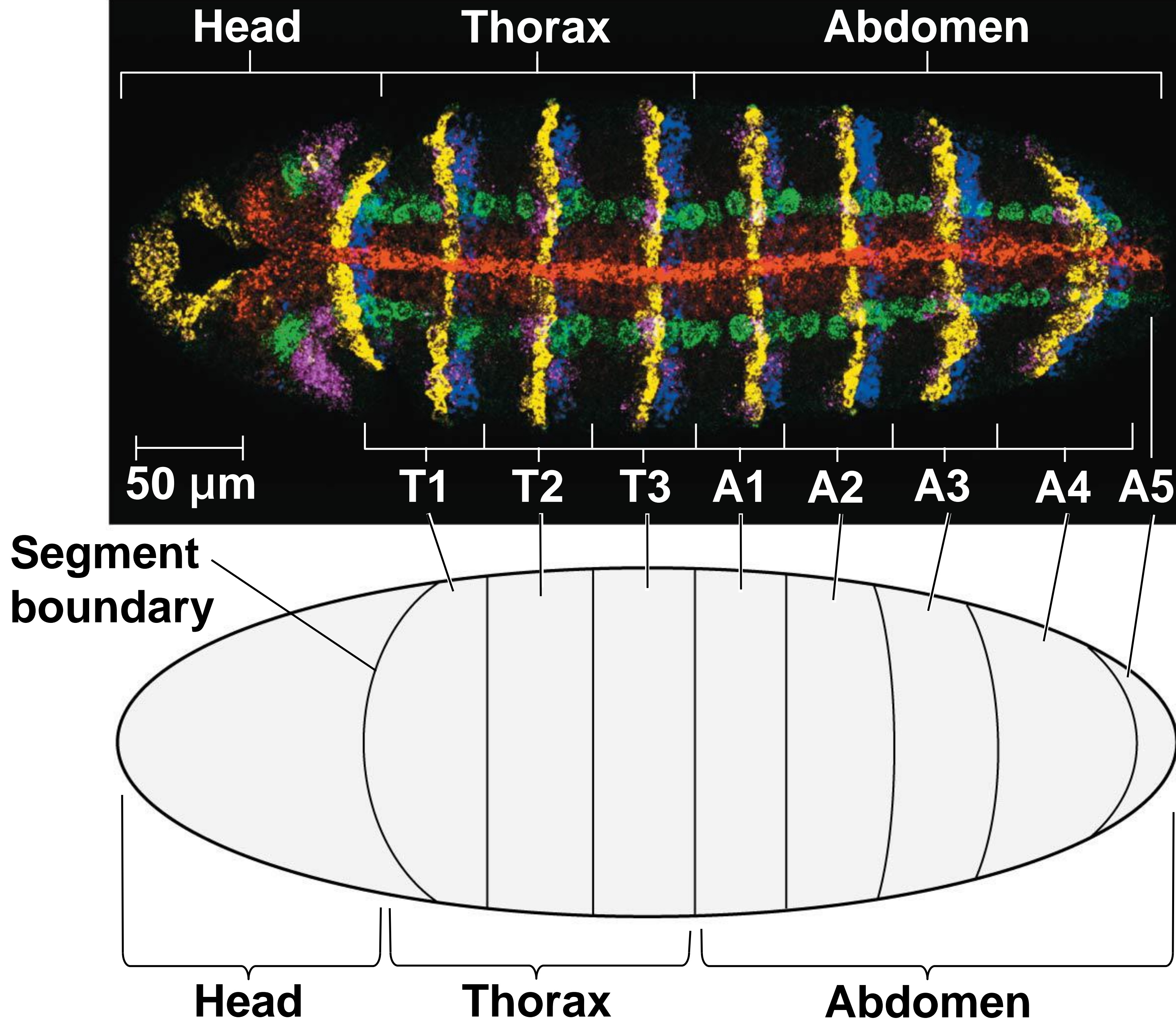


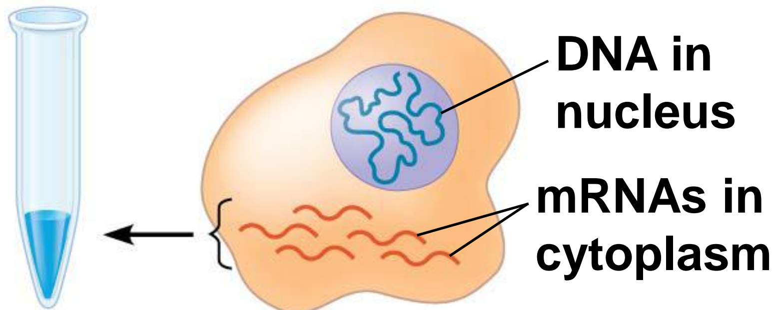
Figure 19.10b



## Reverse transcriptase-polymerase chain reaction (RT-PCR)

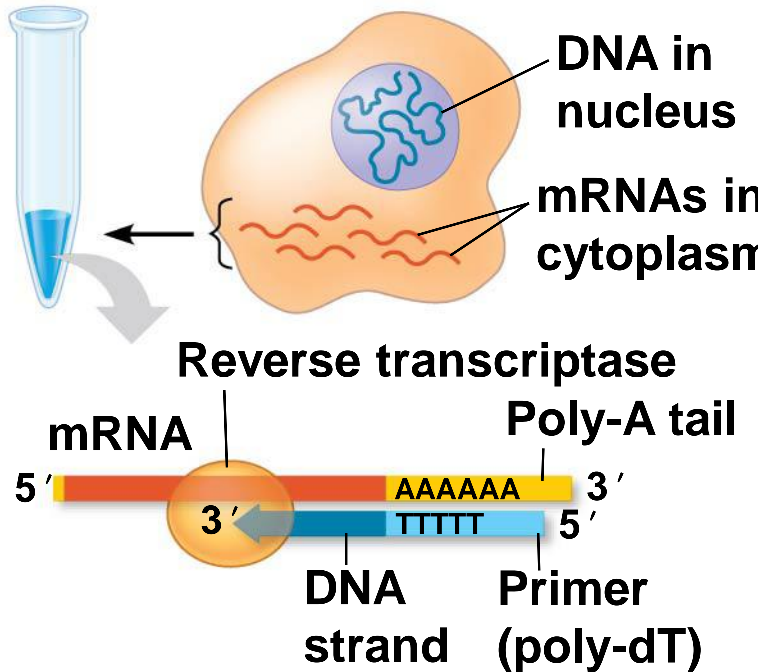
- **Reverse transcriptase-polymerase chain reaction** (**RT-PCR**) is useful for comparing amounts of specific mRNAs in several samples at the same time
- Reverse transcriptase is added to **mRNA** to make **complementary DNA (cDNA)**, which serves as a template for PCR amplification of the gene of interest
- The products are run on a gel and the mRNA of interest is identified

Figure 19.11-1

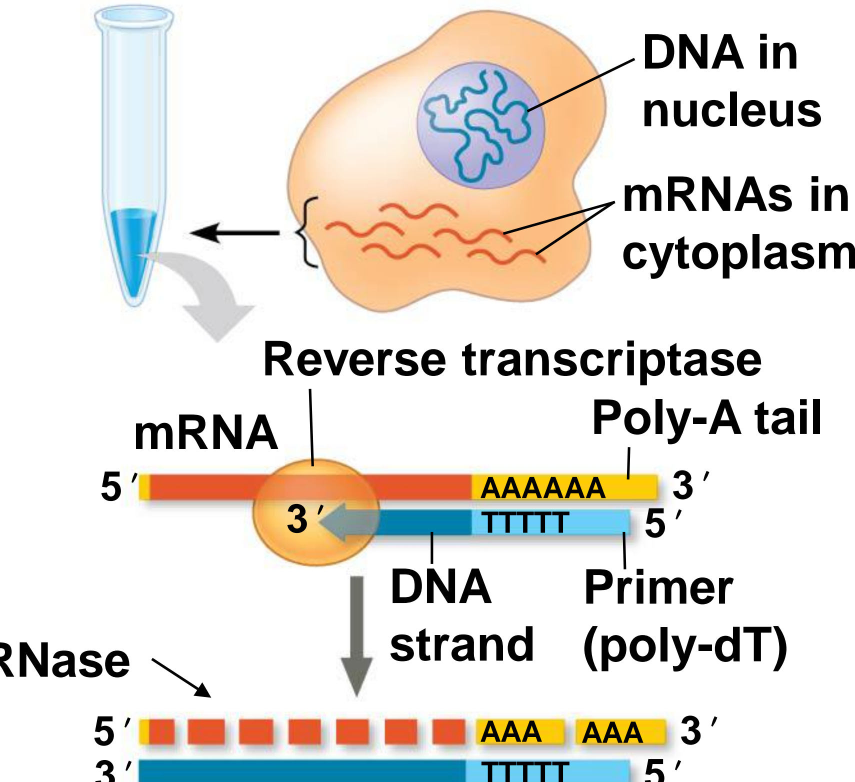



Making complementary DNA (cDNA)

Figure 19.11-2

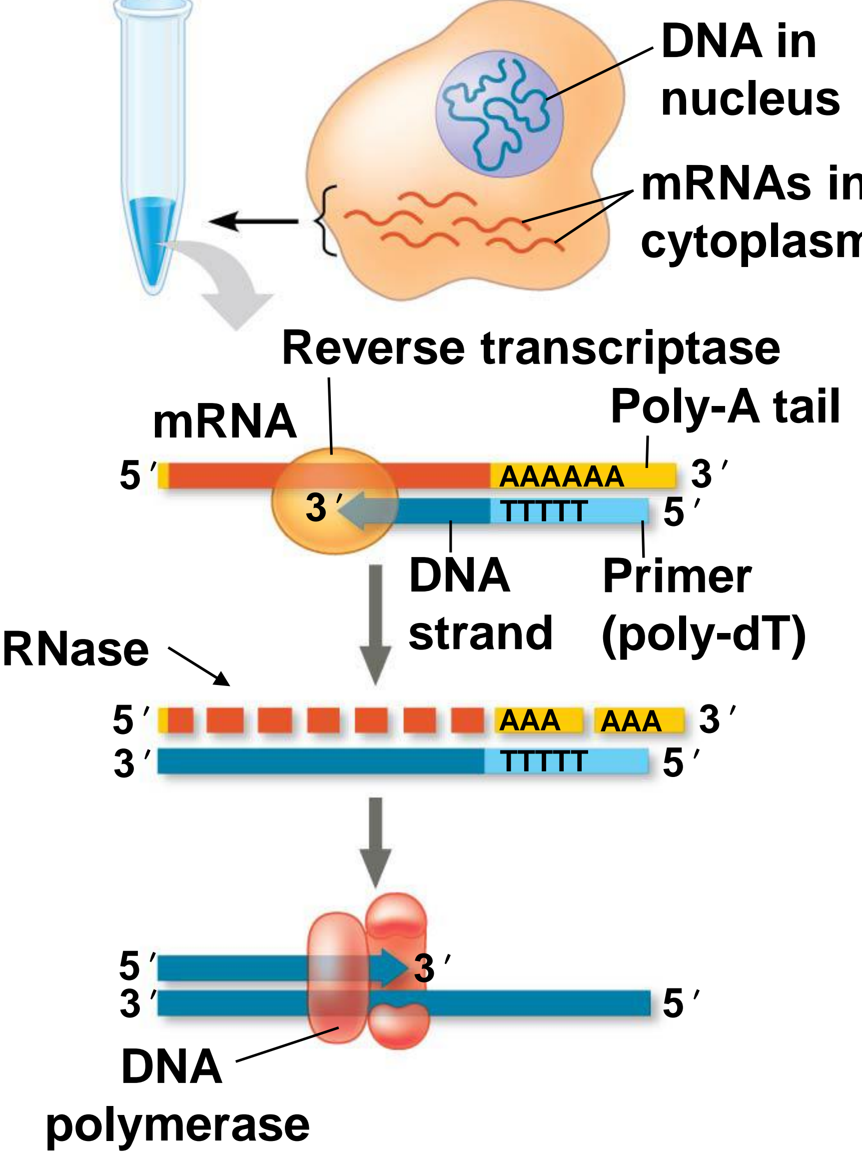



Making complementary DNA (cDNA)



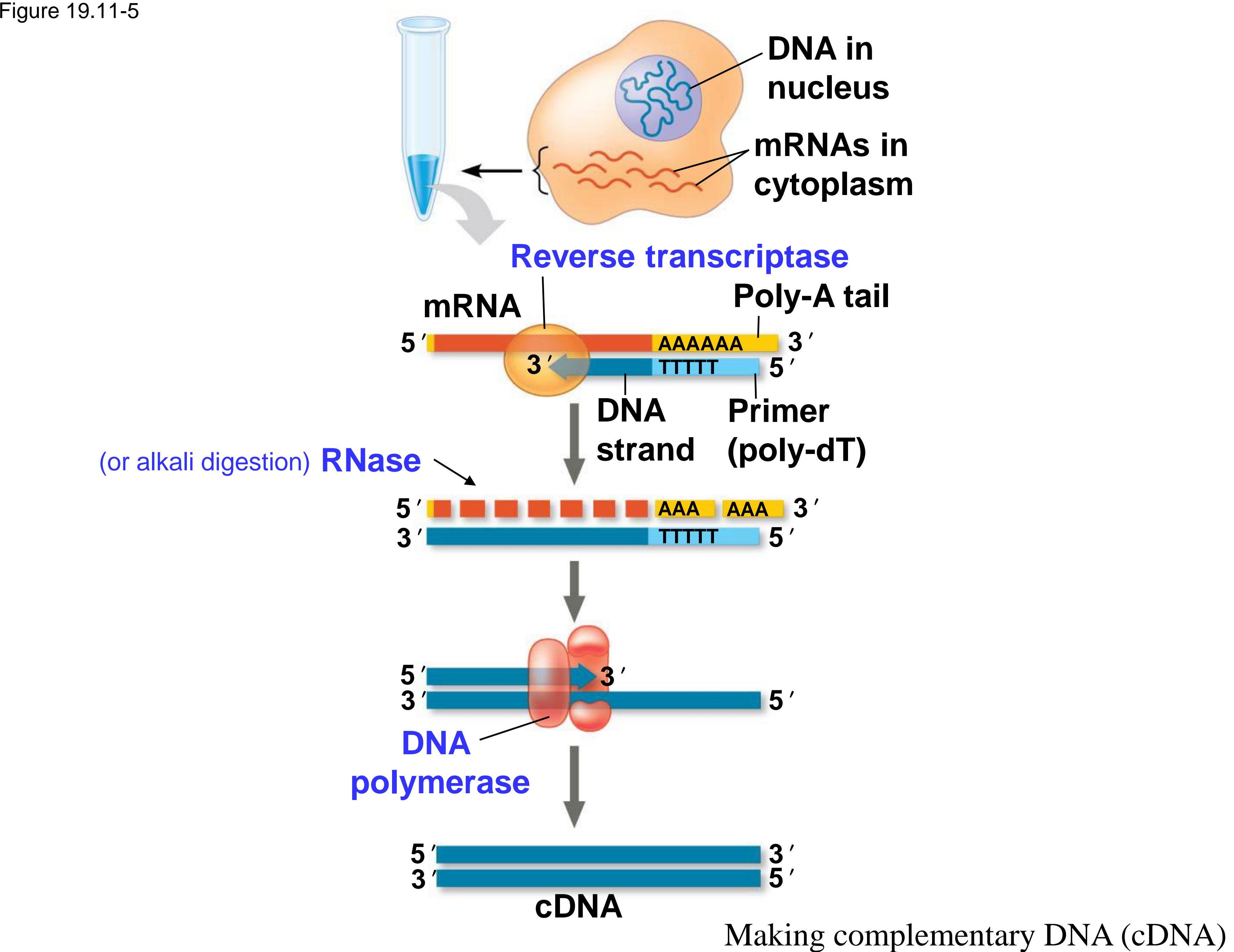

Making complementary DNA (cDNA)

Figure 19.11-4



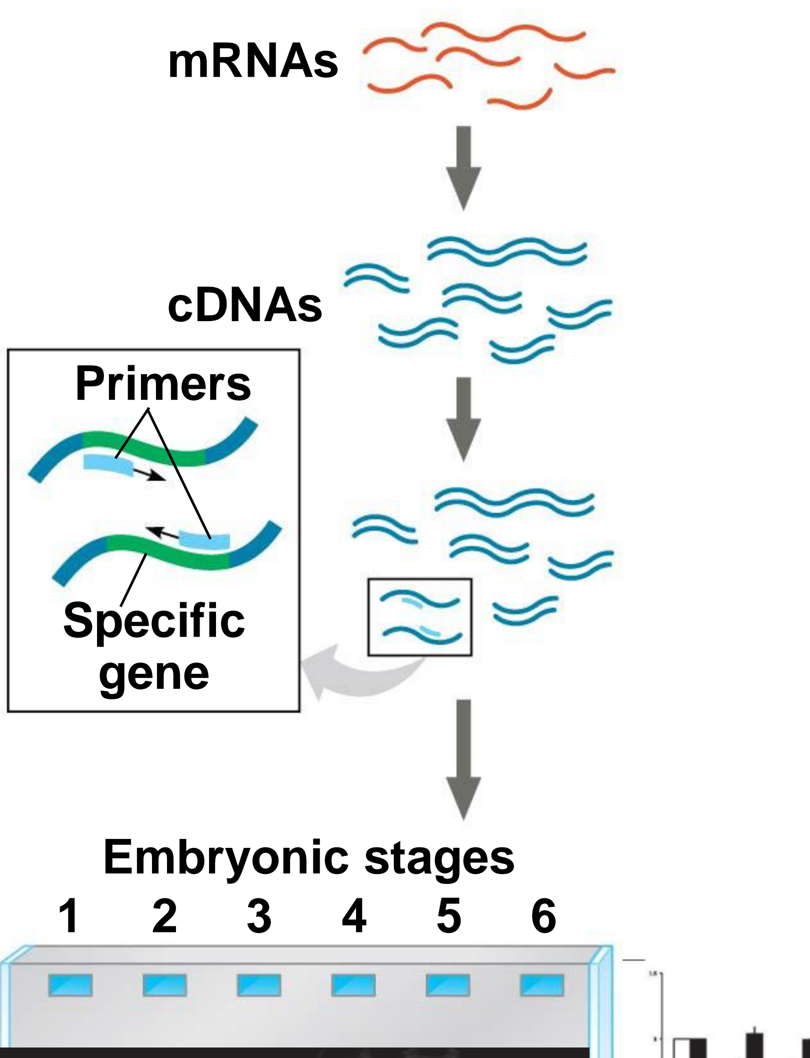

Making complementary DNA (cDNA)

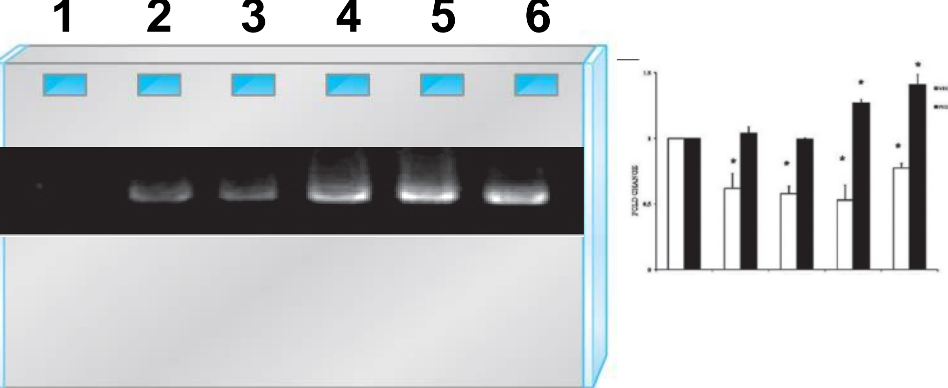
Figure 19.11-5




Making complementary DNA (cDNA)

Figure 19.12




RT-PCR analysis of the expression of genes

# Studying the Expression of Interacting Groups of Genes

- **Automation自動化** has allowed scientists to measure the expression of thousands of genes at one time using DNA microarray assays
- **DNA microarray assays** 微陣列檢定compare patterns of gene expression in different tissues, at different times, or under different conditions

Figure 19.13

Each dot is a well containing identical copies of DNA fragments that carry a specific gene.

Genes expressed in first tissue.

Genes expressed in second tissue.

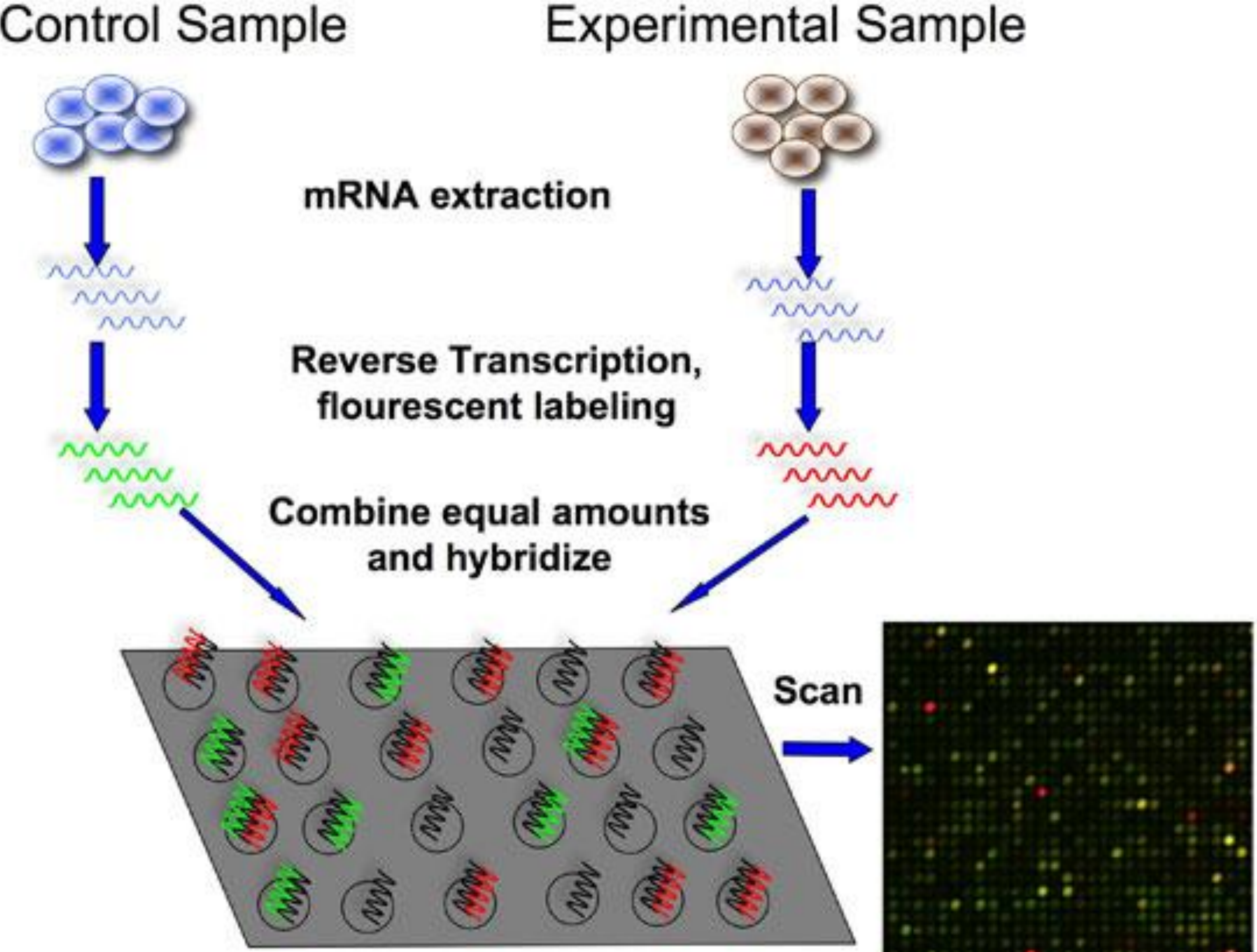
Genes expressed in both tissues.

Genes expressed in neither tissue.

DNA microarray (actual size)

*Protocol next page*

# A typical DNA microarray co-hybridization (2 dye) experiment



# Use of RNA sequencing (RNA-Seq) to analyze expression of many genes

- With rapid and inexpensive sequencing methods, researchers can now just sequence cDNA samples from different tissues or embryonic stages to determine the gene expression differences between them
- This approach is called **RNA sequencing** or **RNA-seq**
- mRNAs are isolated, cut into short, similar-sized fragments, converted into cDNAs, and sequenced

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Figure 19.13

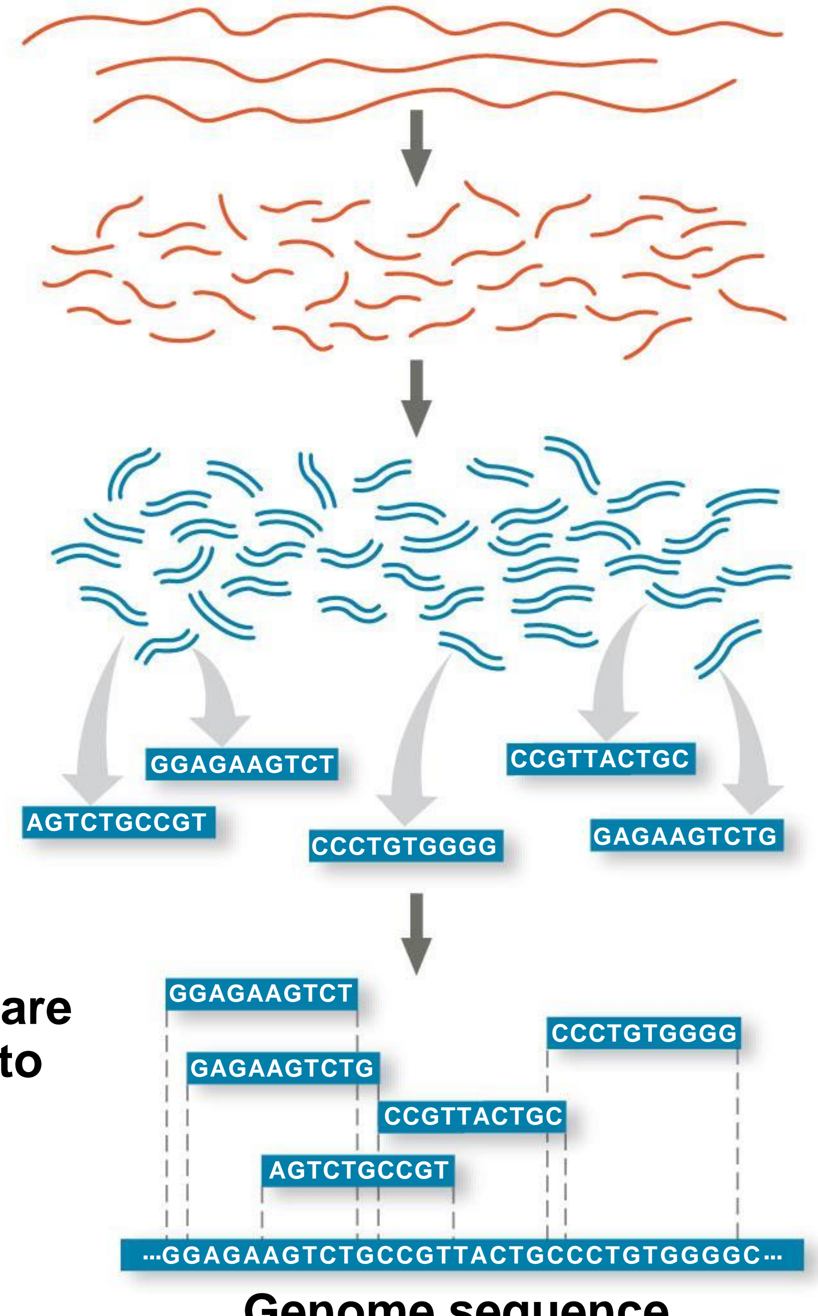
1 mRNAs are isolated from the tissue being studied.

2 mRNAs are cut into similar-sized, small fragments.

3 mRNAs are reverse-transcribed into cDNAs of the same size.

4 cDNAs are sequenced.

5 The short sequences are mapped by computer onto the genome sequence.

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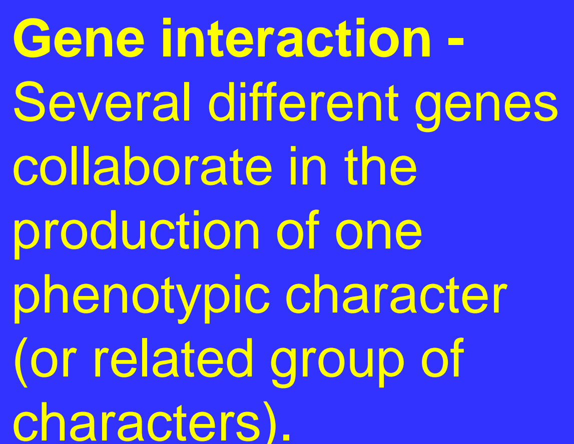
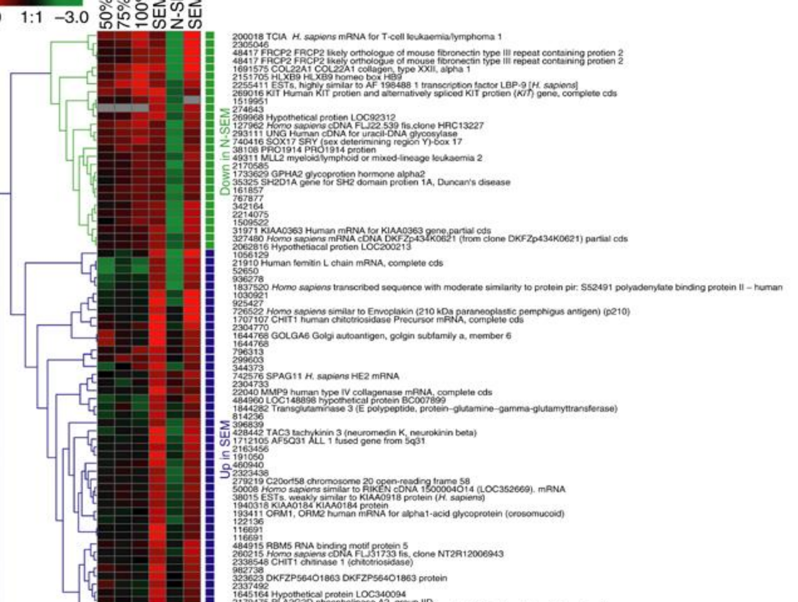
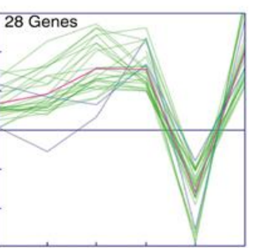
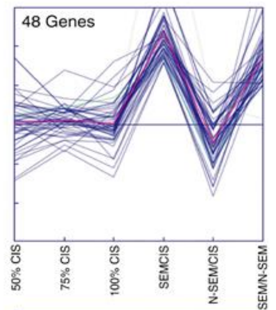
- Technique like RNA-seq enables researchers to determine the gene expression differences between different tissues or embryonic stages
- By uncovering **gene interactions** and **clues to gene function**, gene expression assays may contribute to understanding of disease and suggest new diagnostic targets



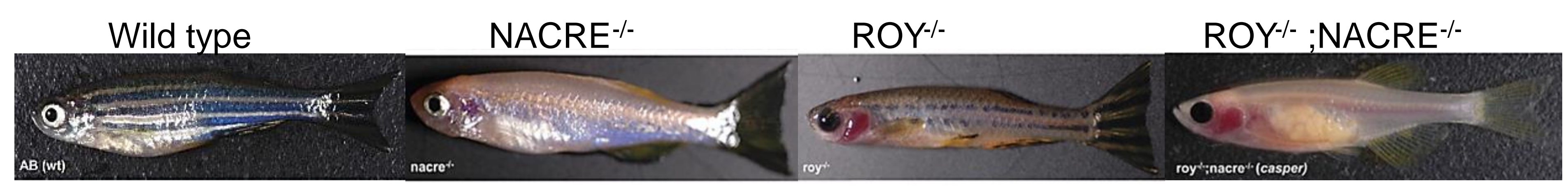




*British Journal of Cancer* (2005) **92**, 1934–1941.

# Example: Determining Gene Function

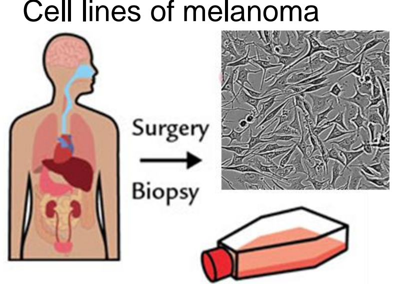
- One way to determine function is to **disable the gene** and observe the consequences – ***loss-of-function*** (vs. ***gain-of-function***)
- How? Using ***in vitro* mutagenesis**,
  - mutations are introduced into a cloned gene, ***altering or destroying* its function**
  - when the mutated gene is returned to the cell, the normal gene's function might be determined by examining the ***mutant's phenotype***

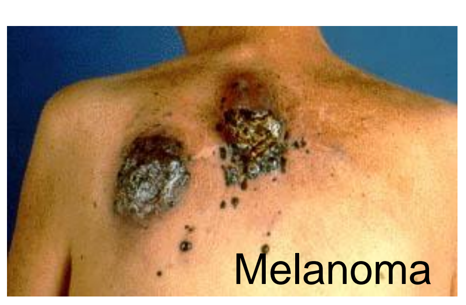


# Animal models of human diseases

| Humans | Animal models |
| --- | --- |
| Mutant Gene ↔ | Mutant Gene |
| ↕ | ↕ |
| Mutant or missing Protein ↔ | Mutant or missing Protein |
| ↕ | ↕ |
| Mutant Phenotype (disease) ⇄ | Mutant Phenotype (disease model) |

Cell lines of melanoma




Mouse model of melanoma

Zebrafish model of melanoma

# Altering or Editing Genetic materials

- Using ***in vitro* mutagenesis** 誘發突變, specific mutations are introduced into a cloned gene, altering or destroying its function
- When the mutated gene is returned to the cell, the normal gene's function might be determined by examining the mutant's phenotype

*in vitro*: 試管內，體外
*in vivo*: 活體內
*In situ*: 原位，正位
*ex vivo:*生物活體之外
*In utero*: 在子宮裡
*In silico*: 經由電腦模擬

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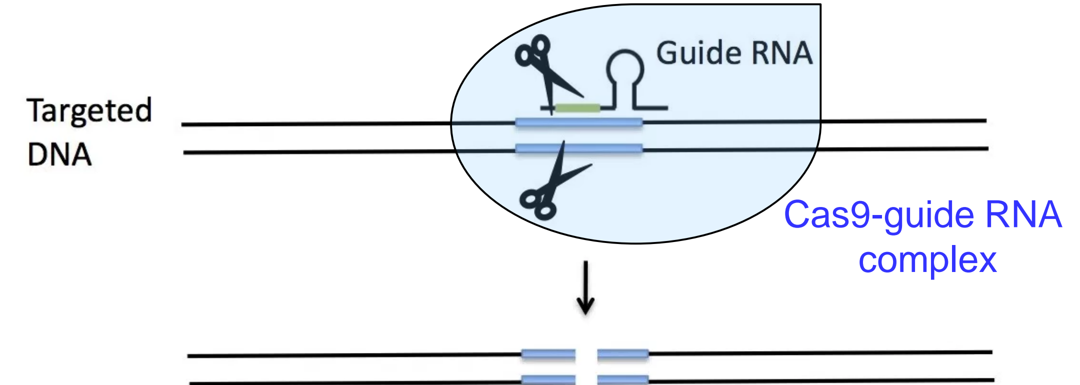
# Genome editing 基因編輯

- **Bacterial defense against bacteriophage**
  - The **CRISPR-Cas9 system** is a powerful new technique for gene editing in living cells and organisms
  - **Cas9** acts together with a "**guide RNA**" made from the **CRISPR region** of the bacterial system; the Cas9-guide RNA complex will bind and cut both strands of any DNA sequence complementary to the guide RNA
- If the guide RNA is engineered to be complementary to the target gene, the target DNA will be cut

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- When the cut DNA is **repaired**, nucleotides may be introduced or removed, causing the gene to be inactivated
- Researchers have also modified the technique so a gene with a mutation in it can be repaired
  - This is done by introducing a segment of the wild-type gene, which may be used as a template to repair the target DNA (i.e. gene therapy)

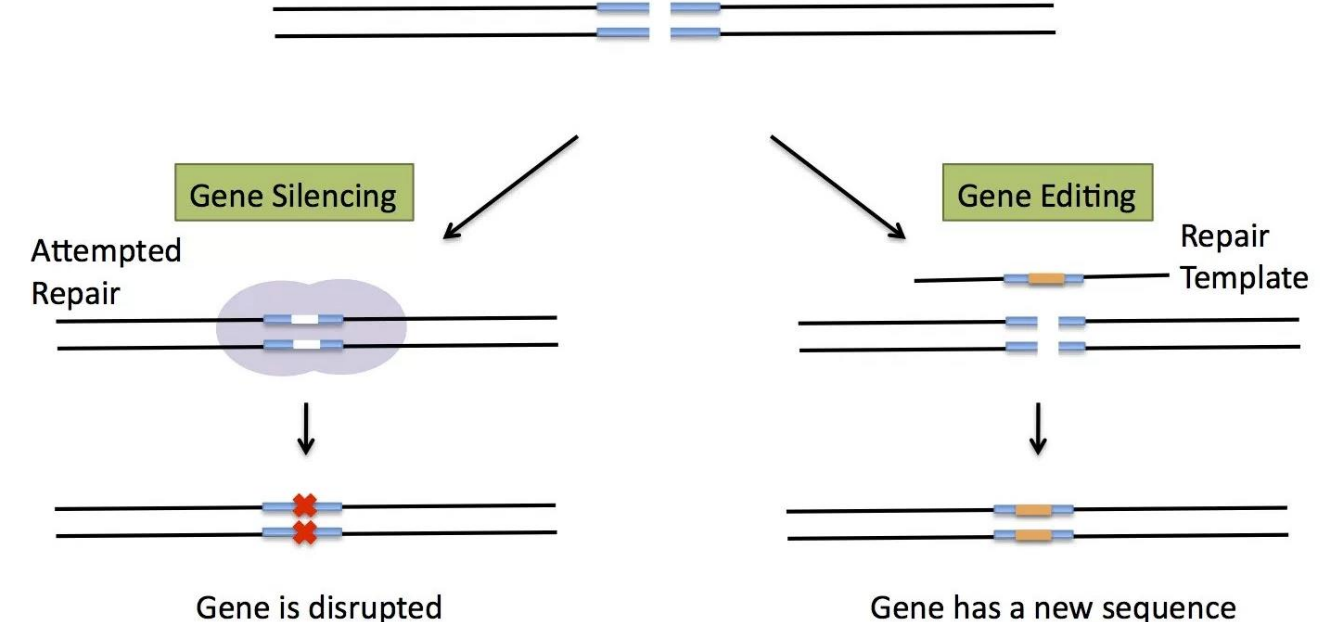


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Figure 19.14a

# Gene editing using the CRISPR-Cas9 system

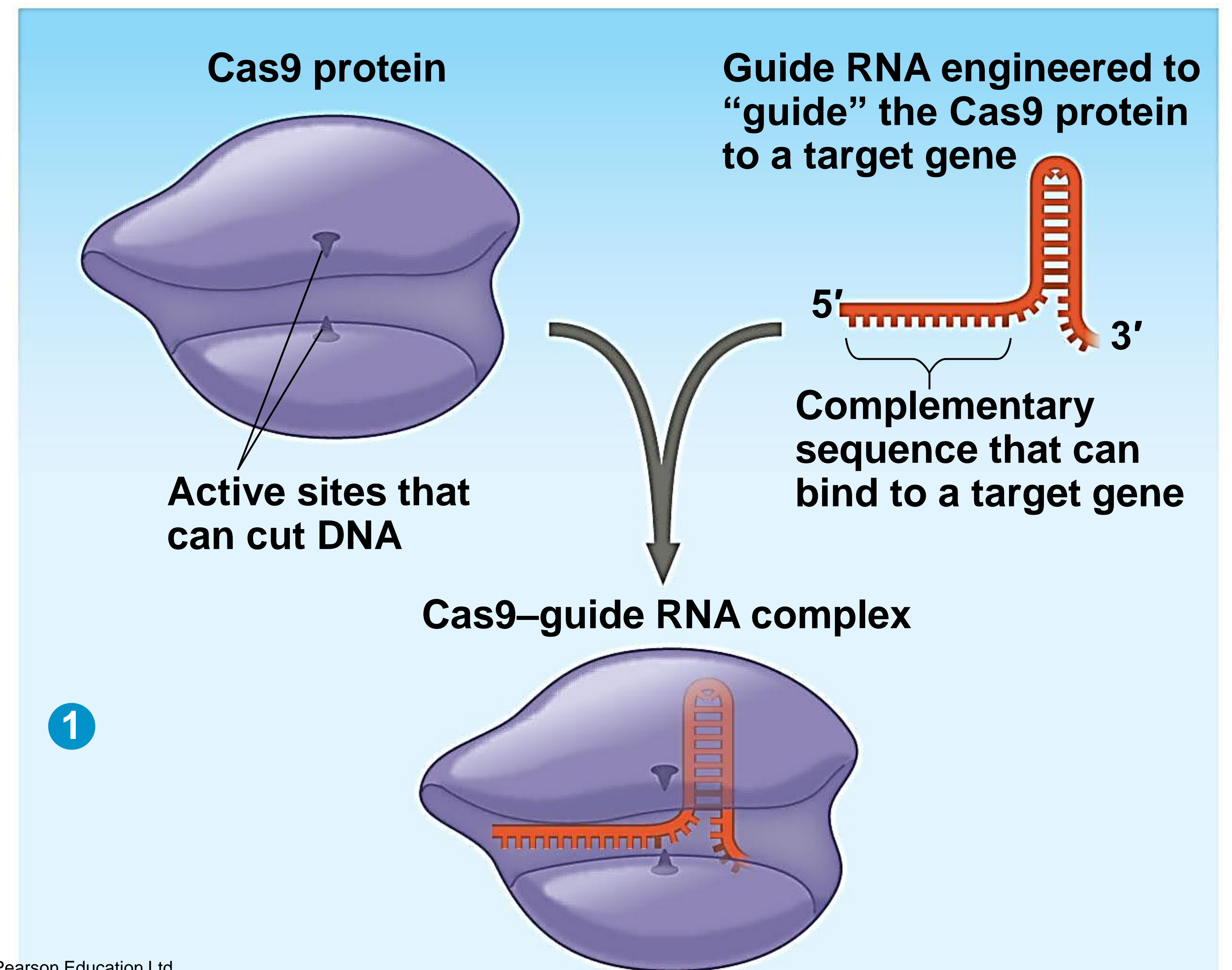


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Figure 19.14b

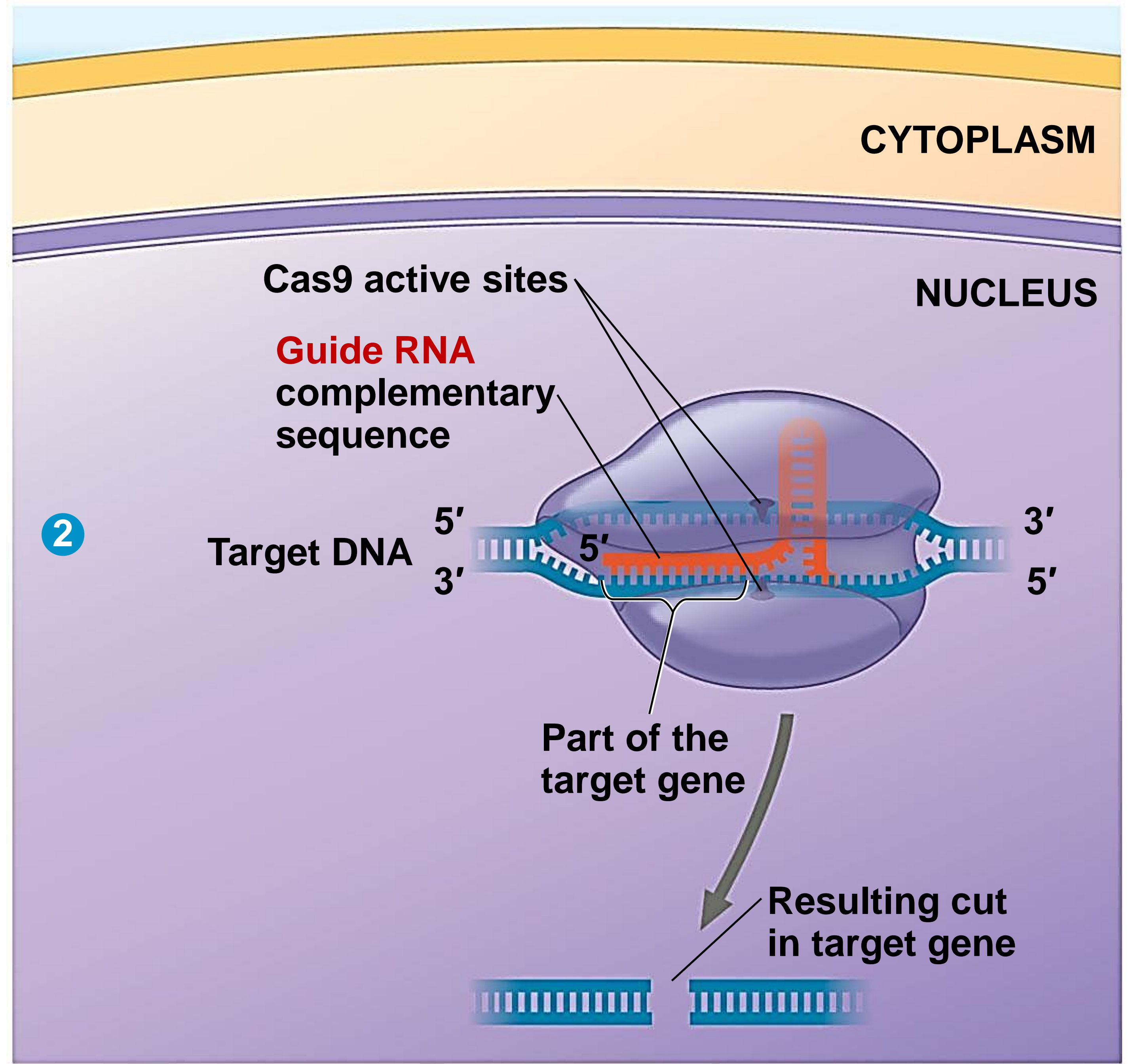


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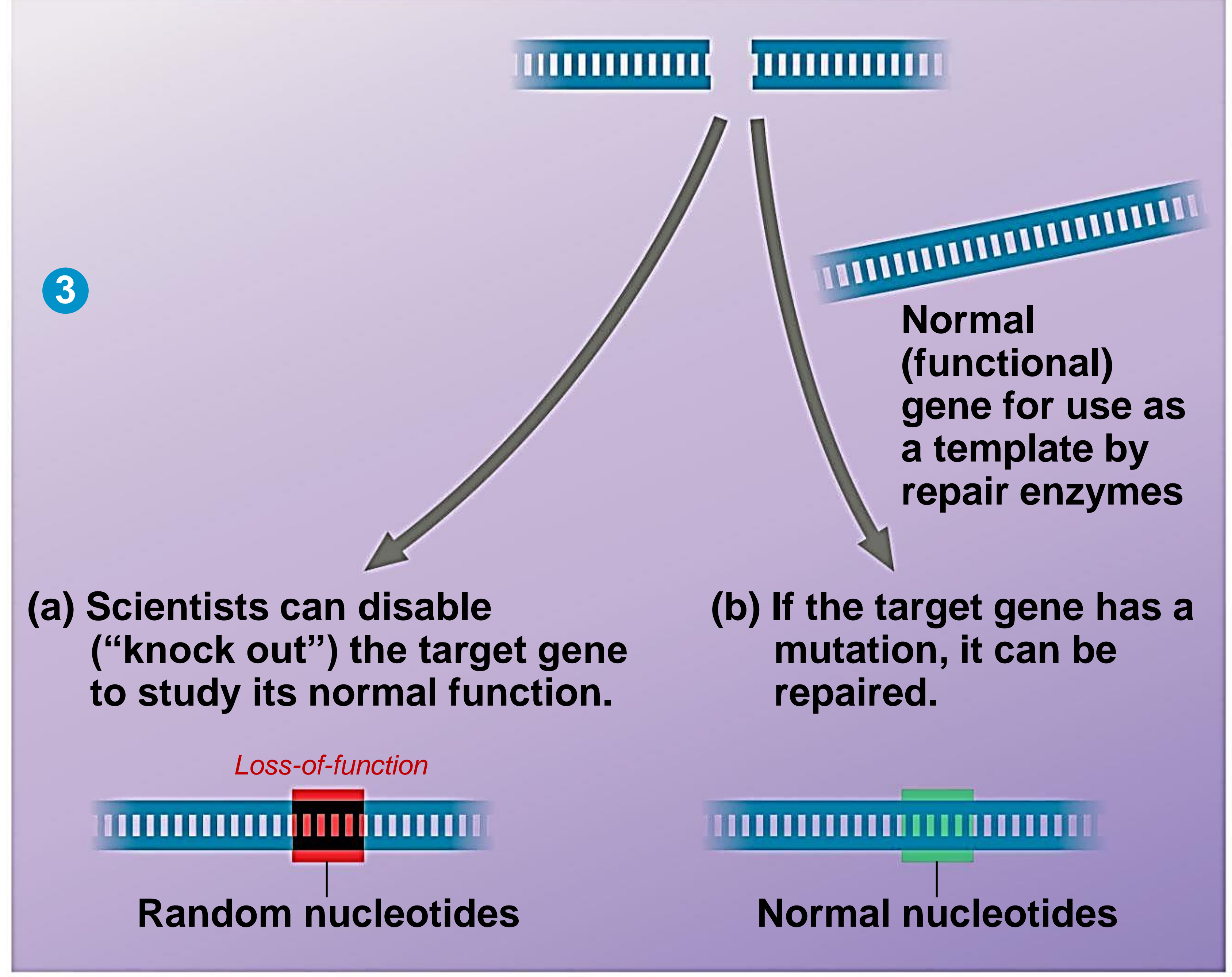
Figure 19.14c

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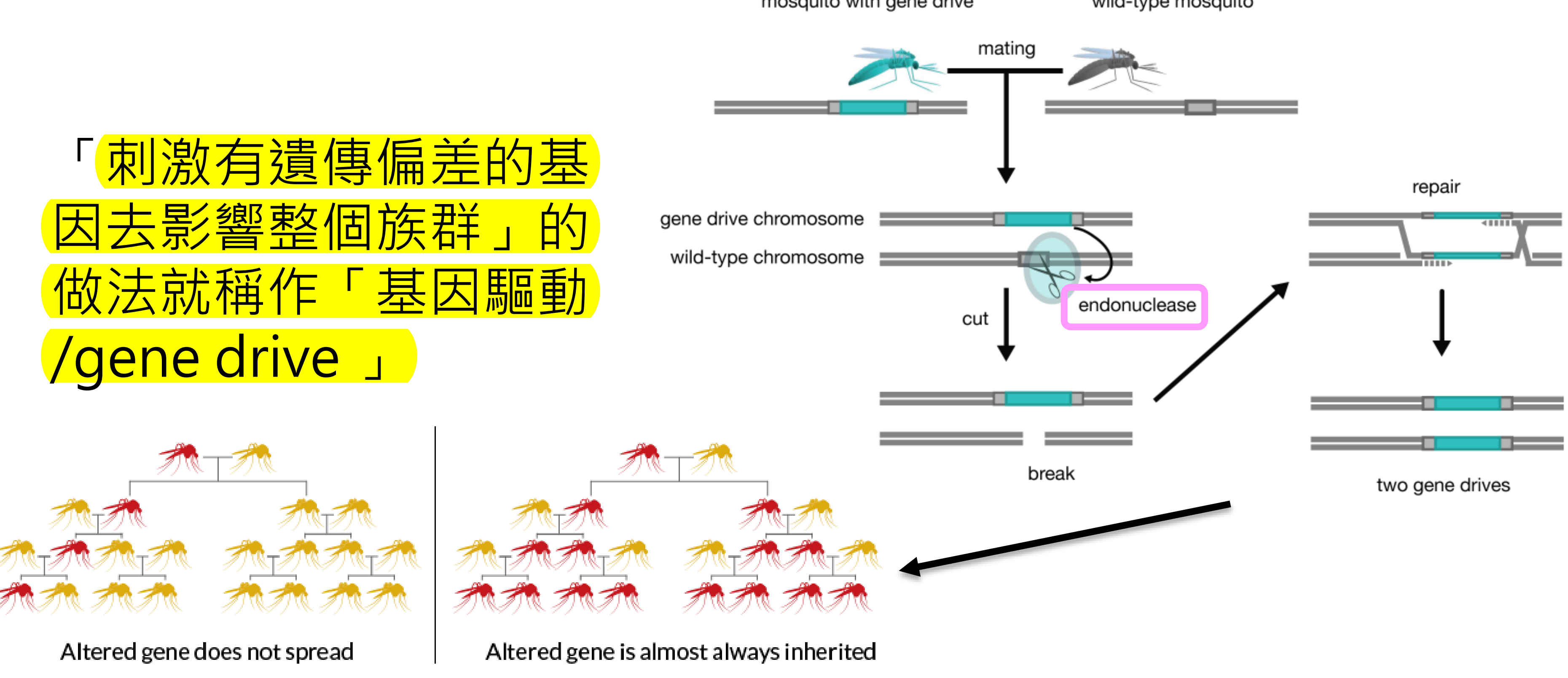
# 補充: Expanding the CRISPR Toolbox

Animation on **CRISPR gene editing and beyond**
https://www.nature.com/collections/txhdfslxzh/videos

The CRISPR-Cas9 system is best known for its ability to knock out or replace specific genes, via targeted cleavage of the genome. But scientists are developing **many more applications**, typically by using an inactive Cas9 to target other enzymes to specific genomic sites.
From transcriptional regulation to base editing, these developments are extending the range of biological questions that can be probed with CRISPR/Cas9.

# Example Application of Gnome Editing

- Alternating genes in insect to stop insect-borne disease (i.e. Mosquito)
- Engineering new allele that is favored for inheritance (i.e. **Gene drive 基因驅動** to introduce new allele through the population )



- 2003 年Imperial College London 的演化生物學家 Austin Burt，想到利用 HEG（homing endonuclease gene）這個自私基因來改變整個族群的基因。HEG 的 H 指的是:「endonuclease gene」會自動返航（homing），跑到同源染色體 (homologous chromosome) 的相對位置。
- Burt 的想法是：首先用基因工程的方法改變 HEG 切割的位置，讓它去切一個對宿主很重要的基因，這個步驟叫基因剔除（gene knockout）。而這個被剔除的基因必須具備一個條件：單一基因被剔除時宿主沒有生存的問題，但當兩個都被剔除時，宿主便無法存活。
- 接著想辦法將 HEG 置於減數分裂啟動子 (meiosis promoter) 的控制下，使得宿主產生大量含 HEG 的配偶子(gametes)，這樣帶有 2 個 HEG 缺陷基因的子代便會全數死亡。估計用這種方法只要放出 1% 的蚊子，20 個世代後便能完全消滅整個族群。
- CRISPR-Cas9: Gene Drives https://wyss.harvard.edu/media-post/crispr-cas9-gene-drives/

# RNA interference (RNAi)

- Gene expression can also be silenced using **RNA interference (RNAi)**
- Synthetic double-stranded RNA molecules matching the sequence of a particular gene are used to break down or block the gene's mRNA
  - **Reduction** in target protein expression
    - **Loss of Function** analysis on specific gene's product
      - Phenotypic effect; Effect on signaling/metabolic pathways, etc.
    - **Potential application for Disease treatment**

# Genome-wide association studies

- In humans, researchers analyze the genomes of many people with a certain genetic condition to try to find nucleotide changes specific to the condition
- **Genetic markers** called **SNPs** (**single nucleotide polymorphisms**) occur on average every 100–300 base pairs
  - SNPs can be detected by **sequencing**, **microarray** or **PCR**, and any SNP shared by people affected with a disorder but not among unaffected people may locate the disease-causing gene

- SNP variants that are found frequently associated with a particular inherited disorder alert researchers to the most likely location for the disease-causing gene
- SNPs are *rarely directly involved in the disease*; they are most often in *noncoding regions of the genome*

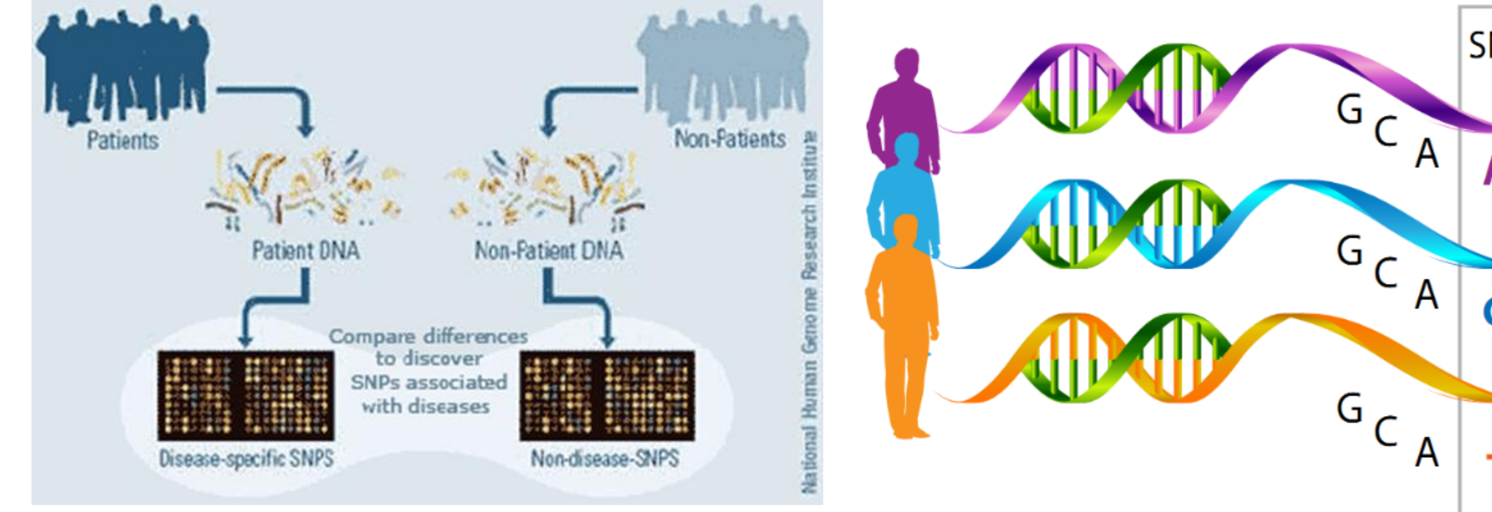
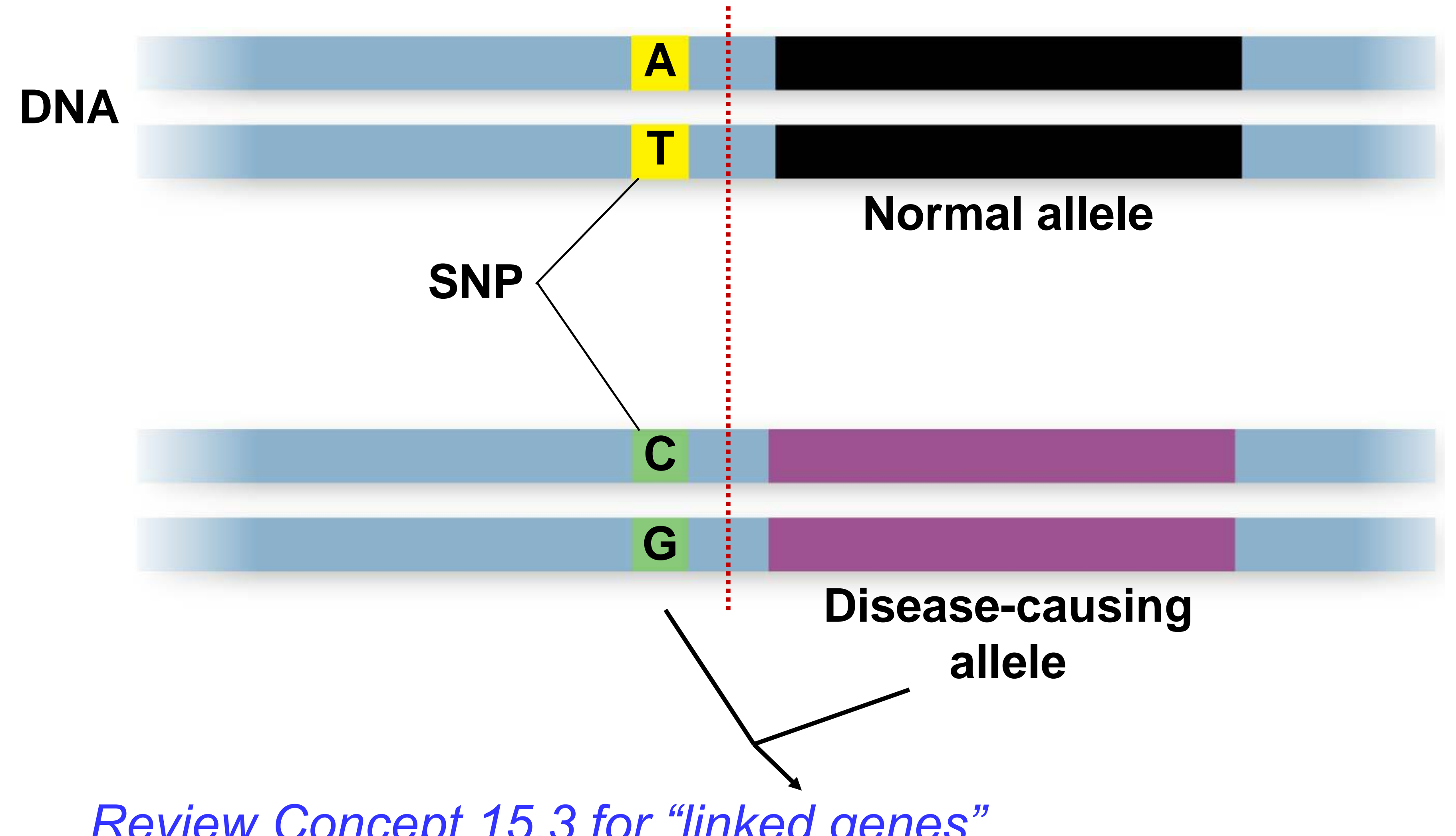


Figure 19.14

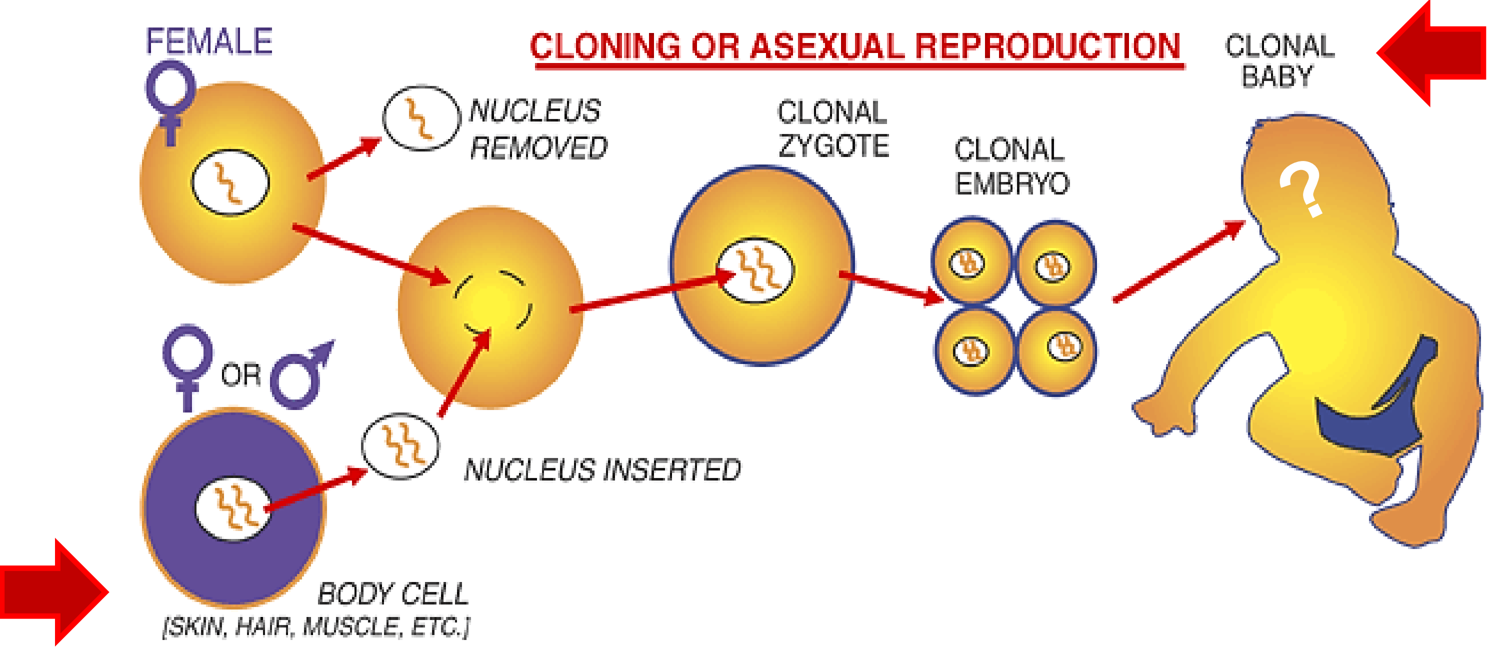
# Single nucleotide polymorphisms (SNPs) as genetic markers for disease-causing alleles




*Review Concept 15.3 for "linked genes"*

# Concept 19.3: Cloned organisms and stem cells are useful for basic research and other applications

- **Organismal cloning** produces one or more organisms genetically identical to the "parent" that donated the single cell

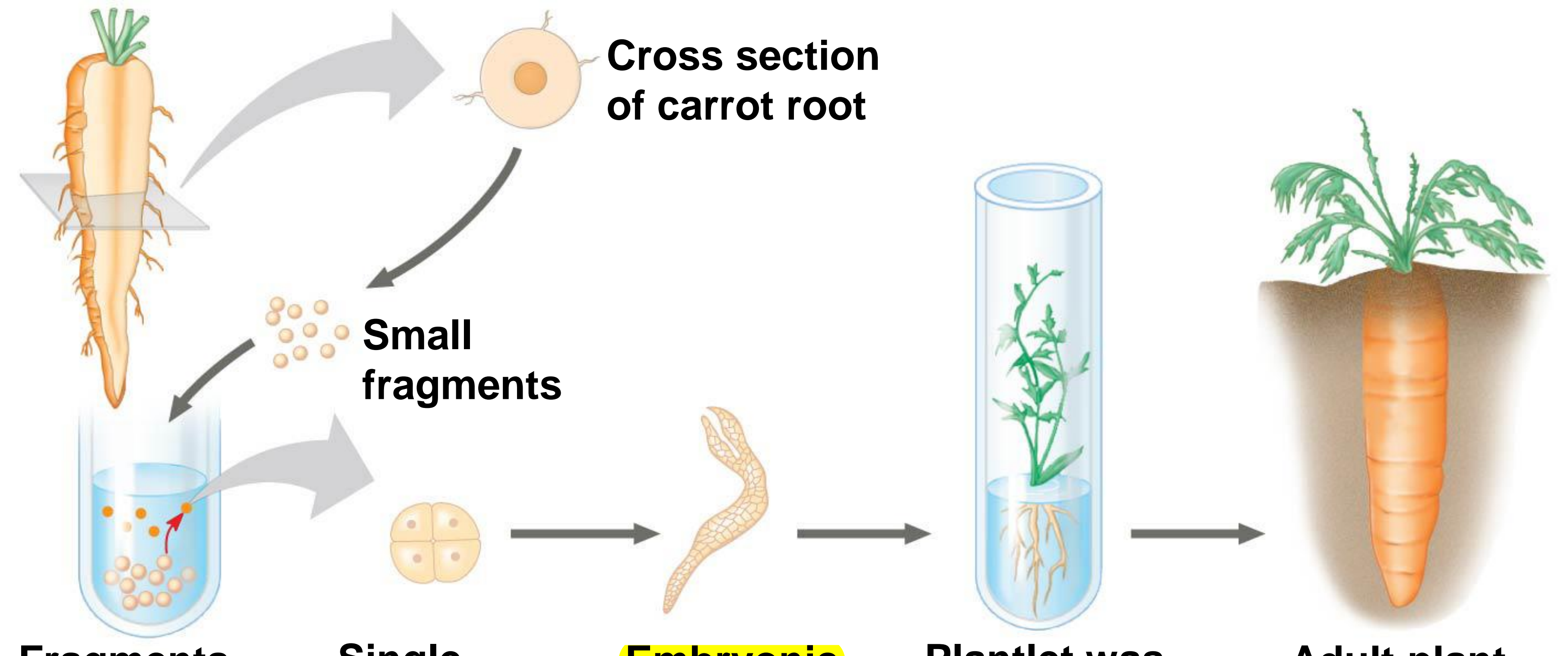


# Cloning Plants: Single-Cell Cultures

- **What is stem cell?**
  - A **stem cell** is a relatively **unspecialized cell** that can reproduce itself indefinitely, or under certain conditions can differentiate into one or more types of specialized cells
- In plants, cells can dedifferentiate and then give rise to all the specialized cell types of the organism - *Plant cloning is used extensively in agriculture*
- A **totipotent** cell (全能細胞) can generate a complete new organism

Figure 19.15

# The cloning of a whole carrot plant from a single carrot cell




Fragments were cultured in nutrient medium; stirring caused single cells to shear off into the liquid.

Single cells free in suspension began to divide.

Embryonic plant developed from a cultured single cell.

Plantlet was cultured on agar medium. Later it was planted in soil.

Adult plant

# Cloning Animals: Nuclear Transplantation

- In **nuclear transplantation**, the nucleus of an unfertilized egg cell or zygote is replaced with the nucleus of a differentiated cell
- Experiments with frog embryos have shown that a transplanted nucleus can often support normal development of the egg
- However, the older the donor nucleus, the lower the percentage of normally developing tadpoles

Figure 19.16

**Experiment**

Can the nucleus from a differentiated animal cell direct development of an organism?

Frog embryo

UV

Frog egg cell

Frog tadpole

Less differentiated cell

Fully differentiated (intestinal) cell

Donor nucleus trans-planted

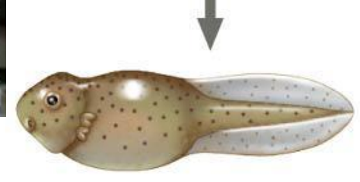
Enucleated egg cell

Donor nucleus trans-planted

Egg with donor nucleus activated to begin development

2012年諾貝爾醫學獎得主
John Gurdon
劍橋大學

**Results**



Most develop into tadpoles.

Most stop developing before tadpole stage.

# Reproductive Cloning of Mammals

- In 1997, Scottish researchers announced the birth of **Dolly**, a lamb cloned from an adult sheep by nuclear transplantation from a differentiated mammary cell
- Dolly's premature death in 2003, as well as her arthritis, led to speculation that her cells were not as healthy as those of a normal sheep, possibly reflecting **incomplete reprogramming** of the original transplanted nucleus

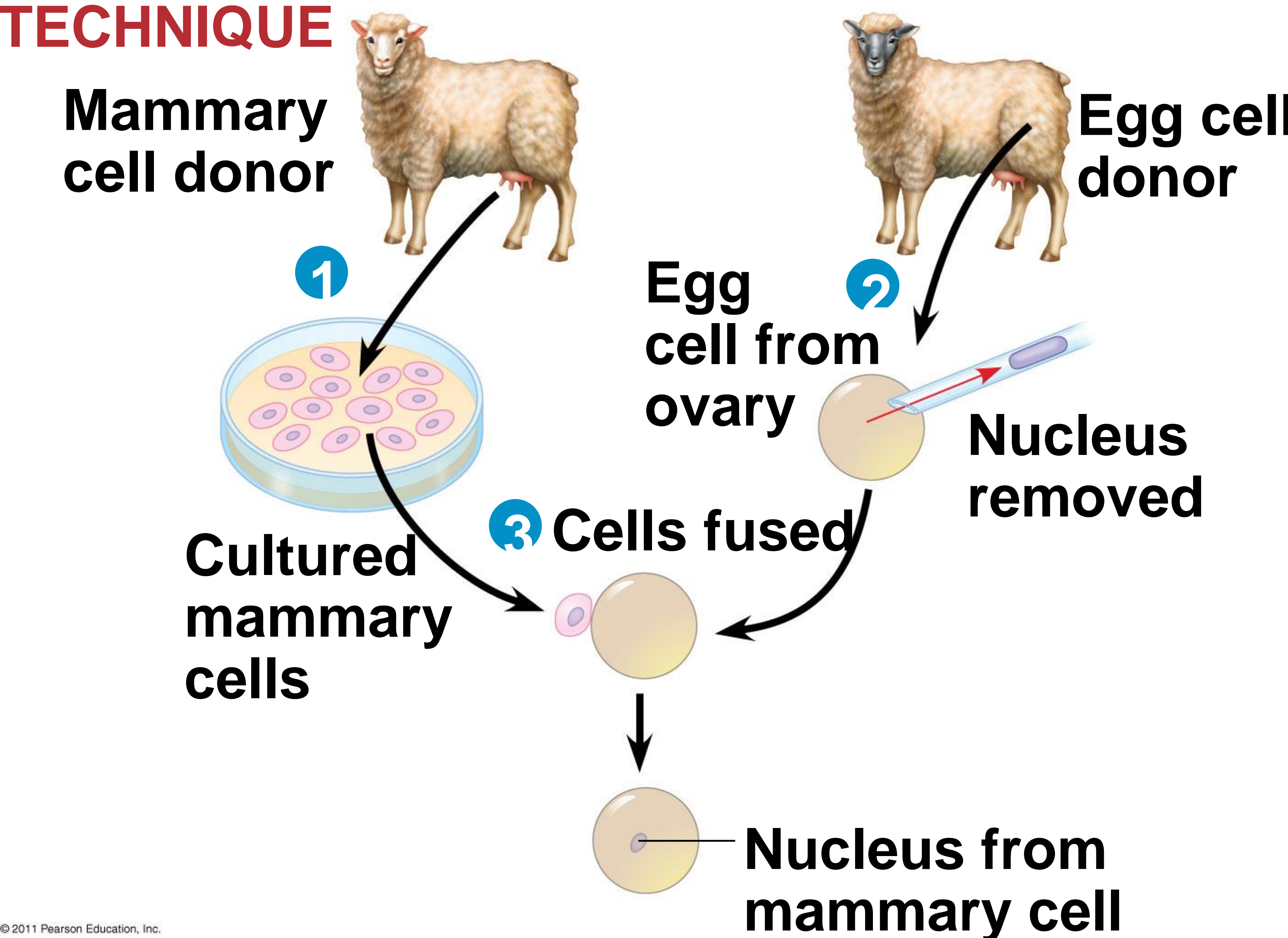



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Figure 19.17

4 Grown in culture

5 Implanted in uterus of a third sheep

6 Embryonic development

**RESULTS**

# CC, the first cloned cat

- Since 1997, cloning has been demonstrated in many mammals, including mice, cats, cows, horses, mules, pigs, and dogs
- CC (for Carbon Copy) was the first cat cloned; however, CC differed somewhat from her female "parent" due to random X chromosome inactivation.

CC

## *Faulty Gene Regulation - Problems Associated with Animal Cloning*

- In most nuclear transplantation studies, only a small percentage of cloned embryos have developed normally to birth
- Many epigenetic changes, such as acetylation of histones or methylation of DNA, must be reversed in the nucleus from a donor animal in order for genes to be expressed or repressed appropriately for early stages of development
- **Human Cloning?! –** Chinese scientists claimed they have done it first @中南大學湘雅醫學院

# Stem Cells of Animals

- Stem cells isolated from early embryos at the blastocyst stage are called *embryonic stem cells*; these are able to differentiate into all cell types
- The adult body (bone marrow) also has stem cells, which replace non-reproducing specialized cells

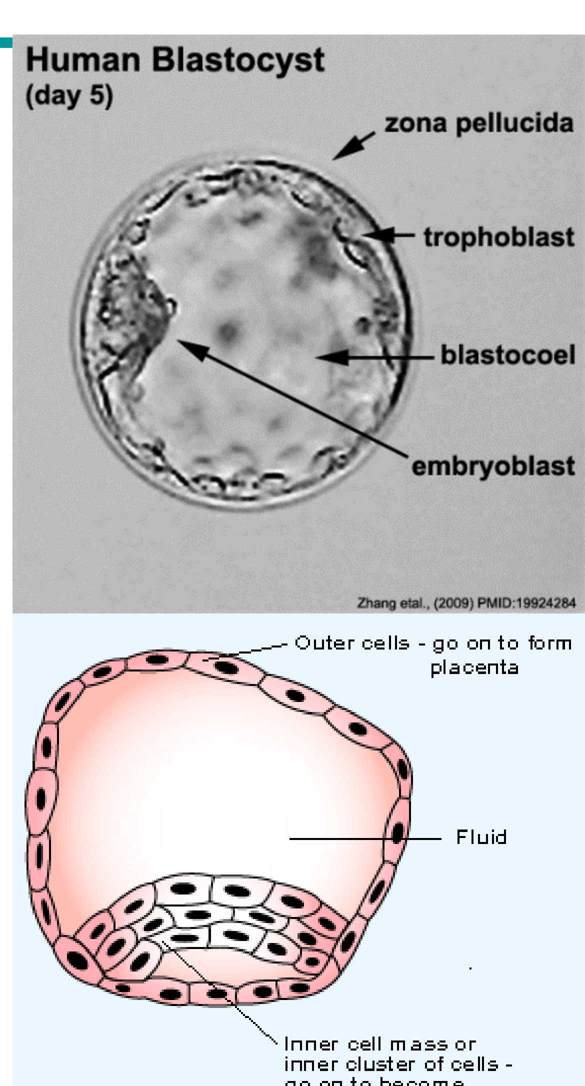


Figure 19.19

How stem cells maintain their own population and generate differentiated cells

(1) A **stem cell** can divide into another stem cell and a precursor cell (or into two stems cells or two precursor cells)

(2) A **precursor cell** can differentiate into one of several cell types, depending on external factors. This example is from a stem cell derived from bone marrow.

**Stem cell**

**Cell division**

**Stem cell** **and** **Precursor cell**

**Fat cells** **or** **Bone cells** **or** **White blood cells**

# Embryonic vs. Adult Stem Cells

- Many early embryos contain stem cells capable of giving rise to differentiated embryonic cells of any type
- Review **Stem Cell** characteristics:
  - In culture, these embryonic stem cells reproduce indefinitely
  - Depending on culture conditions, they can be made to differentiate into a variety of specialized cells
- Adult stem cells can generate multiple (but not all) cell types and are used in the body to replace non-reproducing cells as needed

Figure 19.20

ES cells are **Pluripotent,** they can differentiate into many different cell types

**Embryonic Stem (ES) cells**

**Adult stem cells**

**Cells that can generate all embryonic cell types**

**Cells that generate a limited number of cell types**

**Cultured stem cells**

**Different culture conditions**

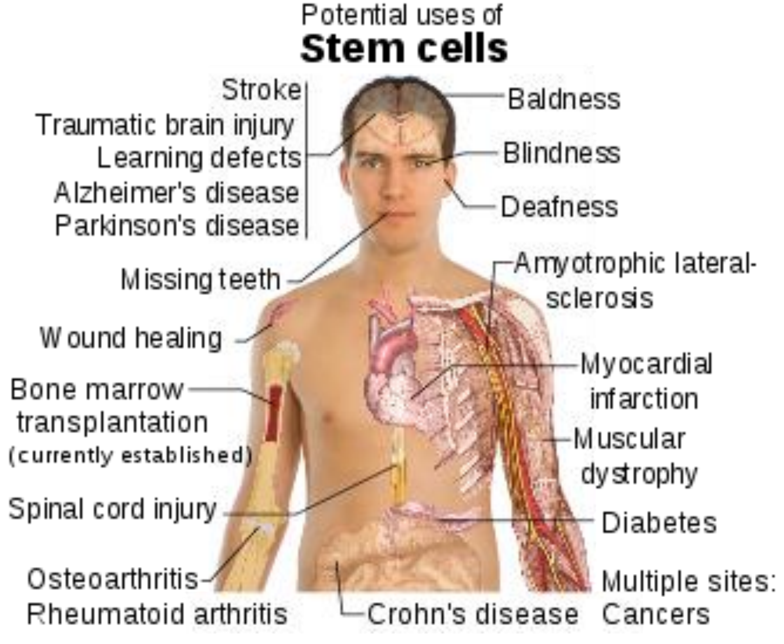
**Liver cells** **Nerve cells** **Blood cells**

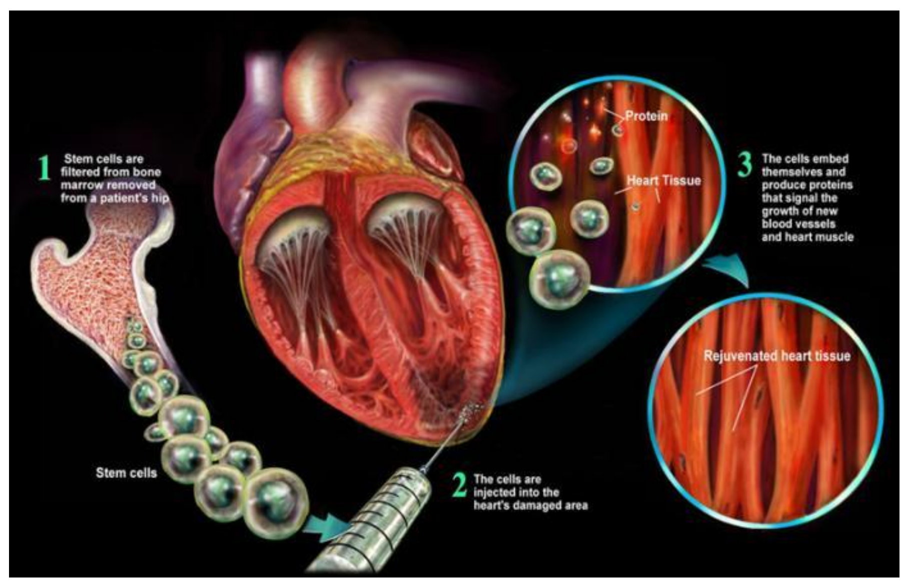
**Different types of differentiated cells**

**Working with stem cells**

# Producing stem cells by therapeutic cloning

- The aim of stem cell research is to supply cells for the **repair of damaged or diseased organs** → **再生醫學**

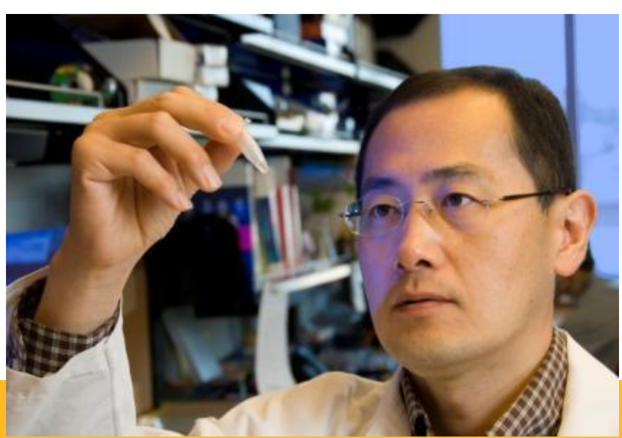



Cell therapy for myocardial infarction

# Induced Pluripotent Stem (iPS) cells

- Researchers can transform differentiated cells into ES cells by using **retroviruses** to introduce stem cell master regulatory genes
- These transformed cells are called **iPS cells (induced pluripotent cells** 誘導多能性幹細胞**)**
- **iPS cells** can perform most of the functions of ES cells, and used as models for the study of certain diseases and potentially be used to replace nonfunctional cells for patients
  - Eliminate many ethical problems!



2012年諾貝爾醫學獎得主
Shinya Yamanaka 山中伸弥
京都大學

Figure 19.21

Can a fully differentiated human cell be "deprogrammed" to become a stem cell?

# One active research topic on iPSCs

## Artificial sperm and egg cells of mice → 人造人? 客製人?

Cell

### Reconstitution of the Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells

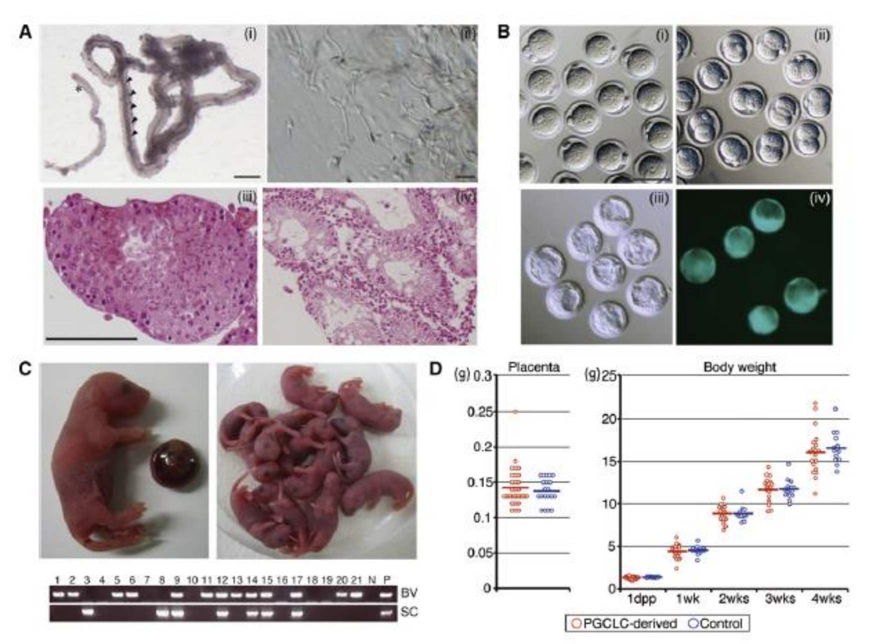
Katsuhiko Hayashi,[1,3] Hiroshi Ohta,[1,3] Kazuki Kurimoto,[1,3] Shinya Aramaki,[1] and Mitinori Saitou[1,2,3,*]
[1]Department of Anatomy and Cell Biology, Graduate School of Medicine
[2]Institute for Integrated Cell-Material Sciences
Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan
[3]JST, CREST, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan
*Correspondence: saitou@anat2.med.kyoto-u.ac.jp
DOI 10.1016/j.cell.2011.06.052

人造精子

SUMMARY

The generation of properly functioning gametes in vitro requires reconstitution of the multistepped

the PGC fate (Kurimoto et al., 2008; Ohinata et al., 2005; Vincent et al., 2005; Yamaji et al., 2008). Essentially all of the epiblast cells from E5.5–E6.0 are competent to express *Blimp1* and *Prdm14* in response to Bmp4, and the PGC-like cells induced




### Offspring from Oocytes Derived from in Vitro Primordial Germ Cell–like Cells in Mice

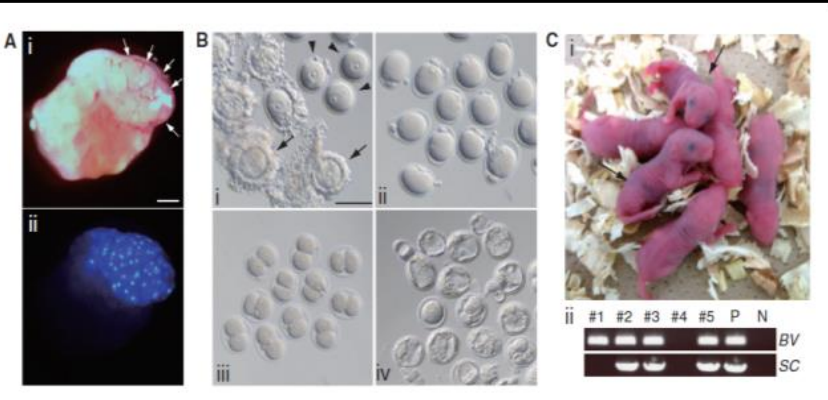
Katsuhiko Hayashi,[1,2,3*] Sugako Ogushi,[1,4] Kazuki Kurimoto,[1,5] So Shimamoto,[1]
Hiroshi Ohta,[1,5] Mitinori Saitou[1,2,5,6*]

Reconstitution of female germ cell development in vitro is a key challenge in reproductive biology and medicine. We show here that female (XX) embryonic stem cells and induced pluripotent stem cells in mice are induced into primordial germ cell–like cells (PGCLCs), which, when aggregated with female gonadal somatic cells as reconstituted ovaries, undergo X-reactivation, imprint erasure, and cyst formation, and exhibit meiotic potential. Upon transplantation under mouse ovarian bursa, PGCLCs in the reconstituted ovaries mature into germinal vesicle-stage oocytes, which then contribute to fertile offspring after in vitro maturation and fertilization. Our culture system serves as a robust foundation for the investigation of key properties of female germ cells, including the acquisition of totipotency, and for the reconstitution of whole female germ cell development in vitro.

The germ cell lineage in mammals originates from pluripotent epiblasts as primordial germ cells (PGCs) and undergoes sexually dimorphic development, generating spermatozoa in males and oocytes in females. These cells fertilize to form zygotes with full developmental

人造卵子





# In a world first, Japanese team creates eggs and sperm of endangered species from iPS cells

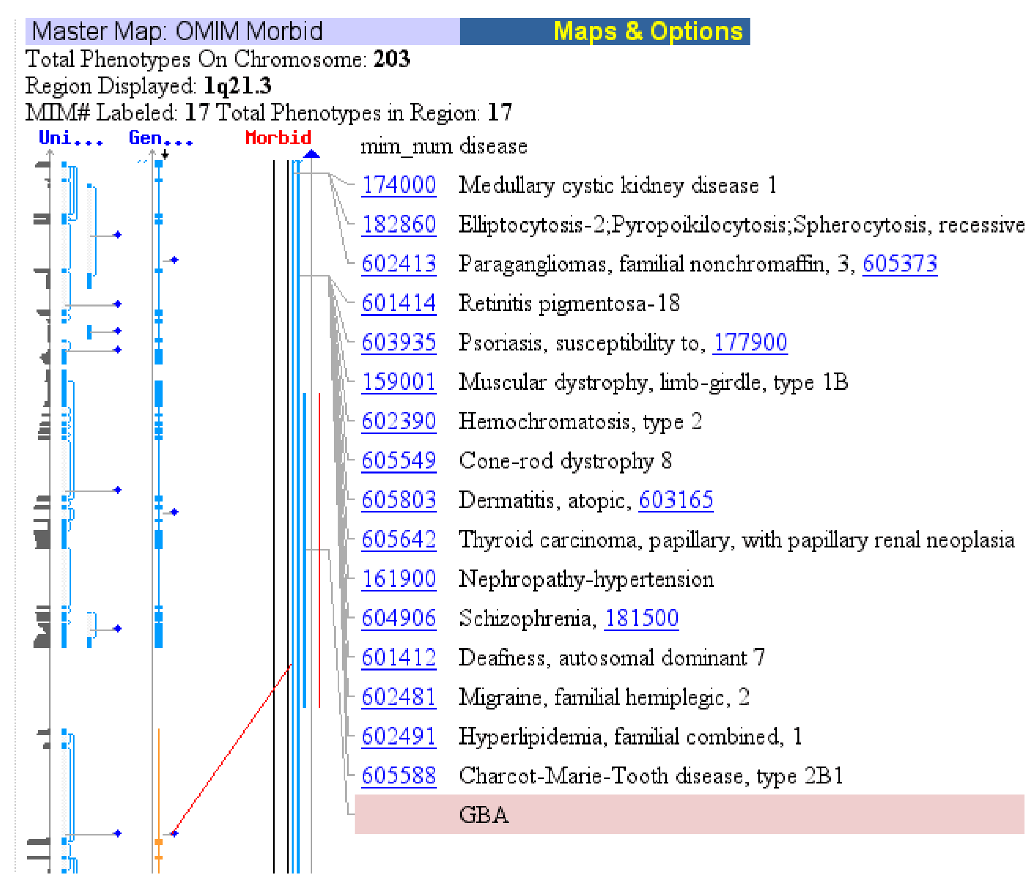
Science Advances 12 May 2017: Vol. 3, no. 5, e1602179, DOI: 10.1126/sciadv.1602179

## Concept 19.4: The practical applications of DNA-based technology affect our lives in many ways

- Many fields benefit from DNA technology and genetic engineering, some examples are:
  - Medical applications
    - Diagnosis of genetics diseases
    - Developing therapy or drug
  - Forensic evidence and genetic profiles
  - Environmental cleanup
  - Agriculture applications

# Medical Applications

- One benefit of DNA technology is identification of human genes in which **mutation** plays a role in genetic diseases




Genes and Diseases

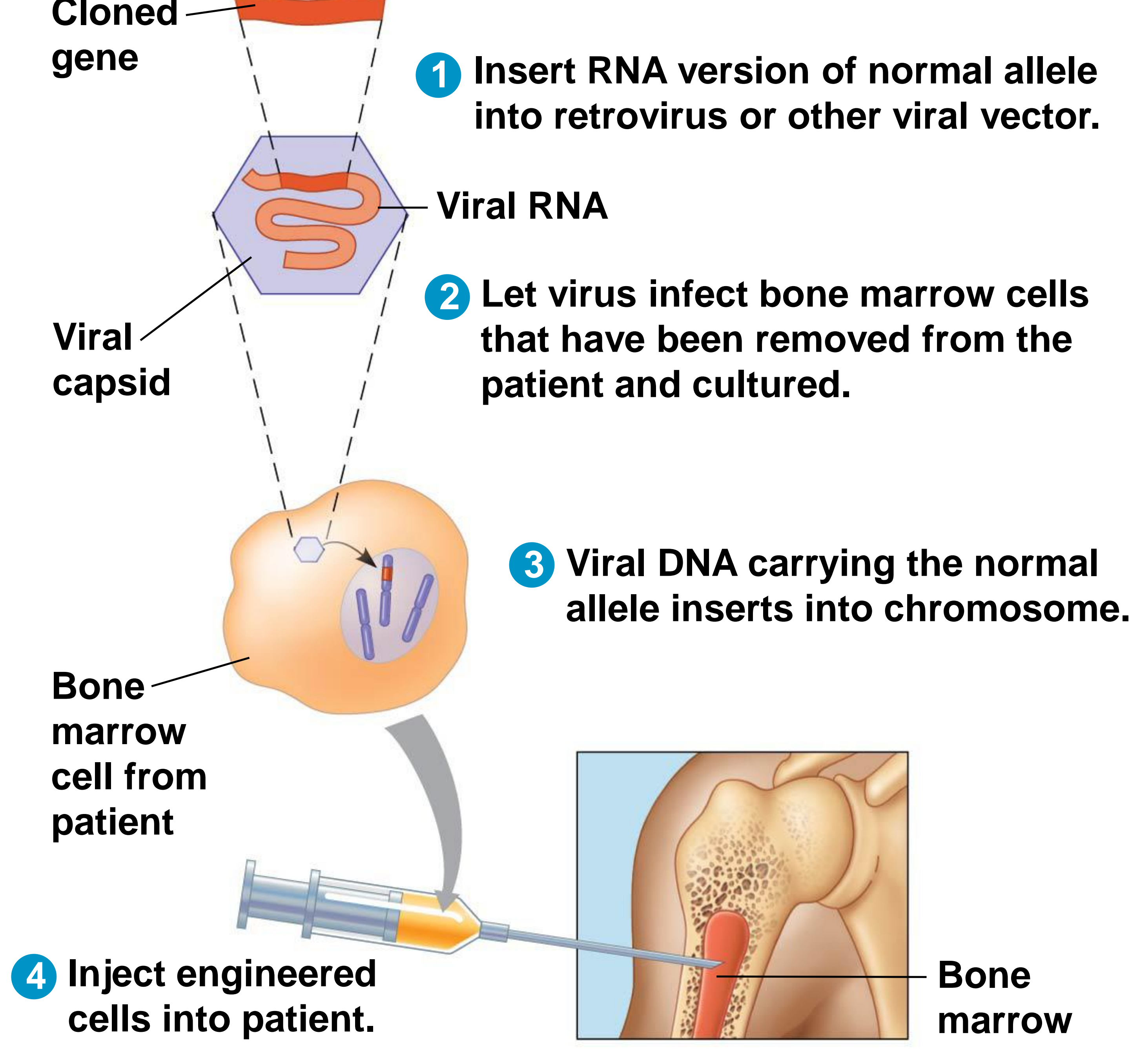
## *Diagnosis of Diseases*

- Scientists can diagnose many human genetic disorders by using **PCR and specific primers corresponding to cloned disease genes**, then sequencing the amplified product to look for the disease-causing mutation
  - SNPs may be associated with a disease-causing mutation
  - SNPs may also be correlated with increased risks for conditions such as heart disease or certain types of cancer

## *Human Gene Therapy*

- **Gene therapy** is the alteration of an afflicted individual’s genes
- Gene therapy holds great potential for treating disorders traceable to a single defective gene
- Vectors are used for **delivery of genes** into specific types of cells, for example bone marrow
- Gene therapy raises ethical questions, such as whether human germ-line cells should be treated to correct the defect in future generations

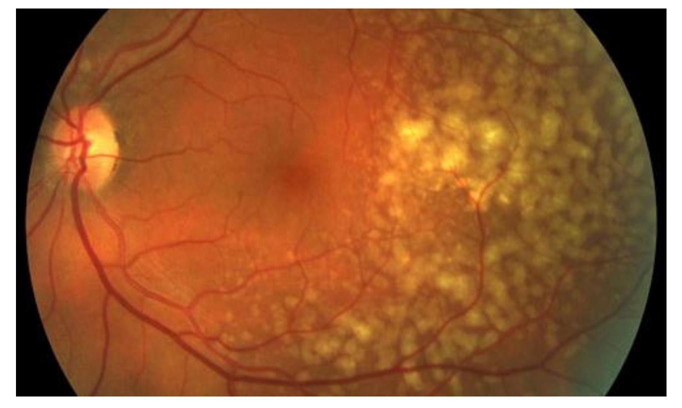
Figure 19.22

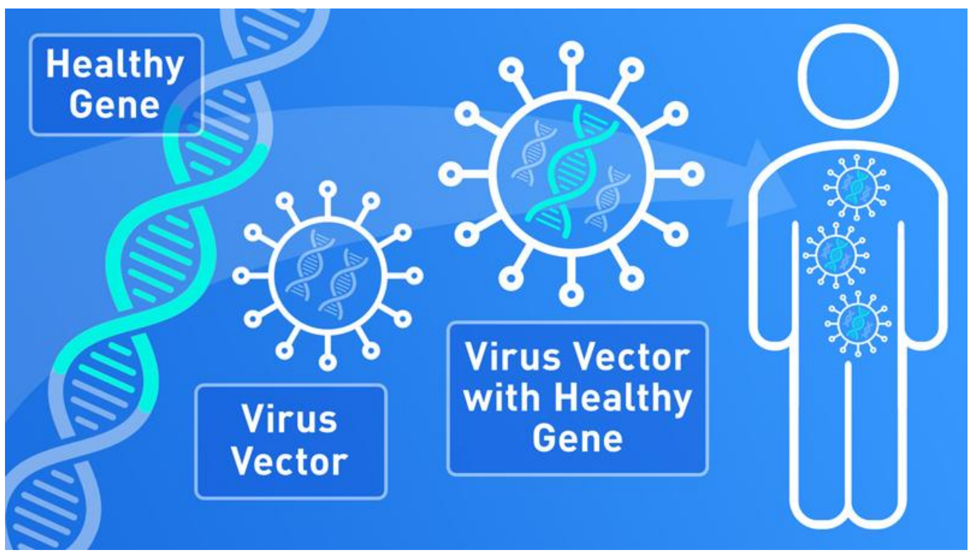


# FDA approves novel gene therapy to treat patients with a rare form of inherited vision loss on 2017/12/19

補充

Hereditary retinal dystrophies are a broad group of genetic retinal disorders of varying severity and with differing inheritance patterns.






- **Luxturna** is the first gene therapy approved in the U.S. to target a disease caused by mutations in a specific gene
- It is an adeno-associated virus vector-based gene therapy indicated for the treatment of patients with confirmed bi-allelic **RPE65** mutation-associated retinal dystrophy. Patients must have viable retinal cells as determined by the treating physician(s).

What is gene therapy? https://www.fda.gov/ForConsumers/ConsumerUpdates/ucm589197.htm

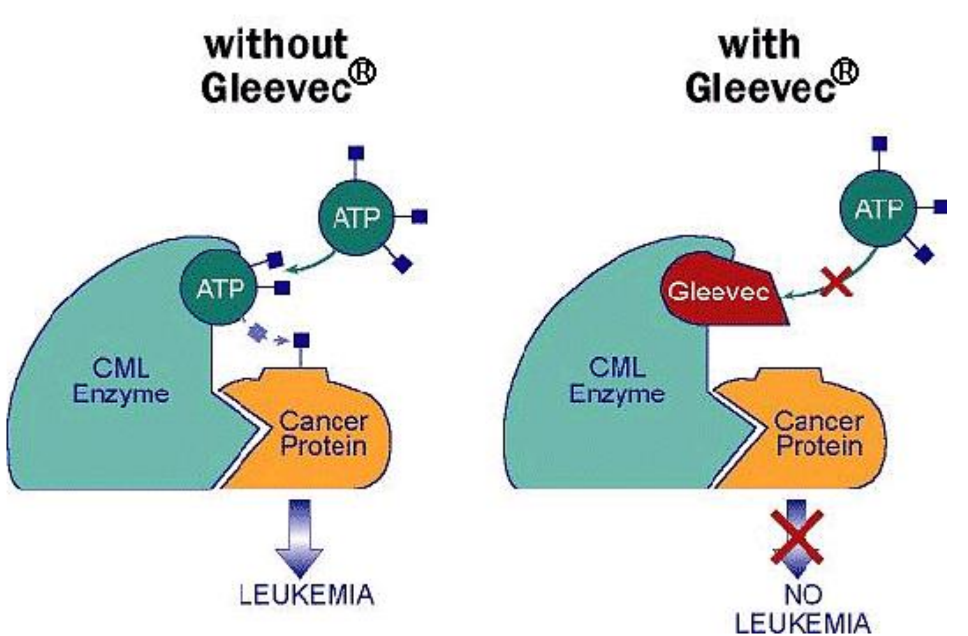
## *Pharmaceutical Products*

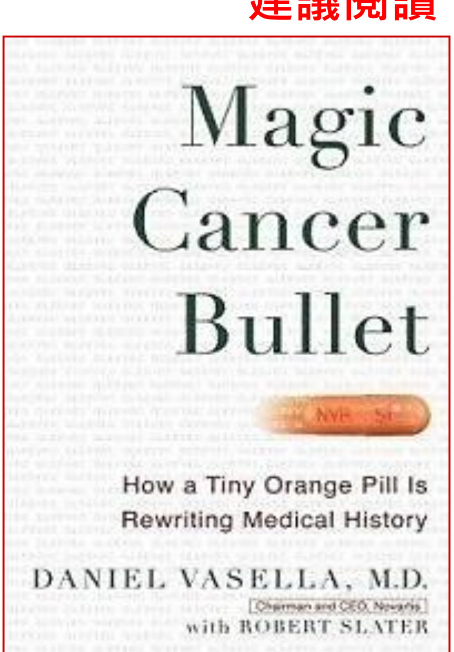
- Advances in DNA technology and genetic research are important to the development of new drugs to treat diseases

# Synthesis of Small Molecules for Use as Drugs

- The drug **imatinib** (Gleevec) is a small molecule that inhibits over-expression of a specific leukemia (CML)-causing receptor tyrosine kinase

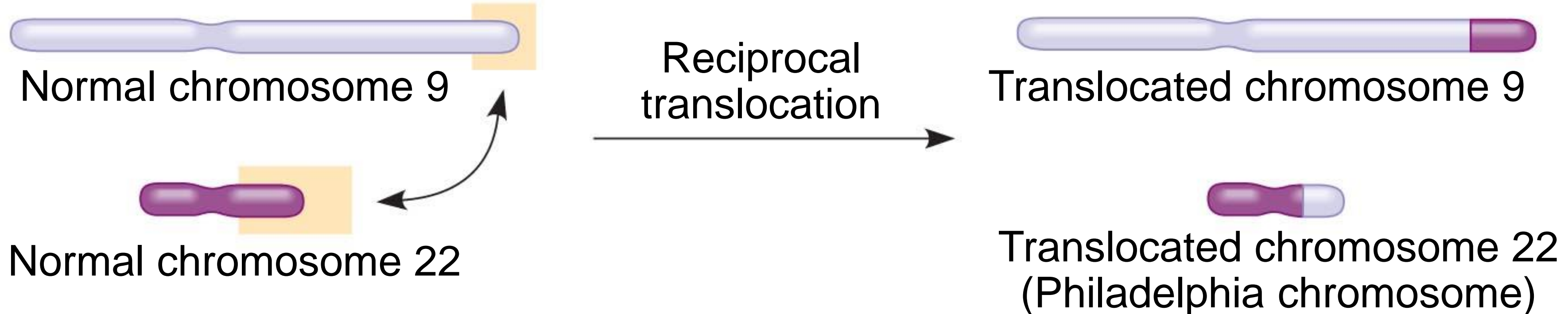
建議閱讀



Review

# Fig.15-16 Translocation associated with chronic myelogenous leukemia (CML)

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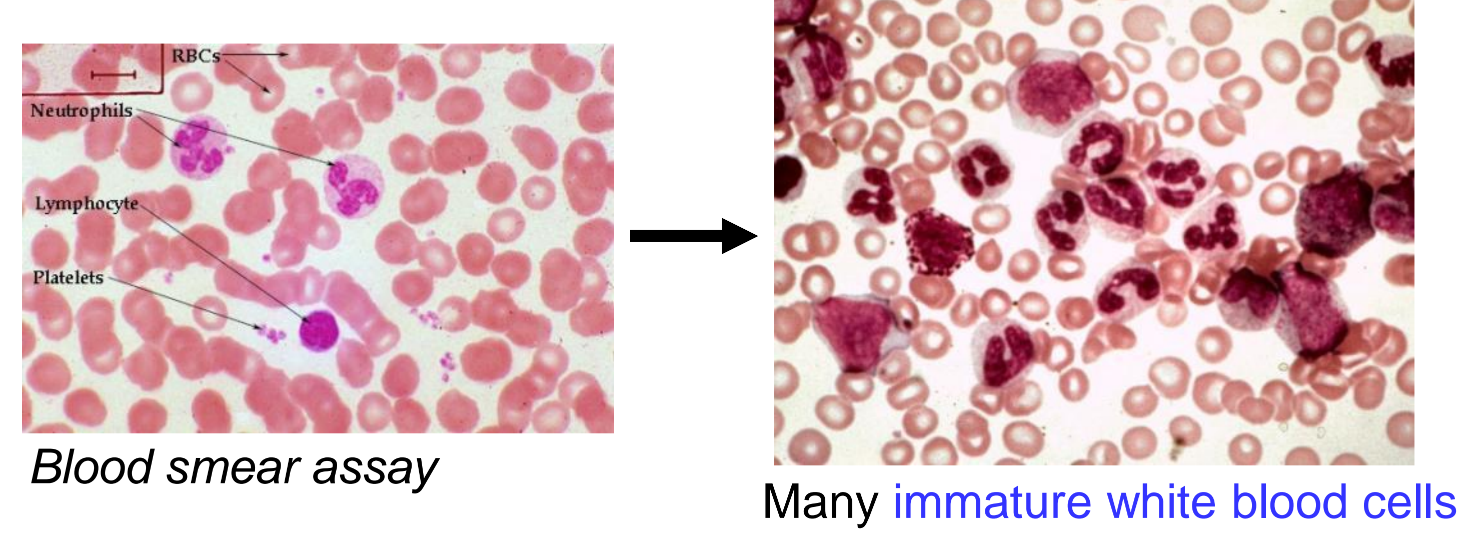



*Blood smear assay*

Many immature white blood cells

# Protein Drug Production in Cell Cultures

- Pharmaceutical products that are proteins can be synthesized on a large scale
- Host cells in culture can be engineered to secrete a protein as it is made
- This is useful for the production of **insulin**, **human growth hormones**, **antibodies** and **vaccines**

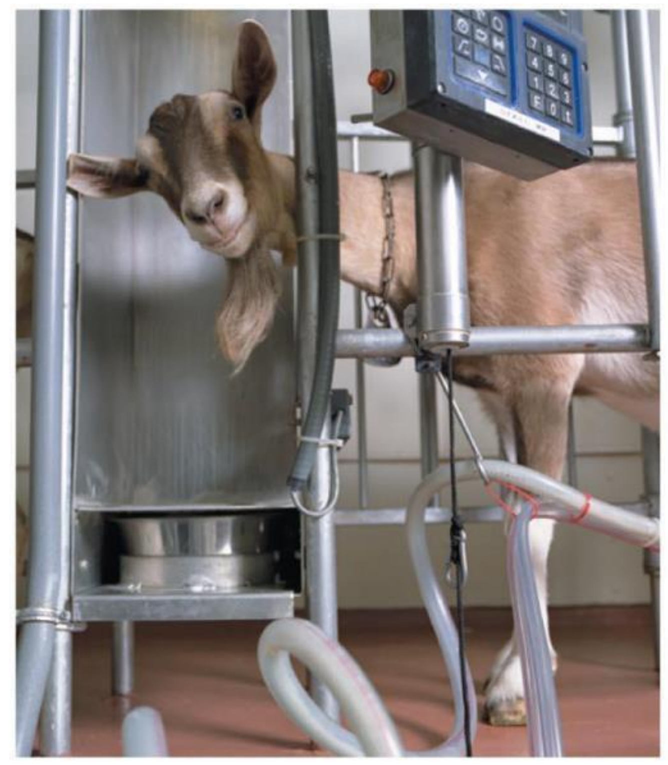
# “Pharm” Animals and Plants

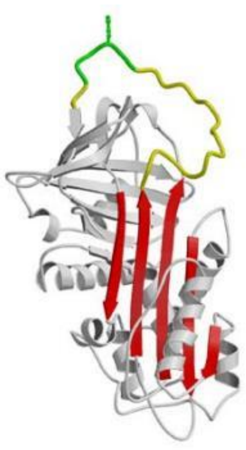
## Protein Production by “Pharm” Animals and Plants

- **Transgenic** animals/plants are made by introducing genes from one species into the genome of another animal/plant
  - Transgenic animals/plants are pharmaceutical “factories,” producers of large amounts of otherwise rare substances for medical use

Fig. 20-24

# Goats as “pharm” animals – milking “antithrombin”





Protein structure of **Antithrombin**

**Transgenic Goat** bred to secret antithrombin into its milk - Such goat was produced by **Nuclear transfer**, performed in a laboratory dish, entails taking a cell carrying the new DNA, which codes for the desired therapeutic protein, and fusing it to a donor goats egg (that has had its genetic material removed). These eggs are then implanted into a surrogate female goat which she will carry to term.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Printed in U.S.A.
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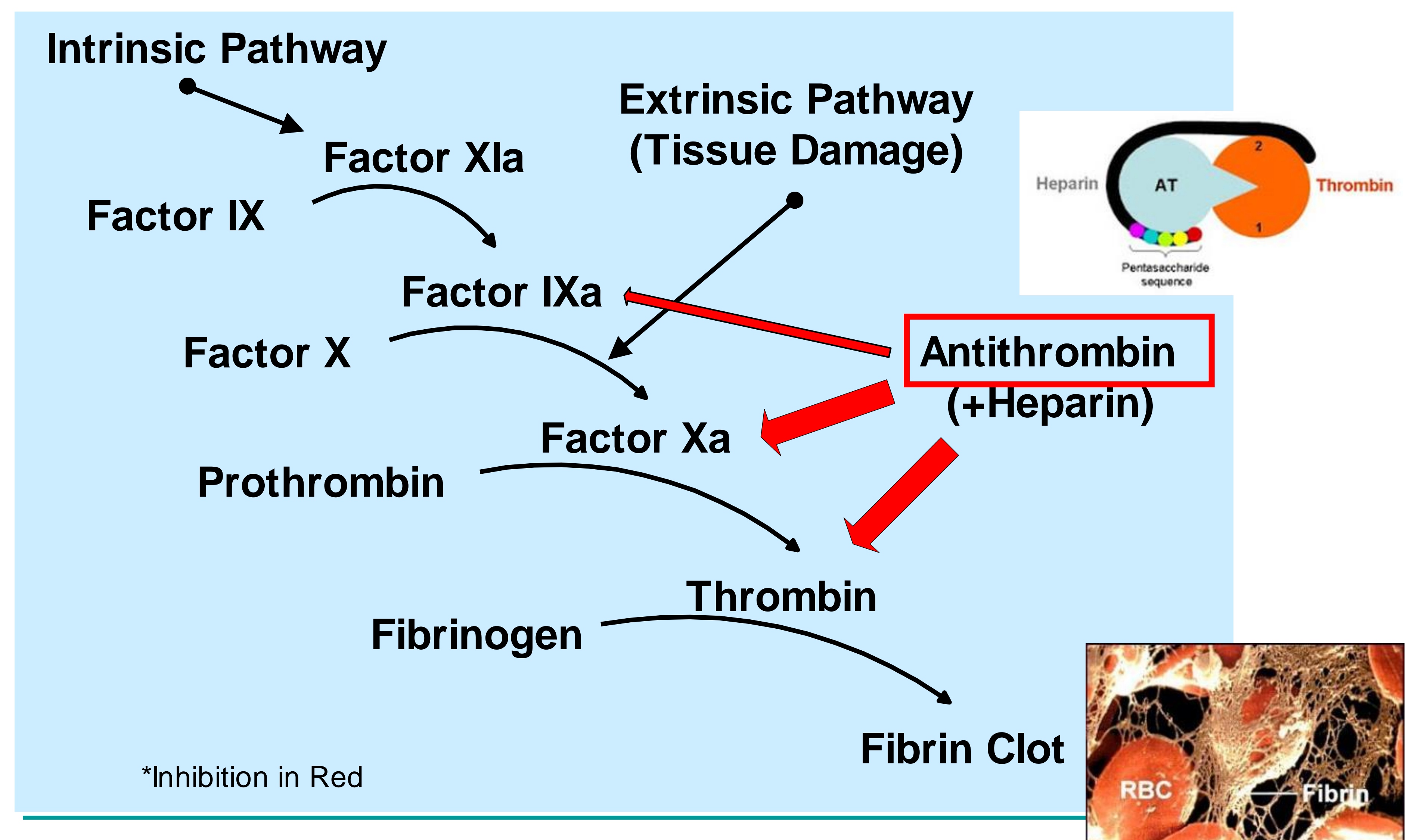
**Heparin Enhances the Specificity of Antithrombin for Thrombin and Factor Xa Independent of the Reactive Center Loop Sequence**

EVIDENCE FOR AN EXOSITE DETERMINANT OF FACTOR Xa SPECIFICITY IN HEPARIN-ACTIVATED ANTITHROMBIN*

Received for publication, December 21, 2000
Published, JBC Papers in Press, February 7, 2001, DOI 10.1074/jbc.M011550200

Yung-Jen Chuang, Richard Swanson, Srikumar M. Raja, and Steven T. Olson‡
*From the Center for Molecular Biology of Oral Diseases, College of Dentistry, University of Illinois, Chicago, Illinois 60612*

# Regulation of the **Blood Clotting Cascade** (凝血 級聯機制) By Antithrombin



# Forensic Evidence and Genetic Profiles

- An individual's unique DNA sequence, or **genetic profile**, can be obtained by analysis of tissue or body fluids
- DNA testing can be used to provide evidence in criminal and paternity cases and to identify human remains
- Genetic profiles are currently analyzed using genetic markers called **short tandem repeats (STRs)**

# Short tandem repeats (STRs)

- **STRs** are variations in the number of repeats of specific DNA sequences (Explanation on next page)
  - PCR and gel electrophoresis are used to amplify and then identify STRs of different lengths
  - The probability that two people who are not identical twins have the same STR markers is exceptionally small

# Short tandem repeat

- STR is a series of repeating units of 2 to 5 (or 2~16) nucleotides
- The repeat number for STRs can vary among sites (within a genome) or individuals

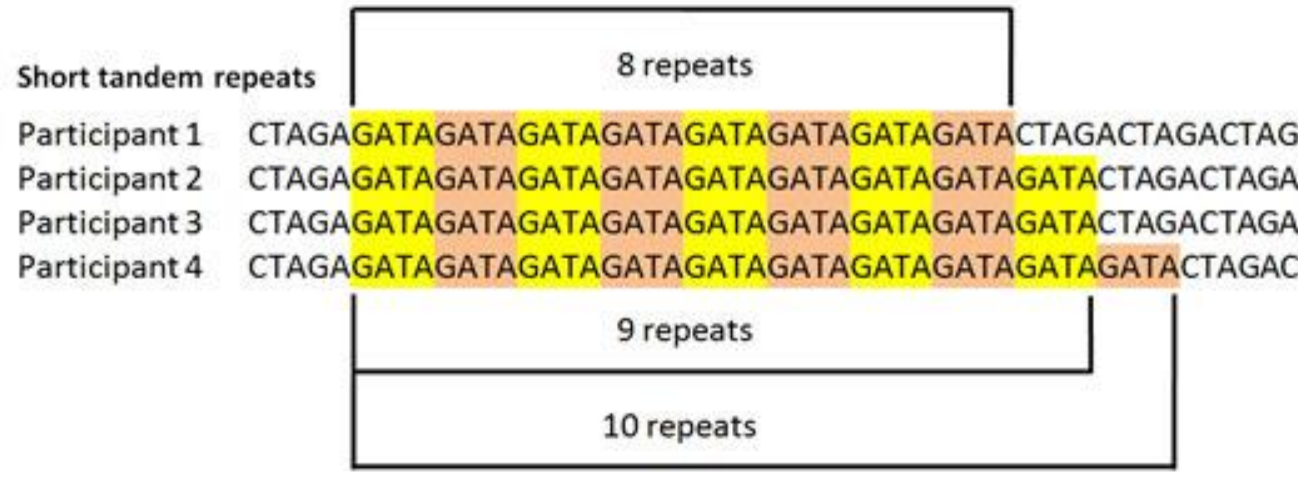


Fig. 19-24

**(a) This photo shows Earl Washington just before his release in 2001, after 17 years in prison.**

Short Tandem Repeats

ACAT

Primer

1 2 3 4 5 6 7 8

Primer

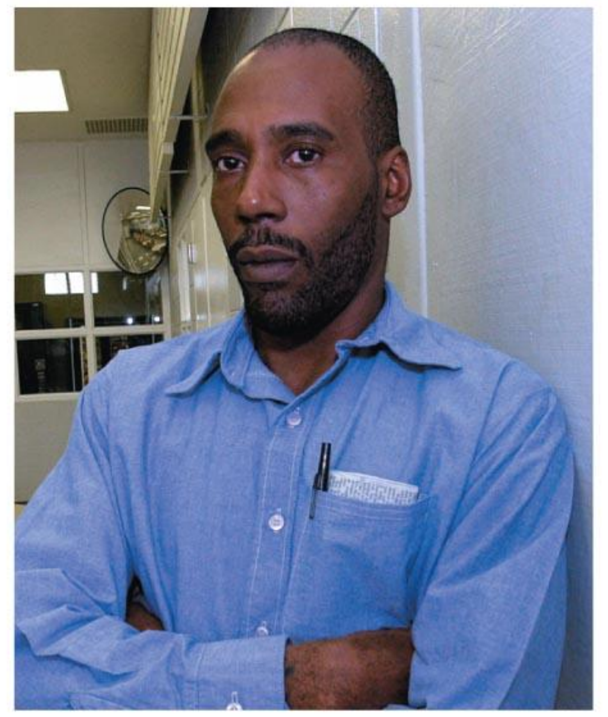
Primer

1 2 3 4 5 6 7

Primer

*The flanking regions where PCR primers bind are constant*

Homozygote = both alleles are the same length
Heterozygote = alleles differ and can be resolved from one another



| Source of sample | STR marker 1 | STR marker 2 | STR marker 3 |
|---|---|---|---|
| Semen on victim | 17, 19 | 13, 16 | 12, 12 |
| Earl Washington | 16, 18 | 14, 15 | 11, 12 |
| Kenneth Tinsley | 17, 19 | 13, 16 | 12, 12 |

No. of repeats

**(b) These and other STR data exonerated Washington and led Tinsley to plead guilty to the murder.**

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# Environmental Cleanup

- Genetic engineering can be used to modify the metabolism of microorganisms
  - Some modified microorganisms can be used to **extract minerals** from the environment or **degrade potentially toxic waste materials ( or oil spills)**
- **Biofuels** make use of crops such as corn, soybeans, and cassava (木薯) to replace fossil fuels

## *Animal Husbandry*

- Genetic engineering of transgenic animals speeds up the selective breeding process
- **Beneficial genes** can be transferred between varieties or species

# Agricultural Applications

- DNA technology is being used to improve agricultural productivity and food quality

Meanwhile, we need to preserve the nature's vanishing **biodiversity** with their DNA codes/Genomes → *Conservation Biology*

李家維教授 熱帶植物保種中心 http://www.kbcc.org.tw/

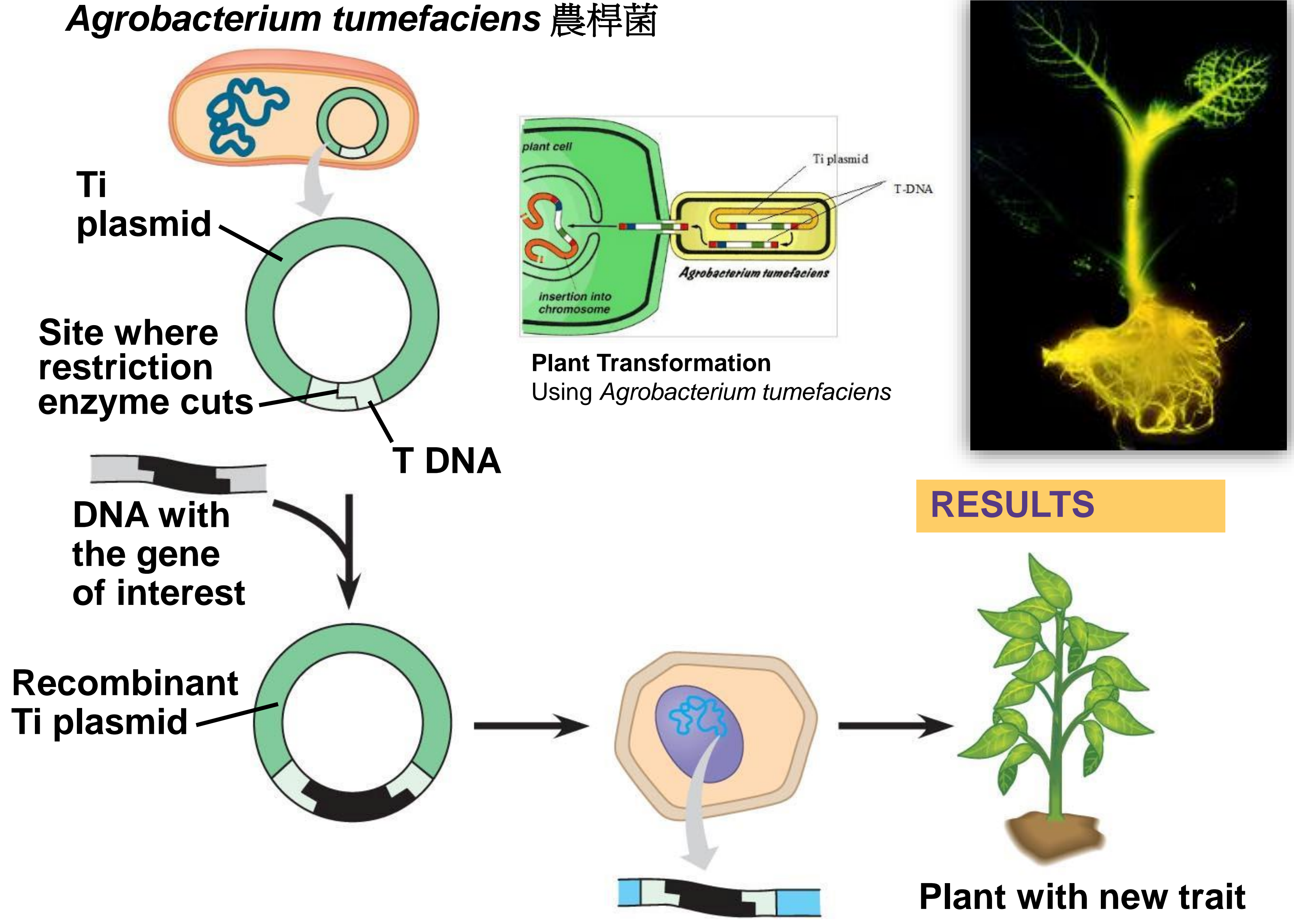
# Genetic Engineering in Plants

- Agricultural scientists have endowed a number of crop plants with genes for desirable traits
- The **Ti plasmid** is the most commonly used vector for introducing new genes into plant cells
  - Recently, CRISPR Cas9 system has been tried for plant genome editing as well
- Genetic engineering in plants has been used to transfer many useful genes including those for herbicide resistance, increased resistance to pests, increased resistance to salinity, and improved nutritional value of crops.

Fig. 20-26

## TECHNIQUE

# Using the Ti plasmid to produce transgenic plants




**Plant Transformation**
Using *Agrobacterium tumefaciens*

# Safety and Ethical Questions Raised by DNA Technology

- Potential **benefits** of genetic engineering must be weighed against potential **hazards** of creating harmful products or procedures
- Guidelines are in place in the United States and other countries to ensure safe practices for recombinant DNA technology

# GMO – genetically modified organism

- Most public concern about possible hazards centers on **genetically modified (GM) organisms** used as food
- Some concerns: (1) the creation of **"super weeds"** from the transfer of genes from GM crops to their wild relatives; (2) allergic reactions

## **GMO from the nature**:
## Solar-powered sea slug harnesses stolen plant genes

Horizontal gene transfer of the algal 藻 nuclear gene *psbO* to the photosynthetic sea slug *Elysia chlorotica* 海蛞蝓

PNAS (2008) Vol 18 No 46 P17867-17871

# Ethical guidelines

- As biotechnology continues to change, so does its use in agriculture, industry, and medicine
- National agencies and international organizations strive to set guidelines for safe and ethical practices in the use of biotechnology

In May 2016, "No substantiated evidence of a difference in risks to human health between GMO crops & conventional crops" was declared by National Academics of Science, Engineering, and Medicine. https://www.ncbi.nlm.nih.gov/books/NBK424543/

## You should now be able to:

1. Describe the natural function of restriction enzymes and explain how they are used in recombinant DNA technology
2. Outline the procedures for cloning a eukaryotic gene in a bacterial plasmid
3. Define and distinguish between genomic libraries using plasmids, phages, and cDNA
4. Describe the polymerase chain reaction (PCR) and explain the advantages and limitations of this procedure

5. Explain how gel electrophoresis is used to analyze nucleic acids and to distinguish between two alleles of a gene
6. Describe and distinguish between the Southern blotting procedure, Northern blotting procedure, and RT-PCR
7. Distinguish between gene cloning, cell cloning, and organismal cloning
8. Describe how nuclear transplantation was used to produce Dolly, the first cloned sheep

9. Describe the application of DNA technology to the diagnosis of genetic disease, the development of gene therapy, vaccine production, and the development of pharmaceutical products
10. Define a SNP and explain how it may produce a RFLP
11. Explain how DNA technology is used in the forensic sciences

12. Discuss the safety and ethical questions related to recombinant DNA studies and the biotechnology industry

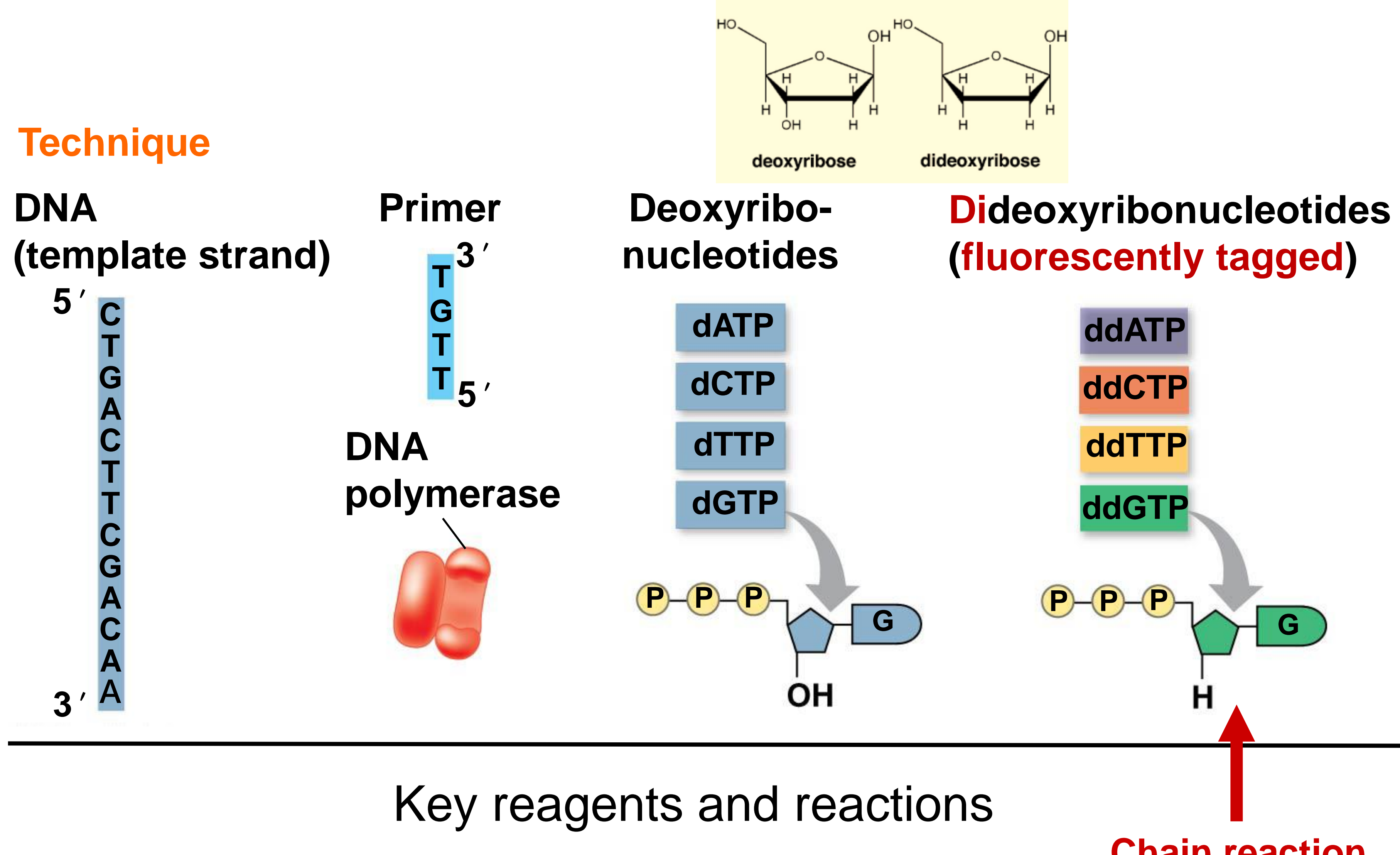
# Supporting information

# DNA Sequencing ( Sanger method)

- Relatively short DNA fragments can be sequenced by the ***dideoxy or chain termination sequencing,*** developed by Sanger
  - Modified nucleotides called dideoxyribonucleotides (ddNTP) attach to synthesized DNA strands of different lengths
  - Each type of ddNTP is tagged with a distinct fluorescent label that identifies the nucleotide at the end of each DNA fragment
  - The DNA sequence can be read from the resulting spectrogram

Figure 19.3a

# DNA sequencing by Dideoxy or Chain Termination method



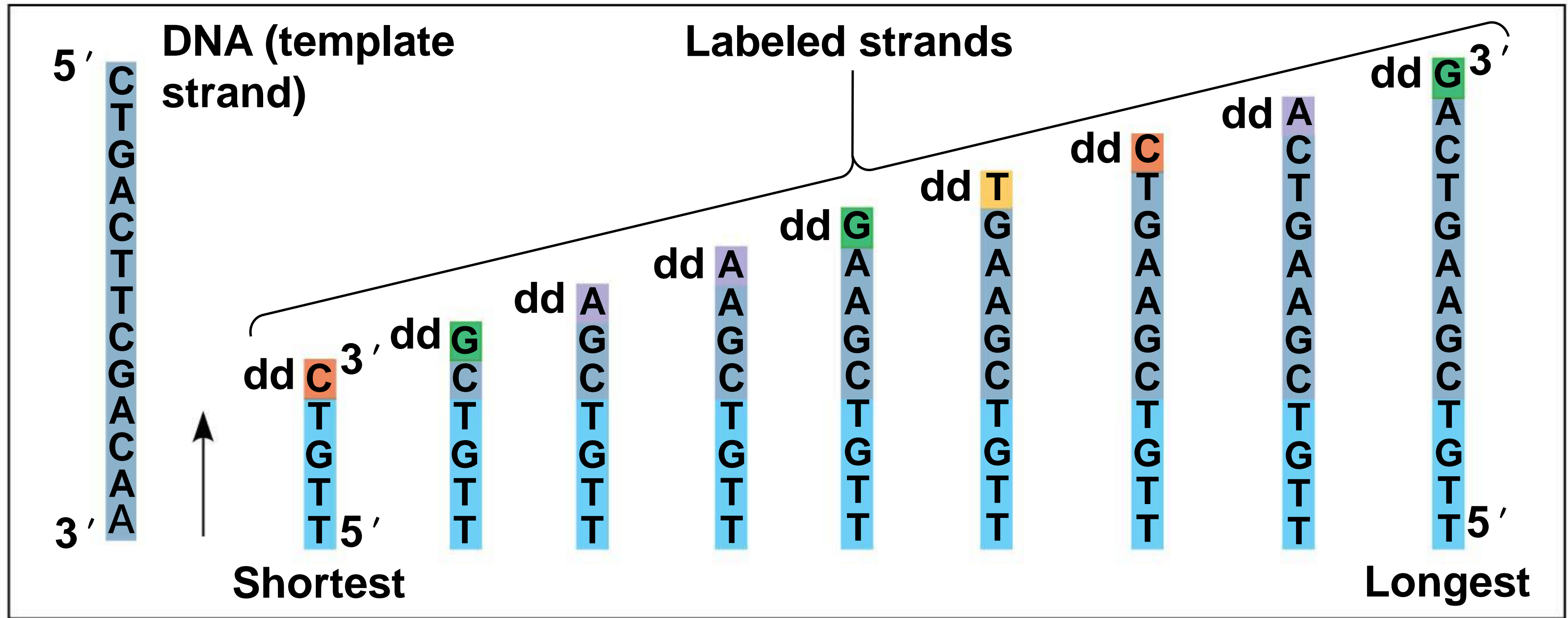

Key reagents and reactions

Chain reaction
terminated

Figure 19.3b

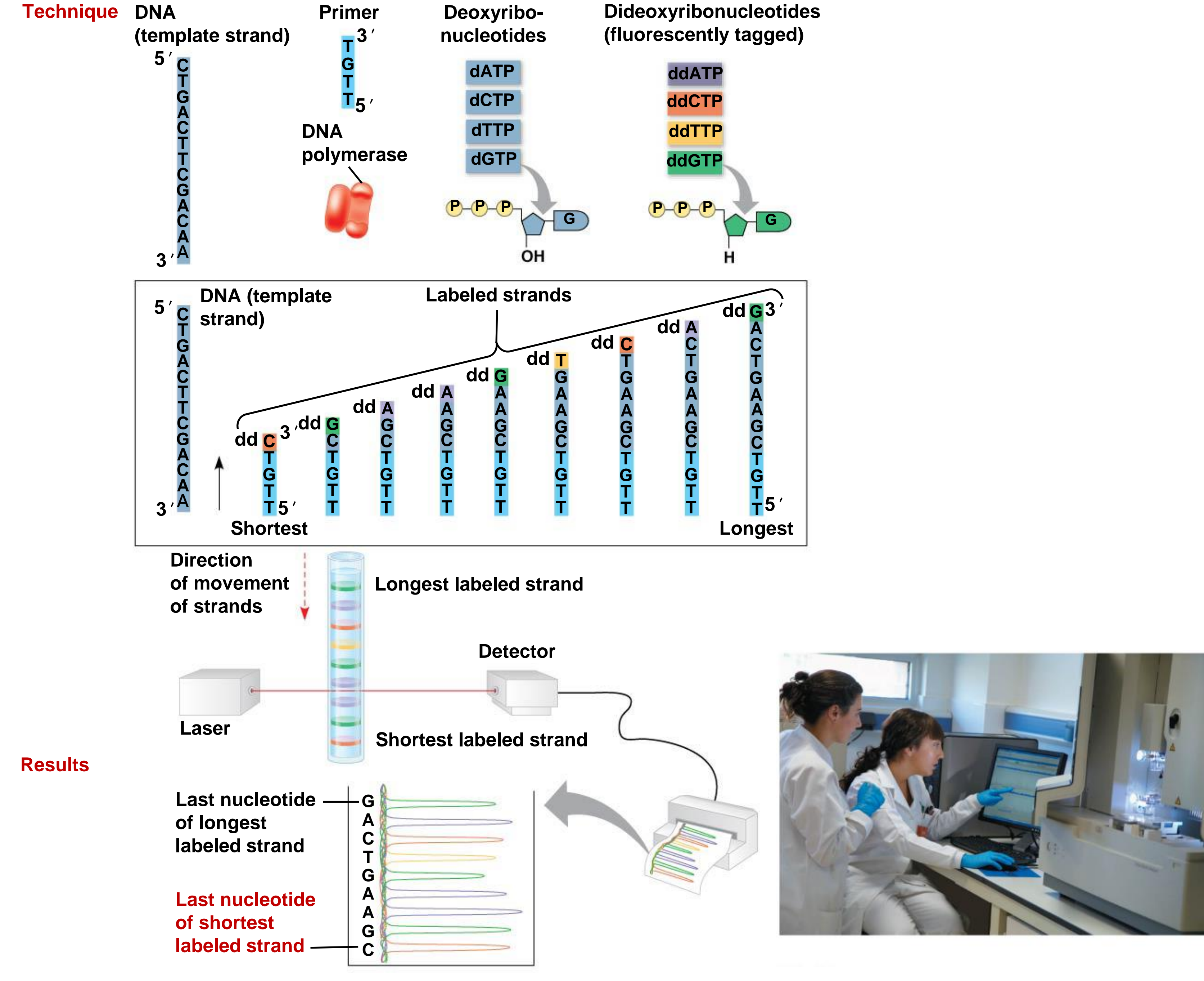
- In the test tube, synthesis of each new strand starts at the same 3' end of the template.
- Due to random termination of the chain reaction, **a set of labeled strands of every possible length** is generation.

## Technique

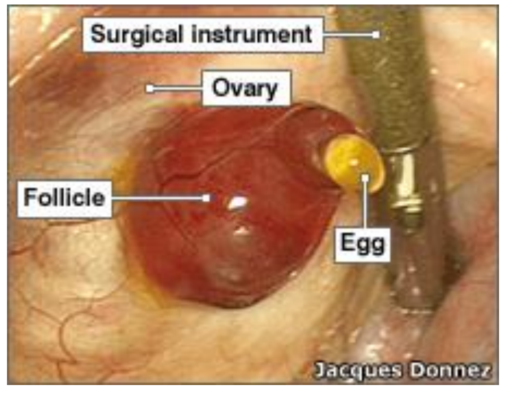


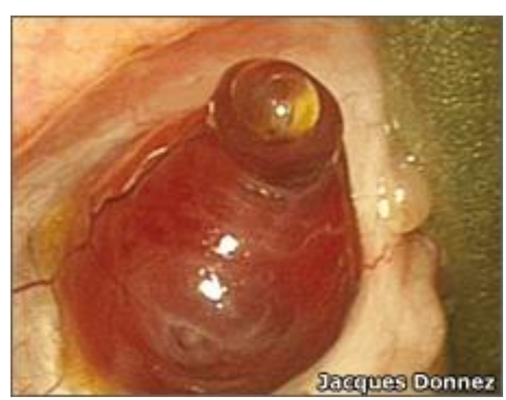

The color of the terminal tag (i.e.. fluorescent molecule) represents the **last nucleotide** in each strand along the sequence.

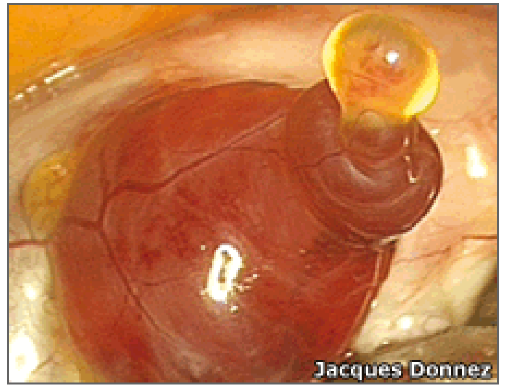
Figure 19.3

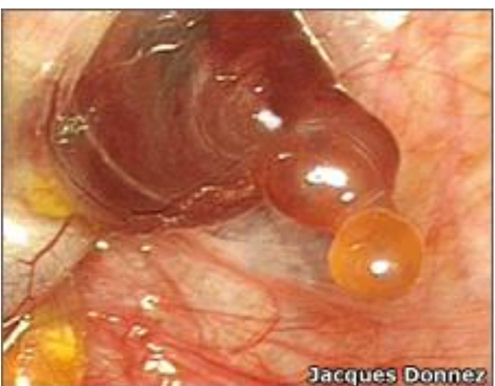


# Human Ovulation

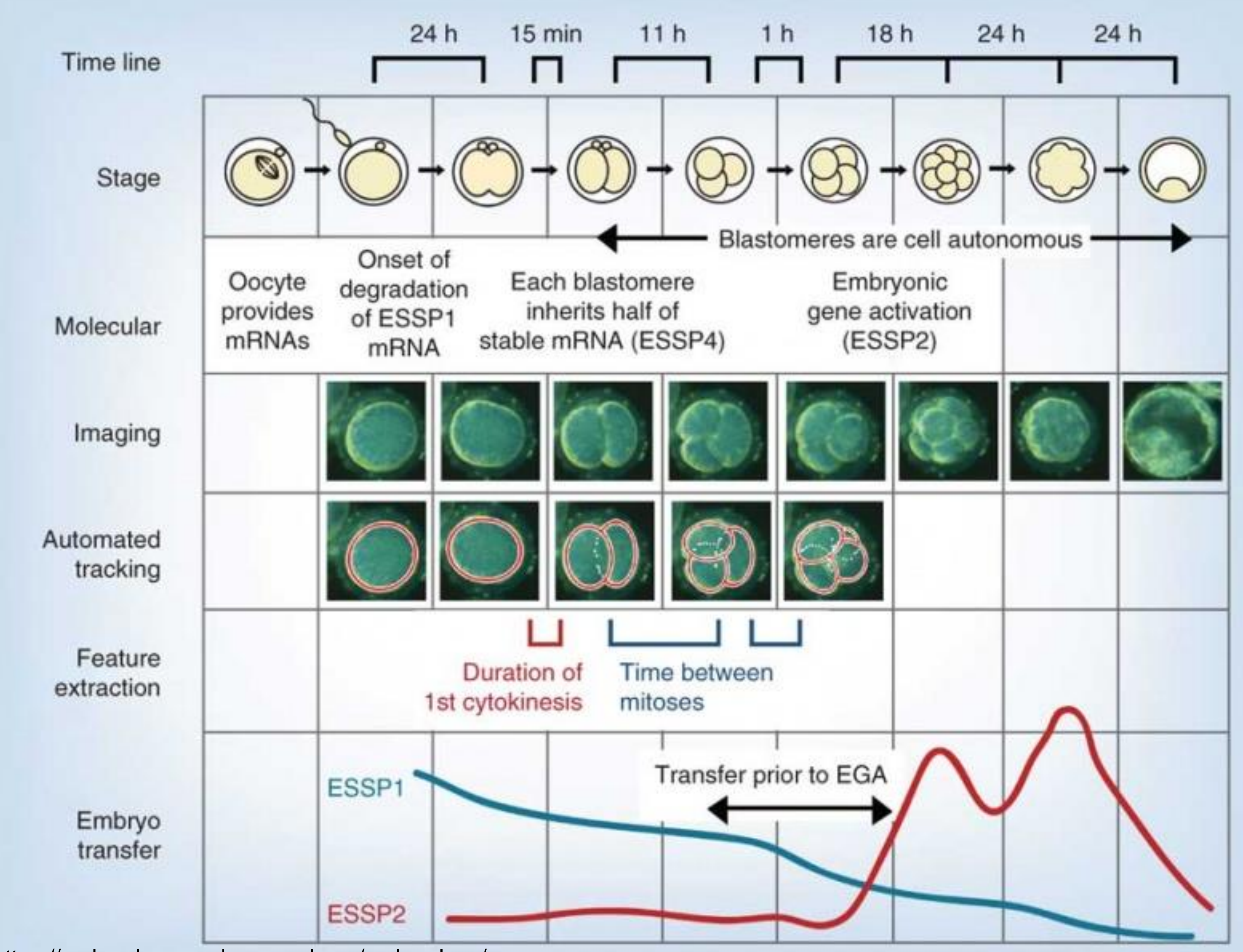


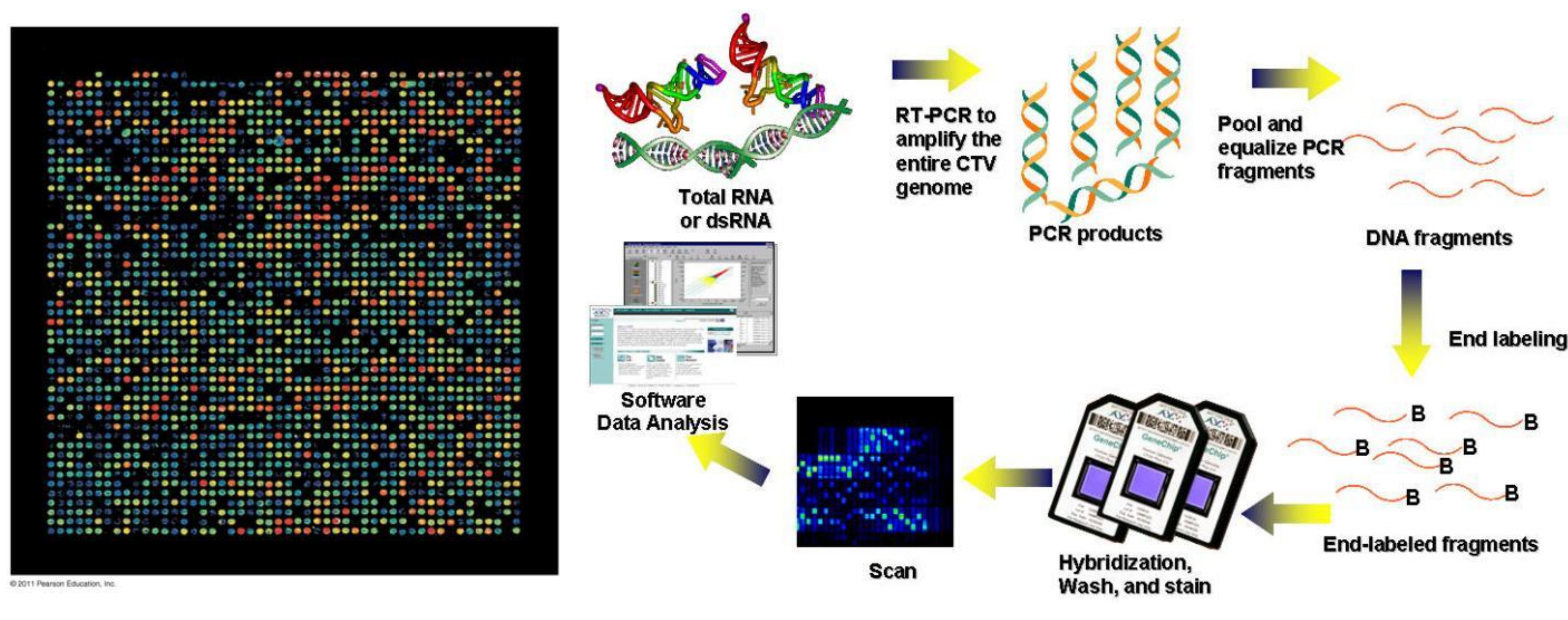




During a hysterectomy of a 45-year-old Belgian woman
Dr. Jacques Donnez, Catholic University of Louvain, Belgium




https://embryology.med.unsw.edu.au/embryology/

An example of DNA technology is the **microarray**, a measurement of gene expression of tens of thousands of different genes



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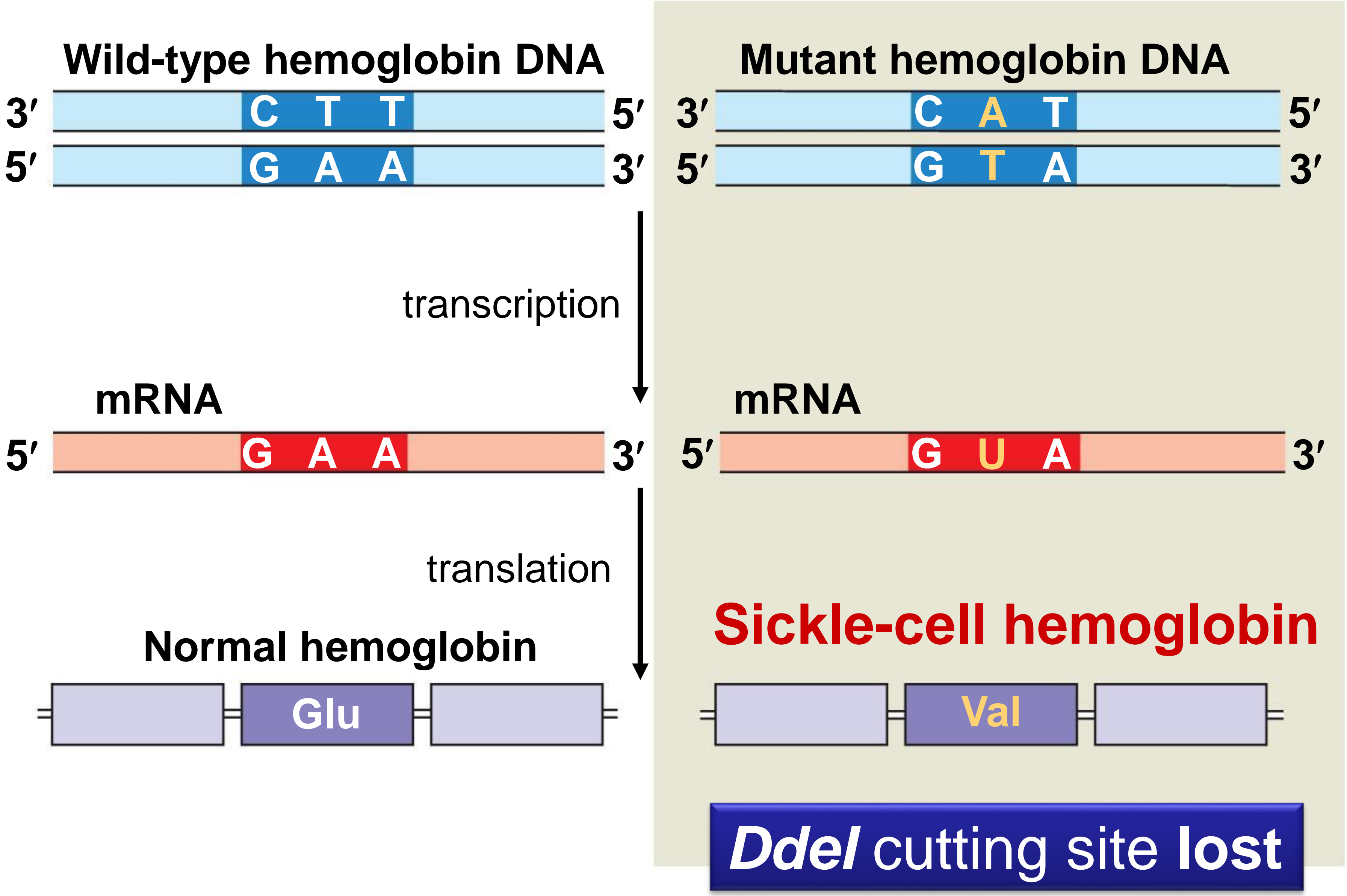
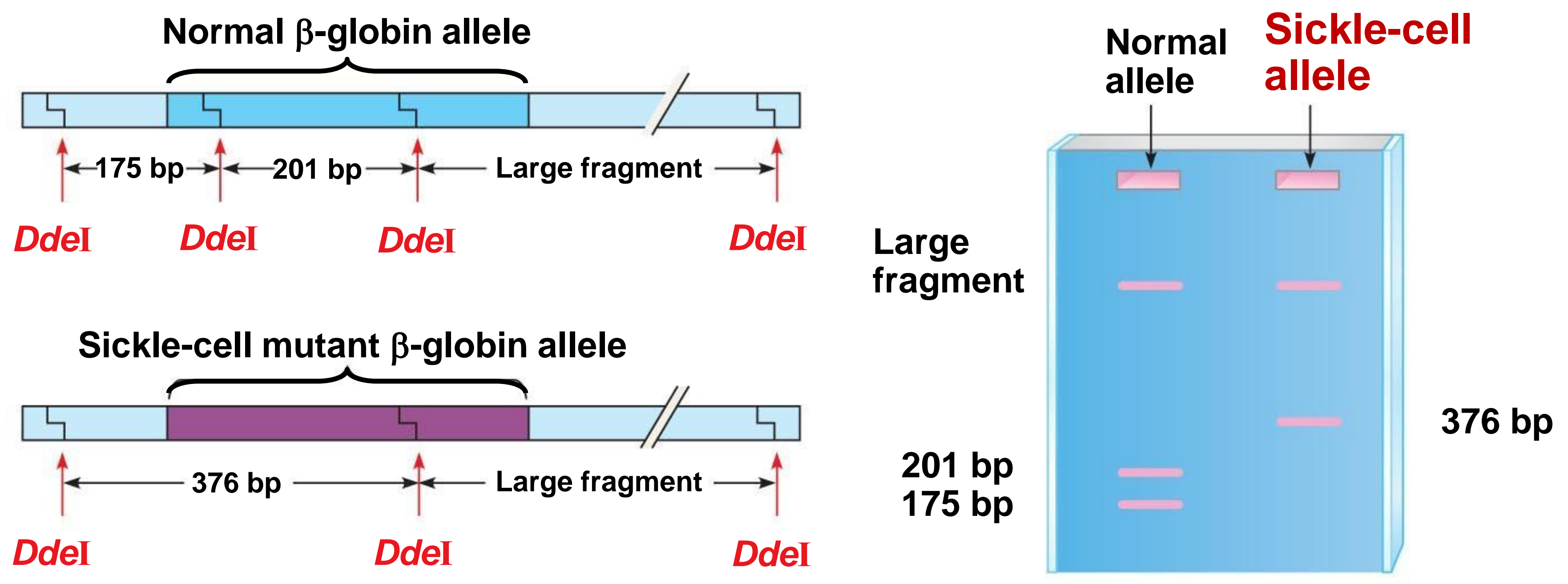
# Restriction fragment analysis (RE digestion diagnostics)

- In *restriction fragment analysis*, DNA fragments produced by **restriction enzyme** digestion of a DNA molecule are sorted by gel electrophoresis
  - A restriction enzyme will usually make many cuts in a DNA molecule
    - Yielding a set of restriction fragments
  - Gel electrophoresis to Separates DNA restriction fragments of different lengths:
  - Small DNA molecules from virus,plasmid– discrete bands
  - Large DNA molecules from eukaryotic chromosome --- smear
- Restriction fragment analysis is useful for comparing two different DNA molecules, such as two alleles for a gene **– restriction fragment length polymorphism (RFLP)**

The molecular basis of sickle-cell disease: a point mutation



# Using restriction fragment analysis to distinguish the normal and sickle-cell alleles of the β-globin gene




(a) *Dde*I restriction sites in normal and sickle-cell alleles of β-globin gene

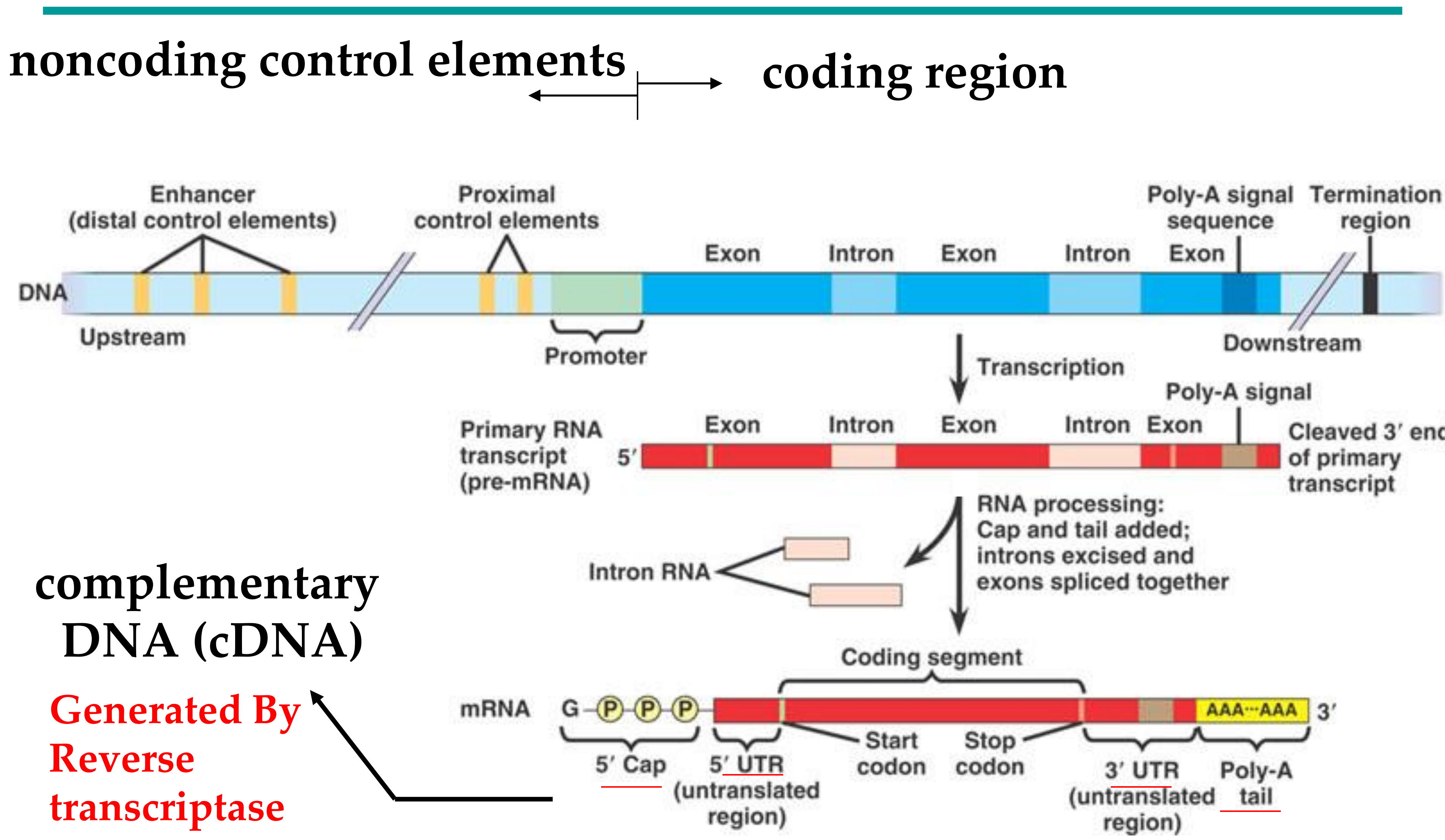
(b) Electrophoresis of restriction fragments from normal and sickle-cell alleles

Molecular Diagnosis (分子診斷)

# *Storing Genetic information in DNA Libraries*

- A **genomic library** that is made using bacteria is the collection of recombinant vector clones produced by cloning DNA fragments from **"an entire genome" -** complete sequence with regulatory region (enhancers), exon, introns, nonconding region, etc.
- A genomic library that is made using bacteriophages is stored as a collection of phage clones

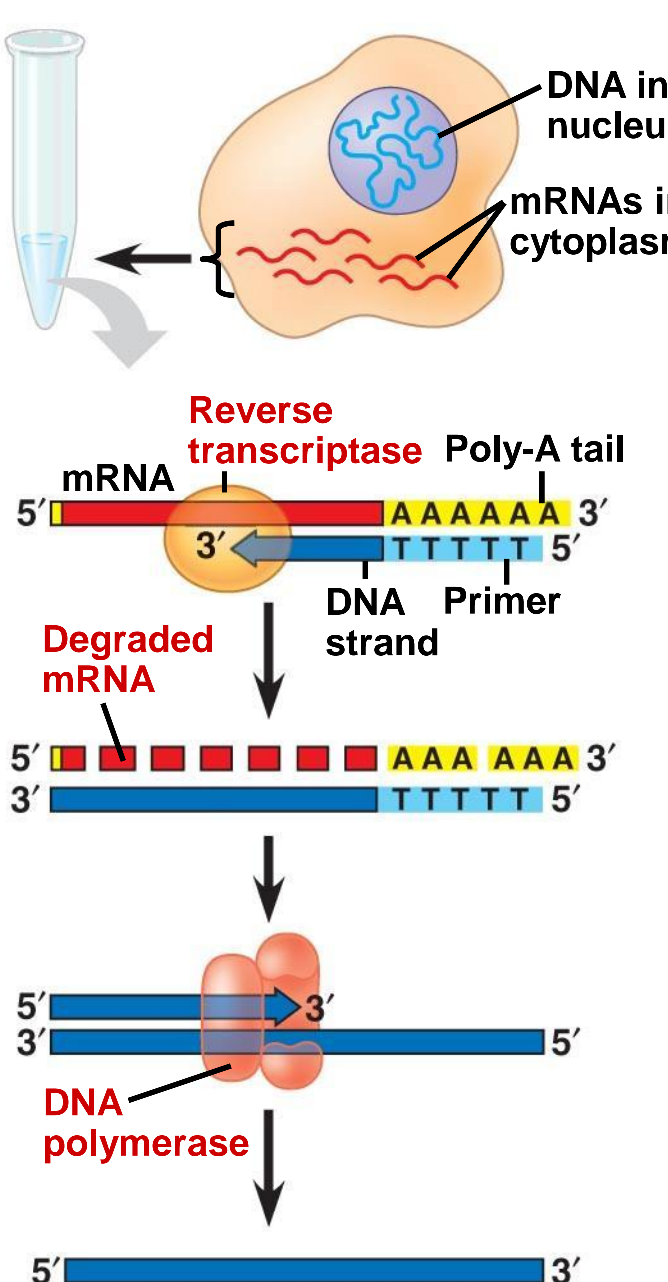
# Organization of eukaryotic genes: with multiple control elements

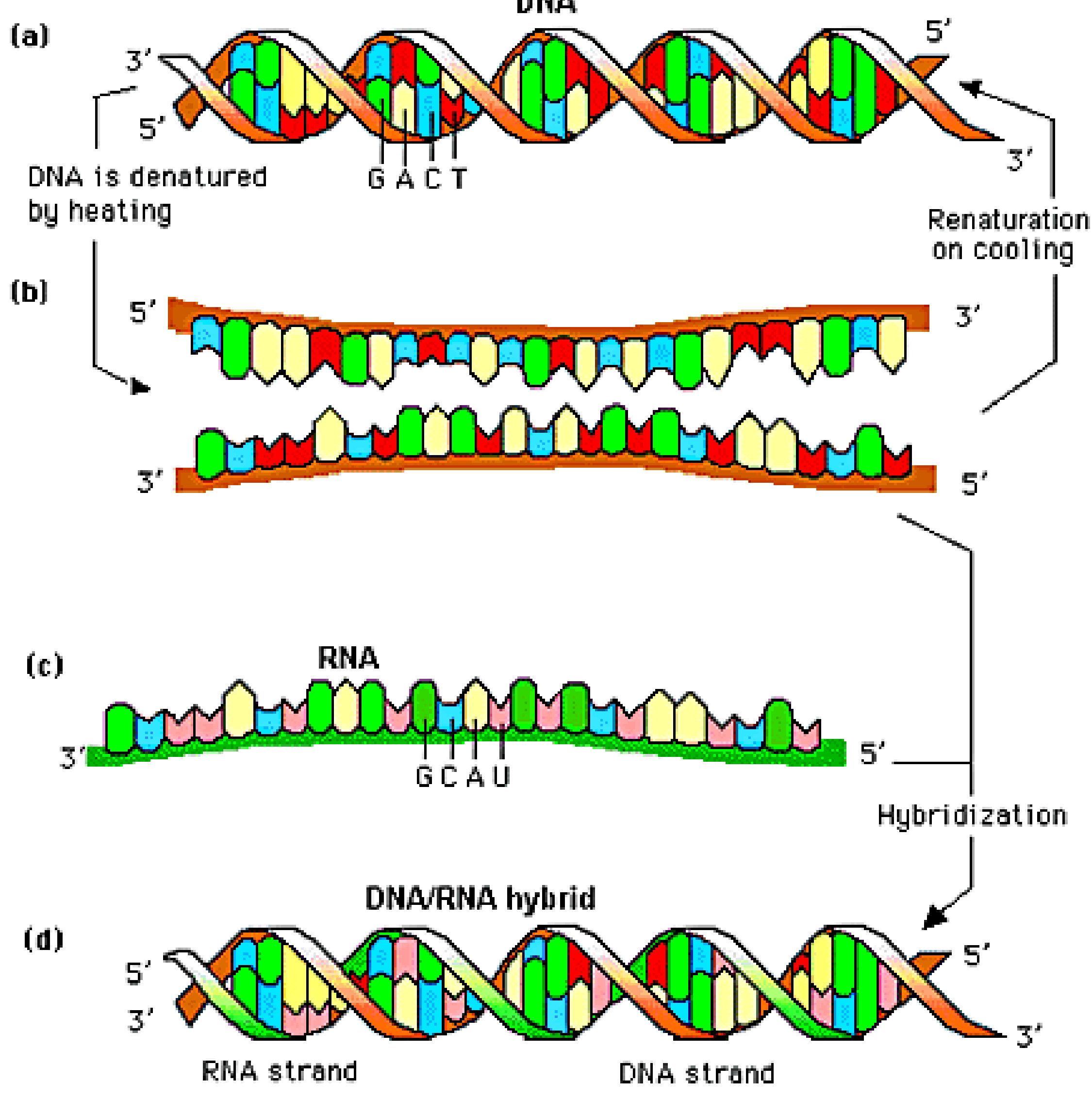


# cDNA library (from mRNAs, 只含已被表現的基因)

- A **complementary DNA (cDNA)** library is made by cloning DNA made *in vitro* by reverse transcription of all the mRNA produced by a particular cell or tissue

- A **cDNA library** represents only part of the genome — only the **subset of genes** transcribed into mRNA in the original cells

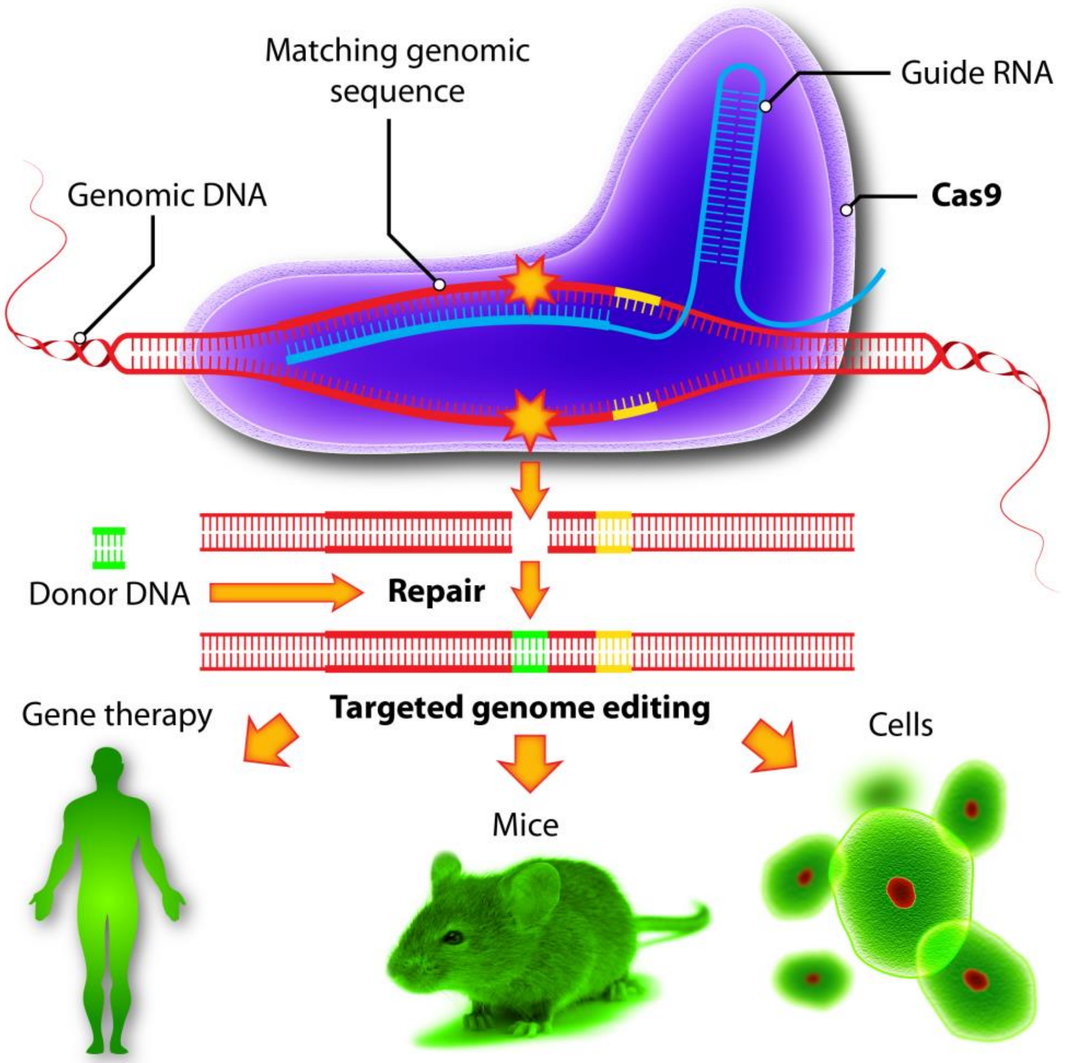

DNA in nucleus
mRNAs in cytoplasm
Reverse transcriptase
Poly-A tail
mRNA
5′
A A A A A A
3′
3′
T T T T T
5′
DNA strand
Primer
Degraded mRNA
5′
A A A A A A
3′
3′
T T T T T
5′
5′
3′
3′
5′
DNA polymerase
5′
3′
3′
5′
cDNA




# Nucleic Acid Hybridization

http://www.accessexcellence.org/RC/VL/GG/nucleic.html

# Genome Editing Technology – CRISPR/Cas9 system




- **CRISPR/Cas9 system** has sparked a revolution in the field of genetic manipulation. Its applications includes targeted gene mutation, transgenic animals/plants production, endogenous gene labeling, and gene therapy.

- CRISPR/Cas system is a form of acquired immune system used by many bacteria and archaea species to provide resistance to viruses and other foreign genetic material.

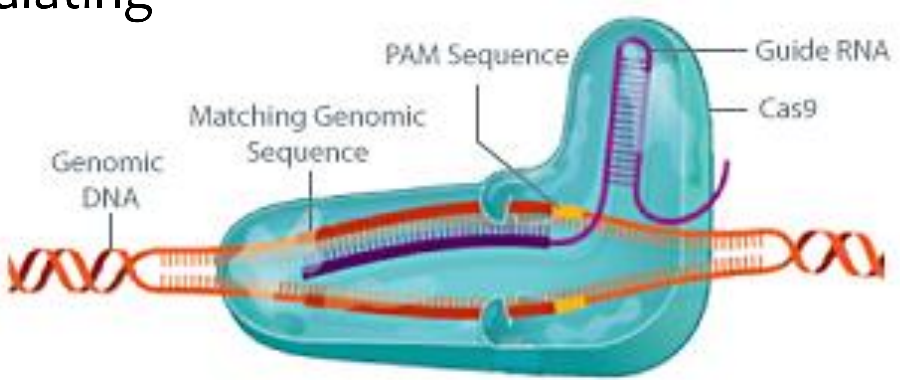
CRISPR/Cas9 technology. Image source: IGTRCN

# CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS (CRISPRS) AND CRISPR-ASSOCIATED (CAS) PROTEINS

# CRISPR/CAS9 SYSTEM

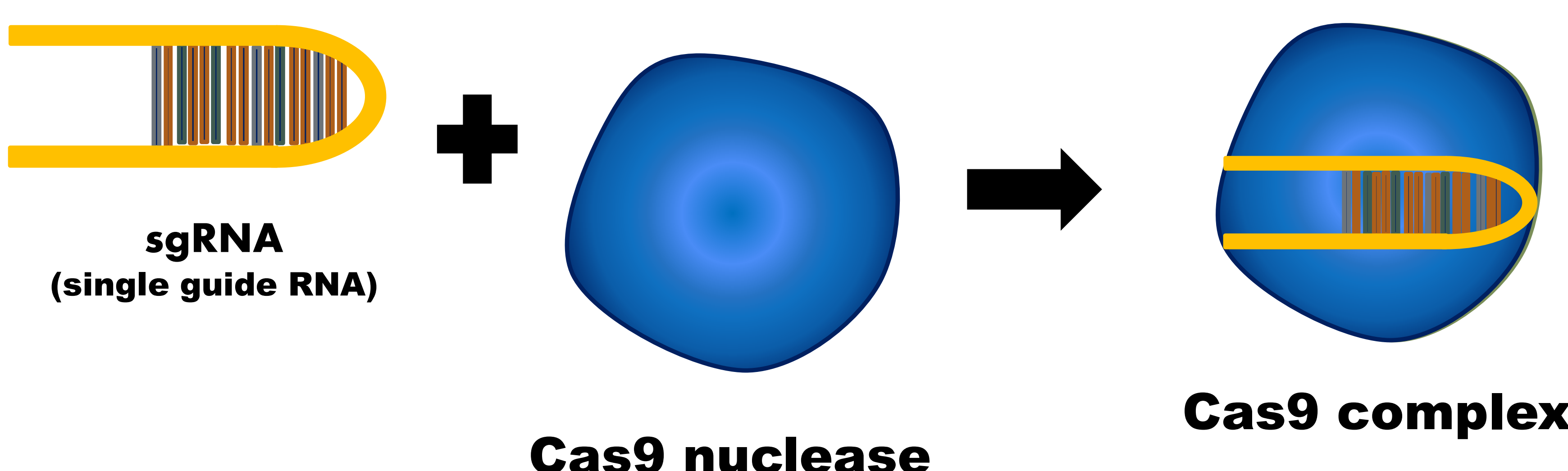
# CRISPR/Cas9 System

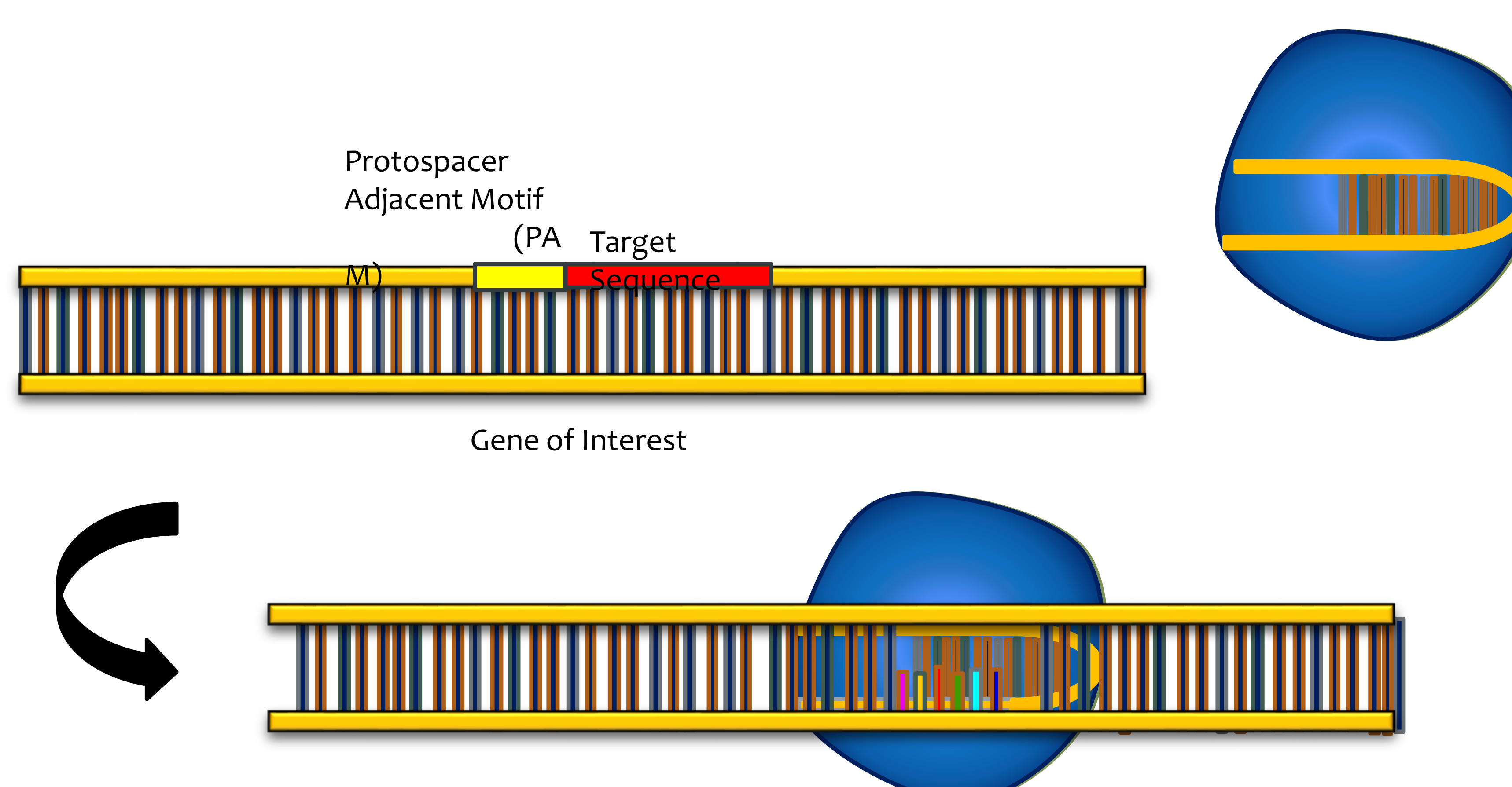
- derived from a natural process found in bacteria to protect themselves from pathogens
- targets genes for editing and regulating
- comparable to Photoshop

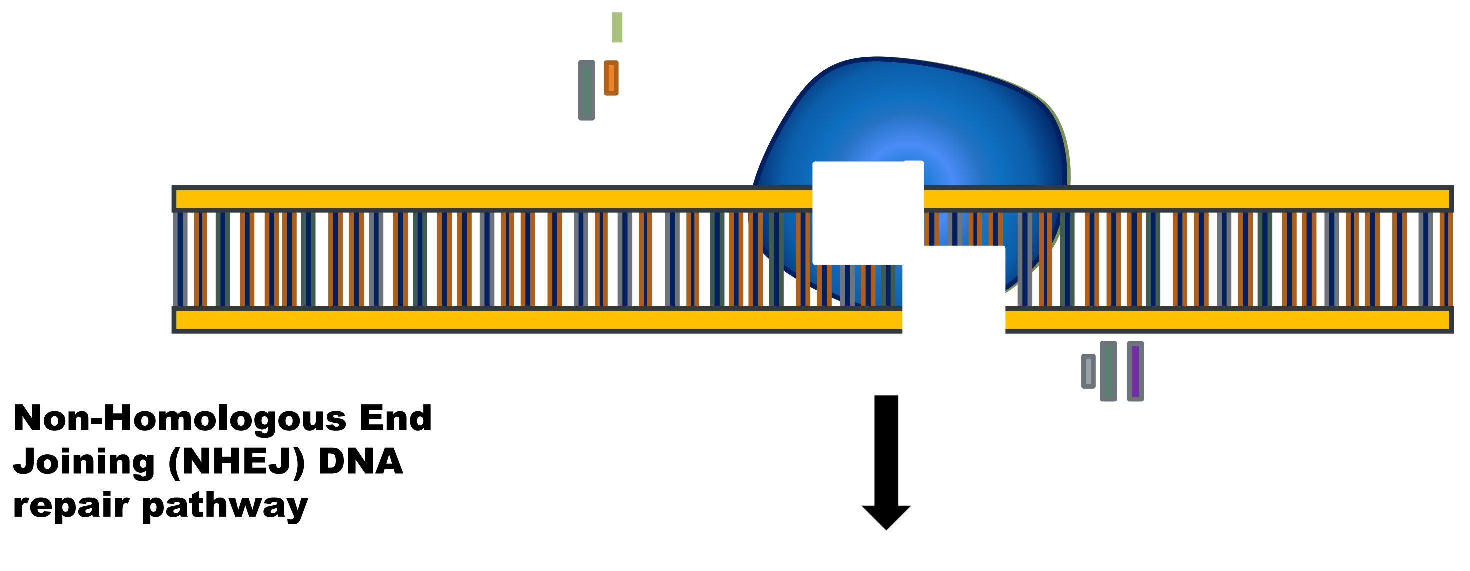



Horizon Licenses Harvard University Gene-Editing Technology. (2013). *Drug Discovery & Development*.

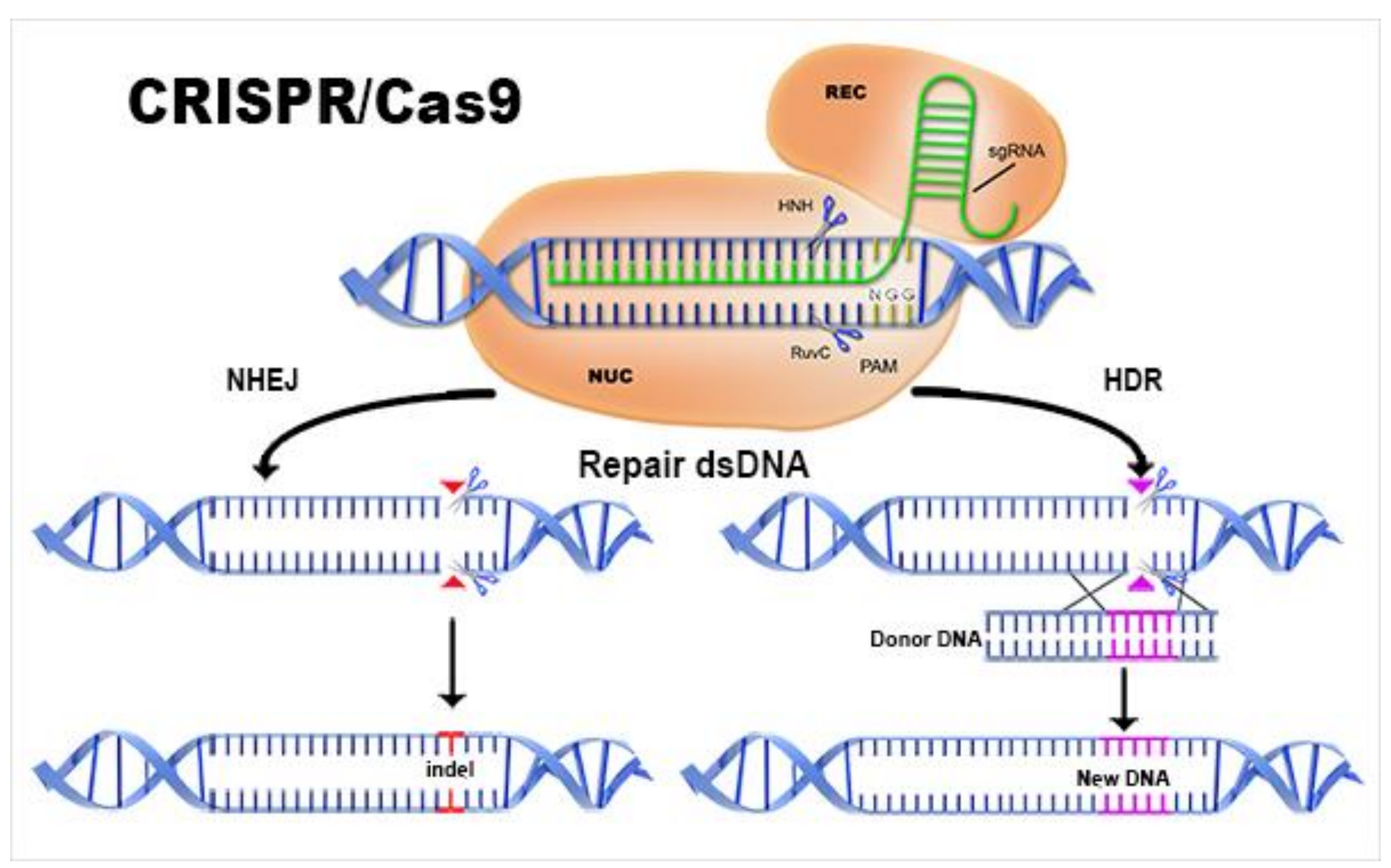
# CRISPR/Cas9 System




Protospacer
Adjacent Motif
(PA
M)
Target
Sequence
Gene of Interest



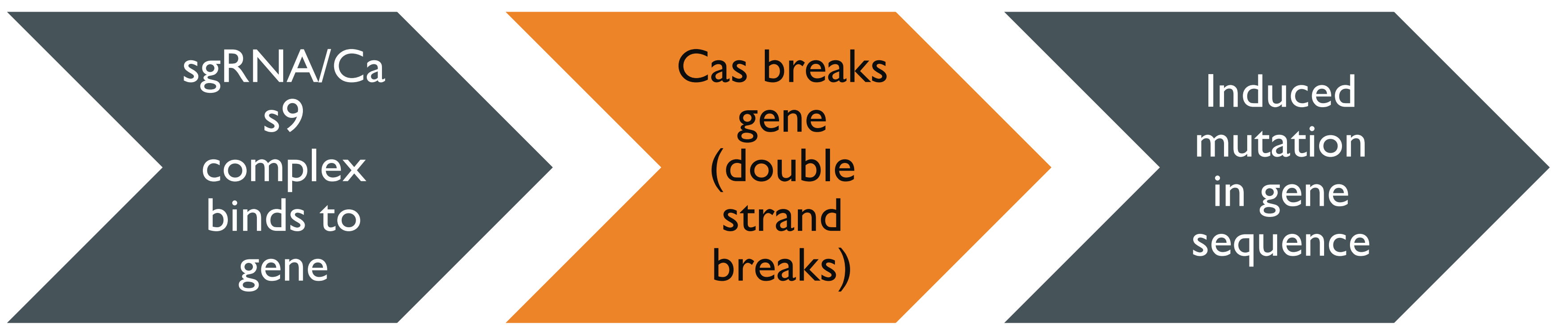
Stop Codon




**Non-homologous end joining** (NHEJ)

**Homology directed repair** (HDR)

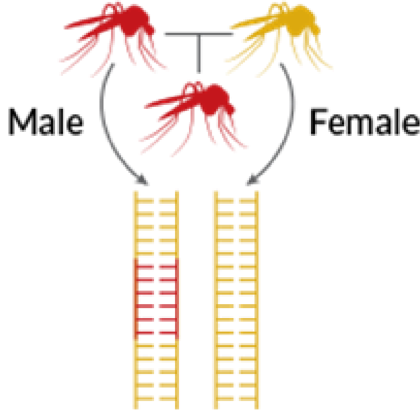
# SUMMATION: CRISPR/CAS SYSTEM



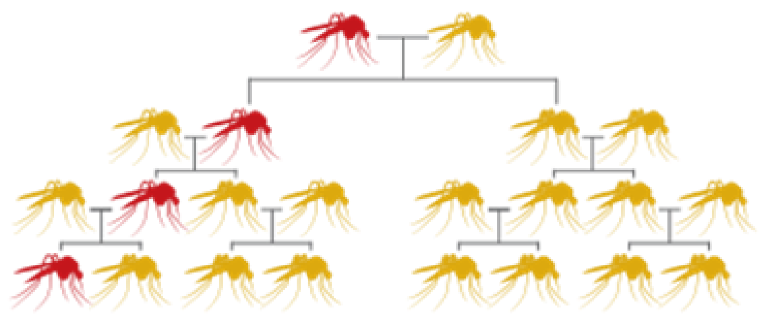

**altered gene sequence → dysfunctional CAD gene**

## Normal inheritance

**Altered gene** **Wild type**



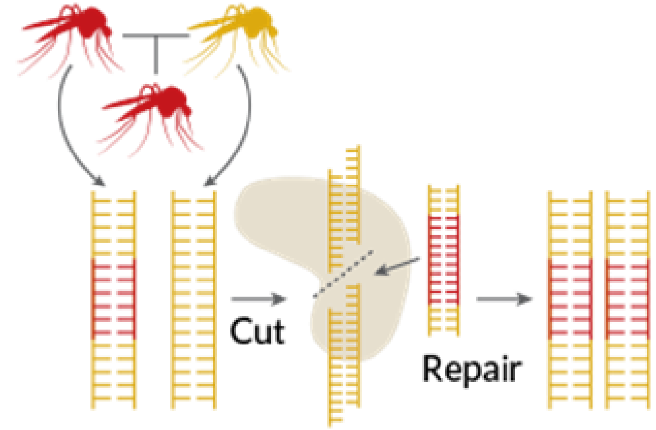

Altered gene without gene drive: One copy inherited from one parent. 50 percent chance of passing it on.



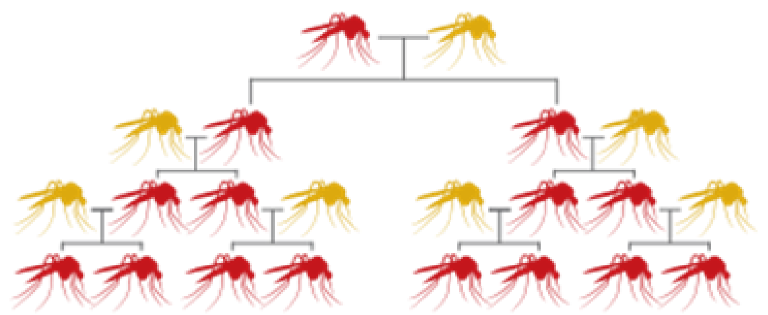
Altered gene does not spread

## Gene drive inheritance

**Gene drive** **Wild type**




Altered gene as gene drive: One copy converts gene inherited from other parent. More than 50 percent chance of passing it on.



Altered gene is almost always inherited