# Molecular technique in radiobiology

黃英彥　醫師

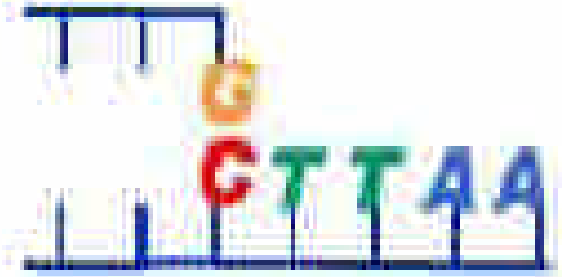
97/04/21

# Restriction endonucleases

- 限制內切酶可對具有特定核苷酸序列的DNA 進行切割。所謂的切割是打斷DNA 的phosphodiester bond，且斷裂點會發生於DNA 的雙股，因此可造成DNA 斷裂。
- 限制酶的生理功能：細菌中存在一種restriction-modification 系統，對於防禦嗜菌體(bacteriophage) 的感染扮演關鍵角色。限制酶 與DNA methylases 是這個系統中的兩個主角，前者可以辨認DNA 上特定的序列，並水解phosphodiesterbonds；後者則對限制酶辨認的序列進行甲基化(methylation) 修飾，一但序列上有甲基化修飾，限制酶即無法對其作用。DNA methylases 可對細菌染色體DNA 上的特定序列進行甲基化修飾，保護染色體DNA 不會受到細菌自己的限制酶水解，但入侵的噬菌體DNA 由於不具有甲基化修飾或甲基化修飾形式不同，細菌的限制酶可將之水解，使其複製與增殖受到限制。

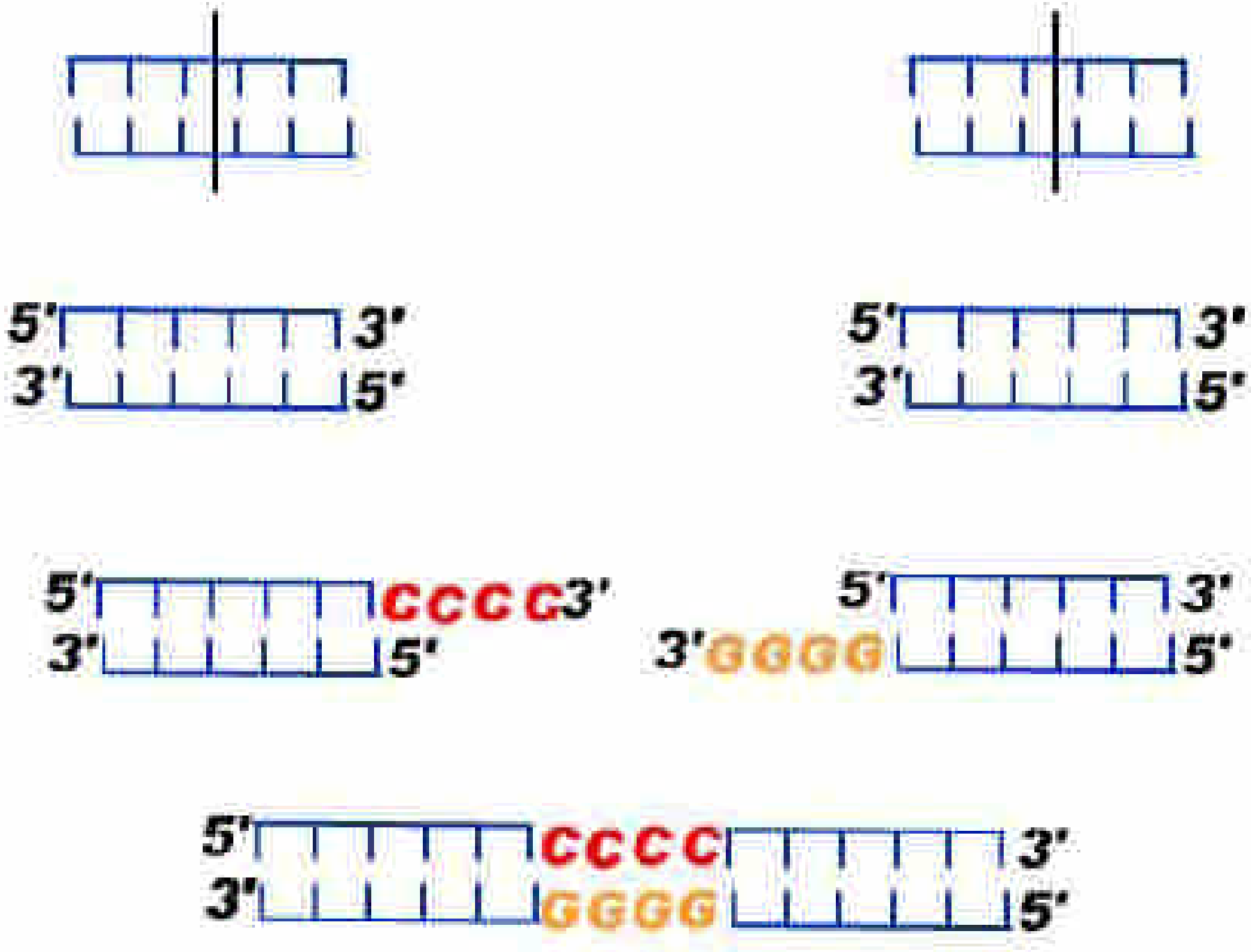
# Restriction endonucleases

- 許多限制內切酶切割的DNA 片段會產生黏性端(sticky ends)。例如*Eco*RI會在每一個DNA 片段的兩端，產生單股的四個鹼基的尾巴。而此特有的單股尾巴會趨向於另一互補的單股尾巴，以鹼基配對的方式接合在一起，故此種特有的單股尾巴被稱爲黏性端。
- 兩個不同的DNA 片段若有相同的黏性端，則有機會互補配對，再加上DNA ligase形成phosphodiester bond，便可進行in vitro site specific 遺傳重組。



# Restriction endonucleases

- 有些限制內切酶切割出齊平端 (blunt end)，進行遺傳重組時，可以用terminal deoxynucleotidyltransferase在切口的3’端接上核苷酸（不需模板，只接上一股），製造出sticky end。


5'
3'
3'
5'
5'
3'
3'
5'
5'
CCCG
3'
3'
5'
5'
3'
3'GGGG
5'
5'
CCCC
3'
3'
GGGG
5'

# Restriction endonucleases

- 限制酶的種類：主要可分爲Type I、Type II 及Type III 三大類。
  - Type I 及Type III：通常是由數個次單元體組成，同時具有endonuclease 及methylase 的活性。這兩類酵素對phoshodiester bond 水解的位置（以下簡稱爲切位）與辨識的序列不同。Type I 酵素需要ATP 的參與，ATP 提供酵素由辨識區移動到距離1 ~ 10kb 之處作用所需的能量；Type III 酵素則不需要ATP 參與反應，其辨識序列與切位通常相距100 bp 以內。
  - Type II：這類酵素僅具有endonuclease 活性，不具methylation 的作用。大部分的Type II 限制　對DNA 之切位就在辨識序列上，不需要ATP 參與反應，但通常需要二價金屬離子Mg2+ 作爲cofactor。三類限制酶中以此類最具實際應用價值。

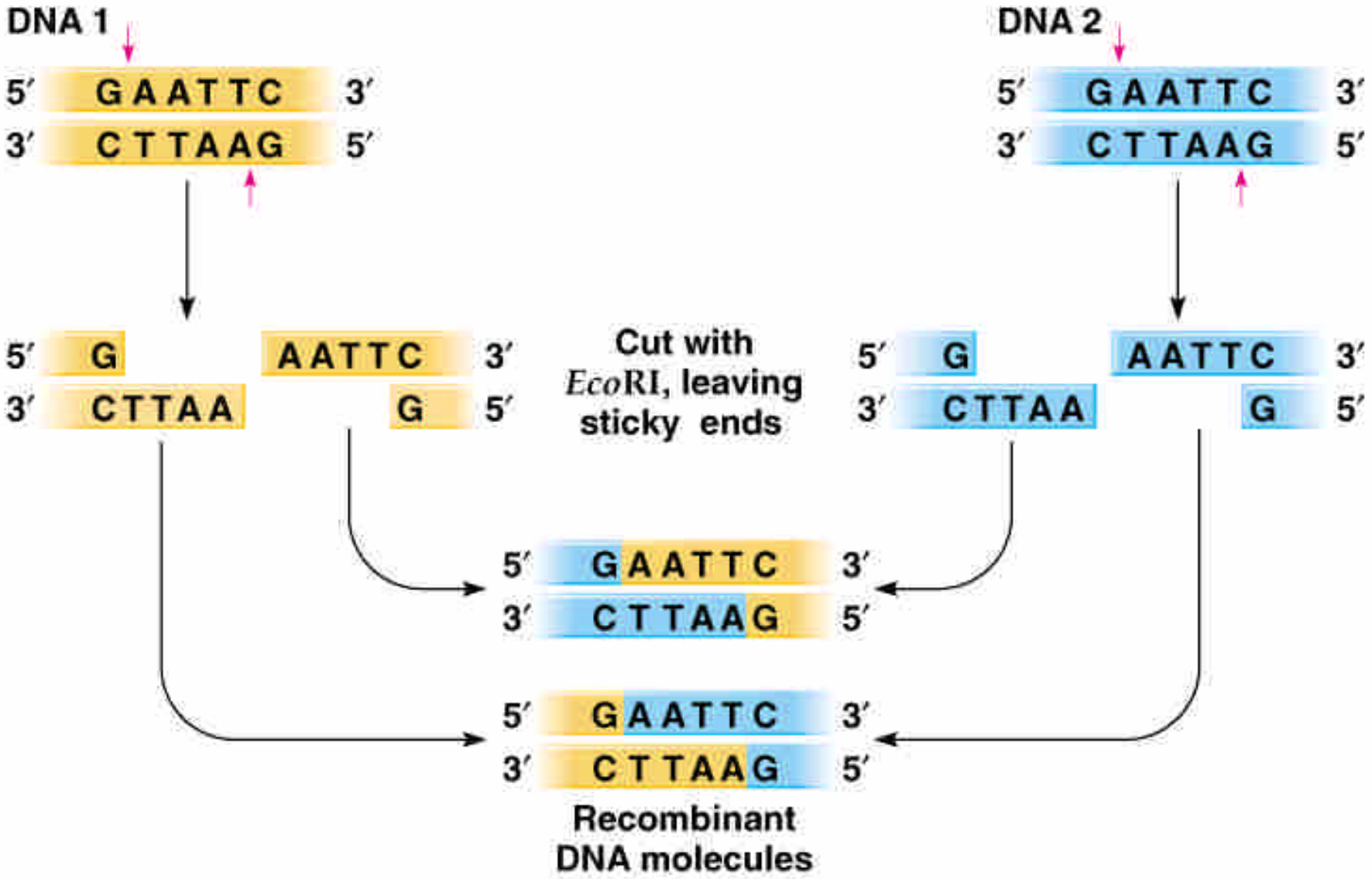
# Restriction endonucleases

- 限制酶之命名：依據其來源細菌命名，由三至四個英文字母及一個羅馬數字組成，前三個字母依序爲來源細菌屬名的第一個字母及種名的前兩個字母，第四個字母來自菌株(strain)名，羅馬數字則代表在同一菌種中被發現的順序。
- 例如，*Eco*RI:
  - *E* = genus *Escherichia*
  - *co* = species *coli*
  - R = strain RY13
  - I = first endonuclease identified
- 例如， *Bgl*II：
  - *B* = genus *Bacillus*
  - *gl* = species *globigii*
  - II = second endonuclease identified

# Restriction endonucleases

- 限制酶的應用：限制酶的應用相當廣泛，例如重組DNA (recombinant DNA) 的建構、DNA 的檢定等都可以利用限制酶來進行。
  - 1) 重組DNA 分子的建構：利用適當的限制酶將兩種不同的DNA 作用成sticky ends，使彼此的5’與3’端序列互補而能黏合在一起，再用DNA ligase 將缺口的phosphodiester bonds 補起來；或是以限制酶將兩種DNA 作用成blunt ends，再以DNA ligase 將兩種DNA 末端接合而形成重組DNA。
  - 2) DNA 的檢定：在DNA 上，限制酶能夠作用的位置及次數隨DNA 序列不同而不同，有時兩種DNA 僅存在1 bp 的差異，但就足以造成限制酶作用結果的不同，因此可以利用限制酶作爲檢定DNA 的工具及建立DNA 的physical map（稱爲限制圖譜，restriction map）。

# Fig. 7.3 Cleavage of DNA by the restriction enzyme *Eco*RI




Peter J. Russell, *iGenetics*: Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.

# Vectors

- Definition: synthetic DNA to clone or delivering foreign DNA by modifying nature plasmid or virus particle or chromosome for recombination purpose.
- Classification:
  - Plasmid: the bacterium origin. insert size 1-10Kb.
  - Bacteriophage: such as lambda phage. insert size 5-40Kb
  - Cosmid: the lambda phage derivative contains the *cos* (stands for cohesive) sites of phage. insert size around 50Kb
  - Artificial chromosome: use in the genomics study
    - yeast artificial chromosome (YAC). insert size 100-2000Kb.
    - bacterial artificial chromosome (BAC). insert size around 300Kb.

Copyright © The McGraw-Hill Companies, Inc. Permission required for reproduction or display.

## Table 14.3
## Some Recombinant DNA Cloning Vectors

| Type | Vector | Restriction Sequences Present | Features |
|---|---|---|---|
| Plasmid (*E. coli*) | pBR322 | *Bam*HI, *Eco*RI, *Hae*III, *Hin*dIII, *Pst*I, *Sal*I, *Xor*II | Carries genes for tetracycline and ampicillin resistance |
| Plasmid (yeast–*E. coli* hybrid) | pYe(CEN3)41 | *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I | Multiplies in *E. coli* or yeast cells |
| Cosmid (artificially constructed *E. coli* plasmid carrying lambda cos site) | pJC720 | *Hin*dIII | Can be packaged in lambda phage particles for efficient introduction into bacteria; replicates as a plasmid; useful for cloning large DNA inserts |
| YAC (yeast artificial chromosome) | pYAC | *Sma*I, *Bam*HI | Carries gene for ampicillin resistance; multiplies in *Saccharomyces cerevisiae* |
| BAC (bacterial artificial chromosome) | pBAC108L | *Hin*dIII, *Bam*HI, *Not*I, *Sma*I, and others | Modified F plasmid that can carry 100–300 kb fragments; has a *cosN* site and a chloramphenicol resistance marker |
| Virus | Charon phage | *Eco*RI, *Hin*dIII, *Bam*HI, *Sst*I | Constructed using restriction enzymes and a ligase, having foreign DNA as its central portion, with lambda DNA at each end; carries β-galactosidase gene; packaged into lambda phage particles; useful for cloning large DNA inserts |
| Virus | Lambda 1059 | *Bam*HI | Will carry large DNA fragments (8–21 kb); recombinant can grow on *E. coli* lysogenic for P2 phage, whereas vector cannot |
| Virus | M13 | *Eco*RI | Single-stranded DNA virus; useful in studies employing single-stranded DNA insert and in producing DNA fragments for sequencing |
| Plasmid | Ti | *Sma*I, *Hpa*I | Maize plasmid |

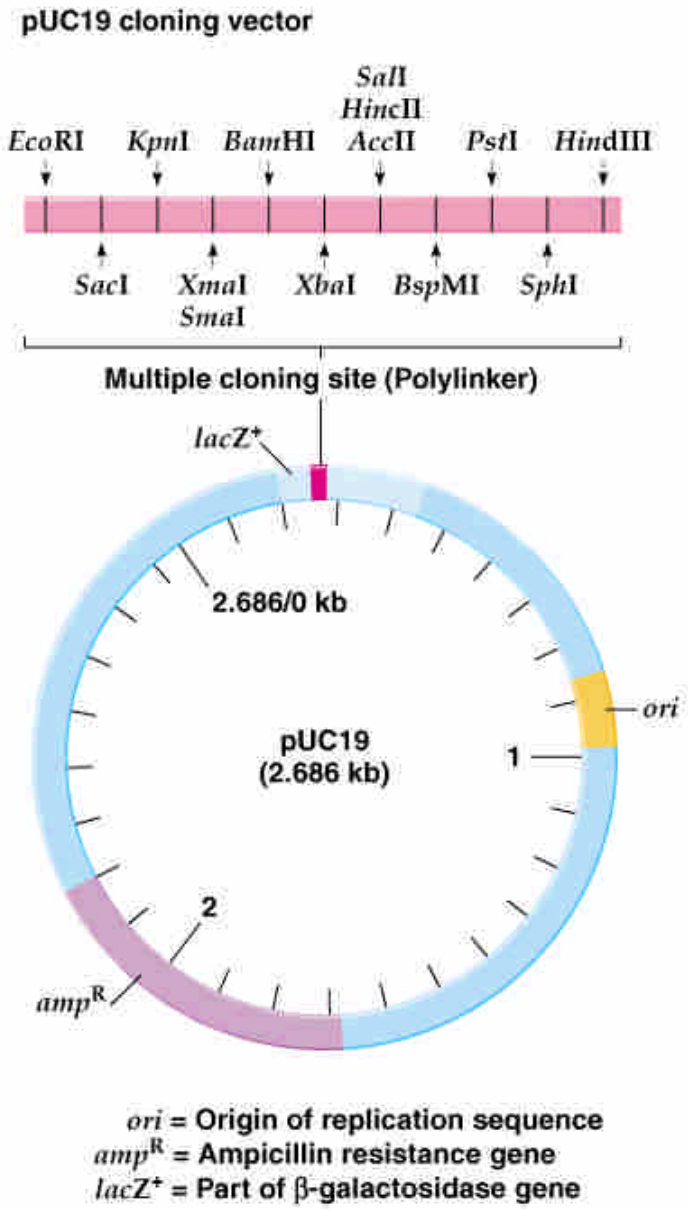
Adapted from G. D. Elseth and K. D. Baumgartner, *Genetics*, 1984 Benjamin/Cummings Publishing, Menlo Park, CA. Reprinted by permission of the author.

# Vector use in recombinant techniques (contd)

C: Feature of recombinant DNA vector.

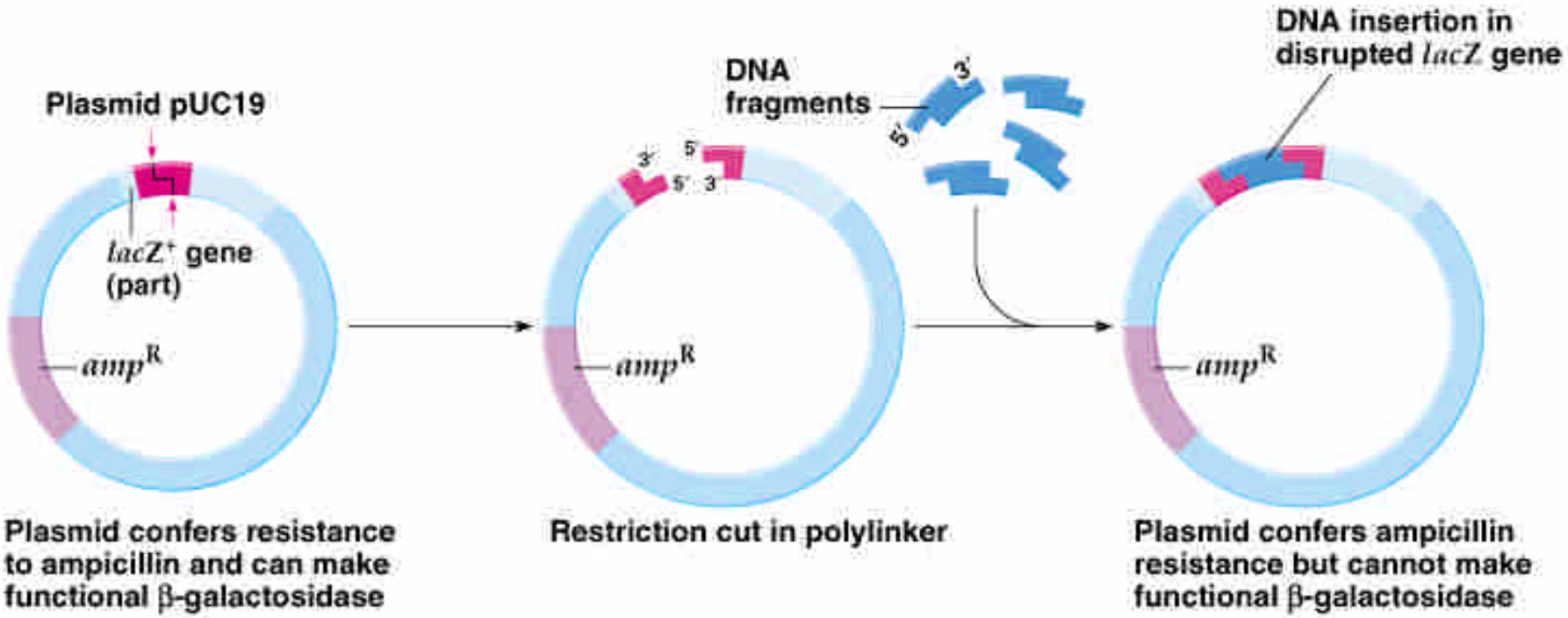
1. multiple cloning sites (MCS): recognized by many restriction endonuclease
2. procar./eucar. origin site of replication (Ori sites)
3. procar./eucar. antibiotic selection marker.
4. under tissue specific promoter or universal promoter. (found in expression vector)
5. appropriate termination.

# The plasmid cloning vector pUC19



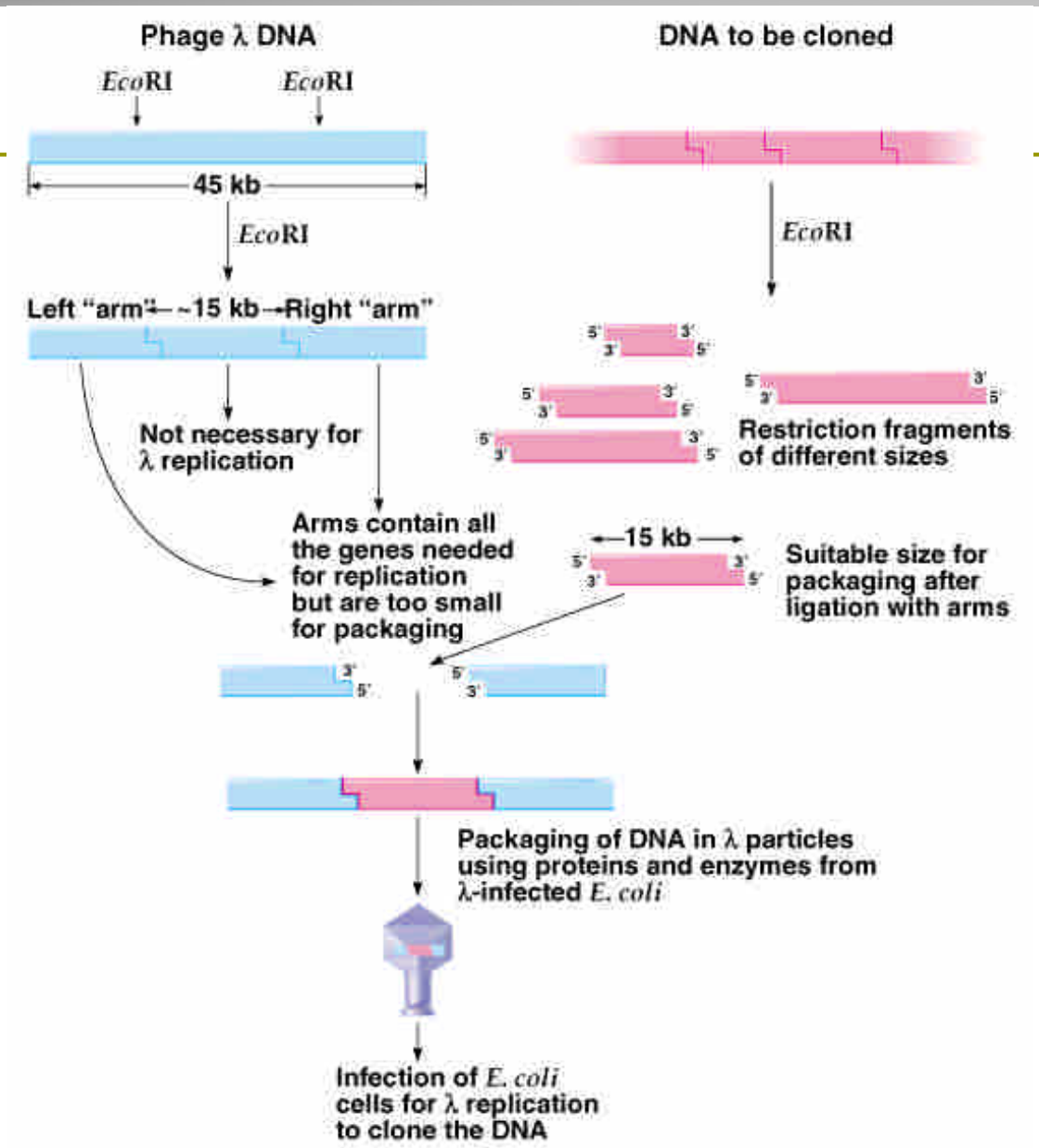

Peter J. Russell, *iGenetics*: Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.

# Insertion of a piece of DNA into the plasmid cloning vector pUC19



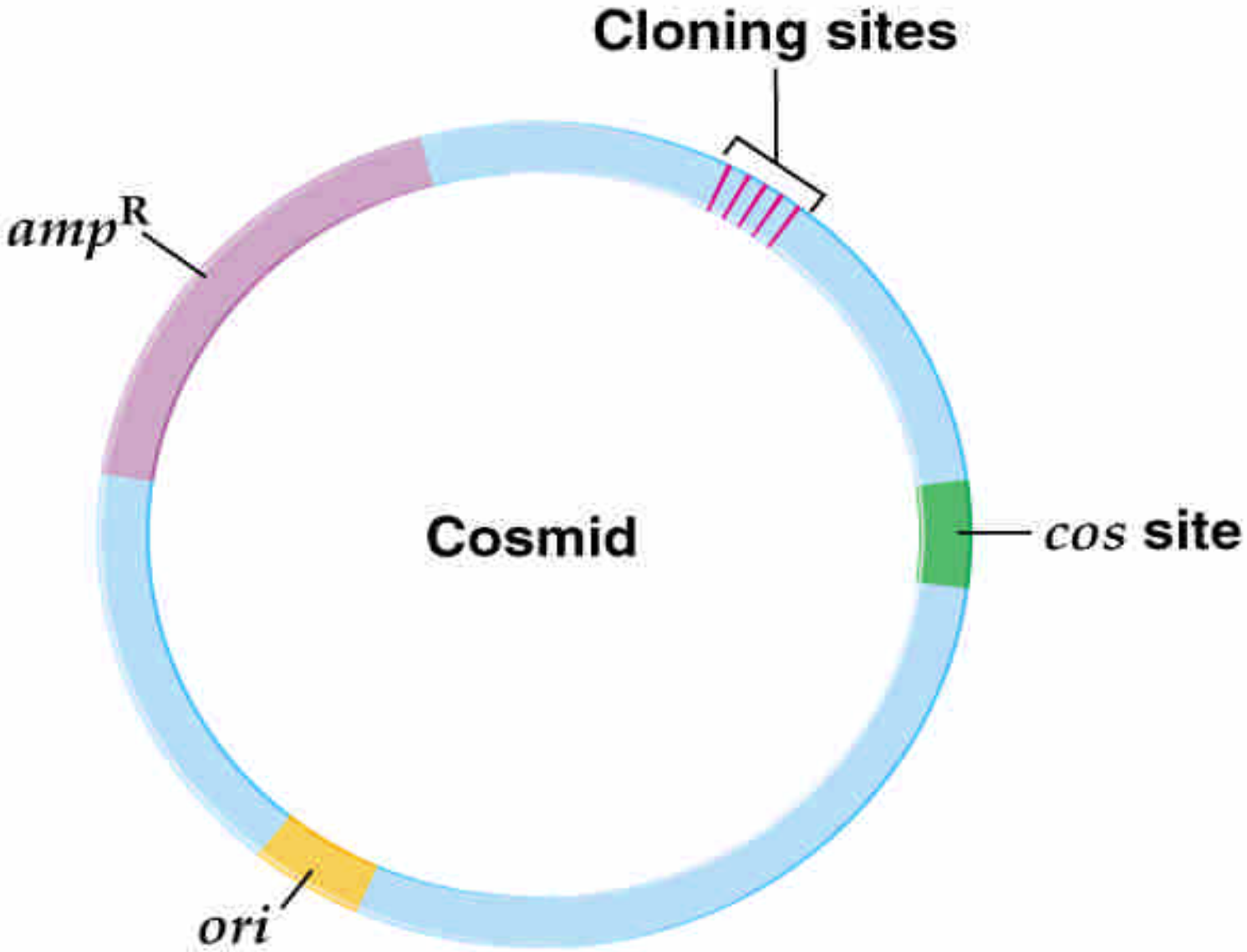

Peter J. Russell, *iGenetics*: Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.

# Scheme for using phage λ DNA as a cloning vector



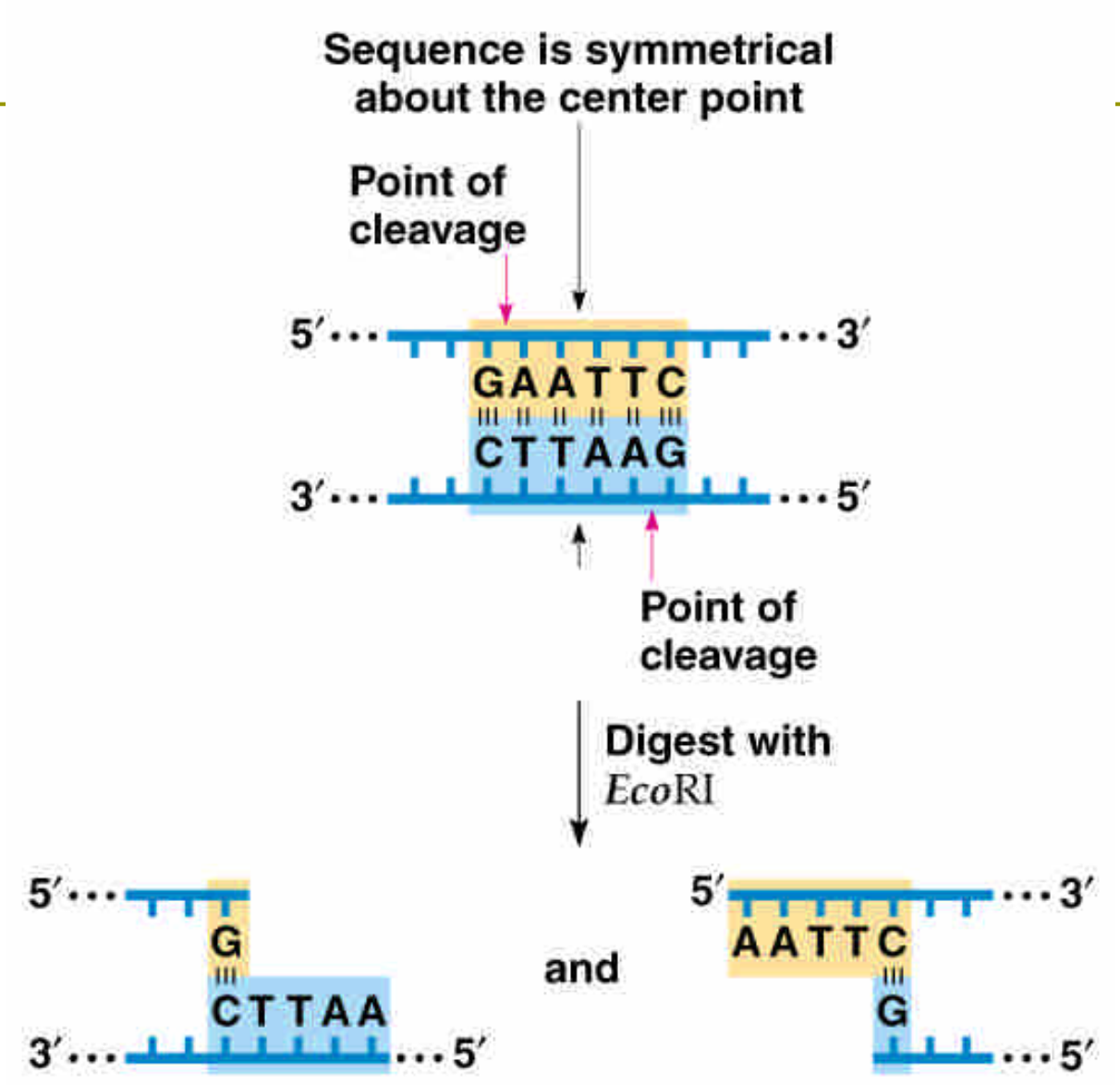

Peter J. Russell, *iGenetics*: Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.

# Cosmid cloning vector




Peter J. Russell, *iGenetics*: Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.

# Example of a yeast artificial chromosome (YAC) cloning vector




Peter J. Russell, *iGenetics*: Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.

# Libraries

- cDNA 基因庫 (cDNA library)：

  mRNA 帶有合成蛋白質的完整信息，以 reverse transcriptase 可逆向翻製成 DNA 分子，稱爲 cDNA (complementary DNA)，不含 intron； cDNA 再植入載體，送入宿主中，即得 cDNA 庫。 但需注意，此種基因庫只代表正在表現中的基因，並不包括所有的基因。 cDNA 可以表現出蛋白質，並以其專一性抗體篩選之。

- 染色體基因庫 (genomic library)：

  染色體 DNA 以限制酵素切成隨意片段後，植入載體，再送入宿主建庫。 此基因庫可能含有所有的基因，包括正在表現的，與休眠中的基因；也包含 intron，以及基因上游的調控區域 (如 promotor, enhancer 等)。 通常使用噬菌體爲載體，以便容納較大的 DNA 片段。

# Hosts

- E. coli
- Yeast
- Mammalian cells

# DNA-mediated gene transfer

- Microinjection: a fine glass needle
- Calcium phosphate precipitation:
- Cationic lipids：high efficiencies
- Electroporation: 用於Calcium phosphate precipitation失敗時
- Viral vectors

# Recombinant DNA techniques

A: gel electrophoresis. 電泳

ex, The chemical properties of DNA.

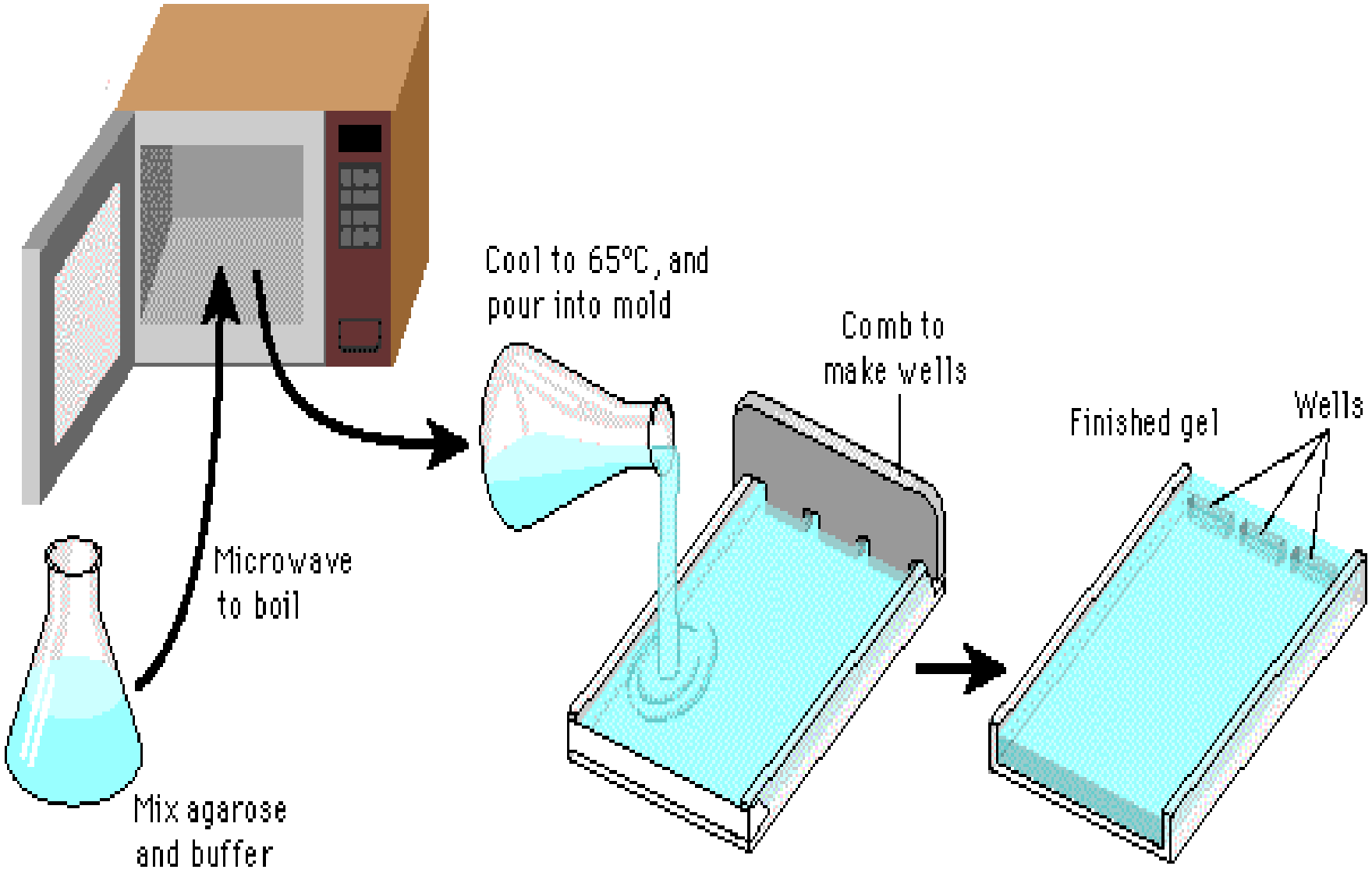
Agarose gel (用於水平電泳)

acrylamide gel (用於垂直電泳)

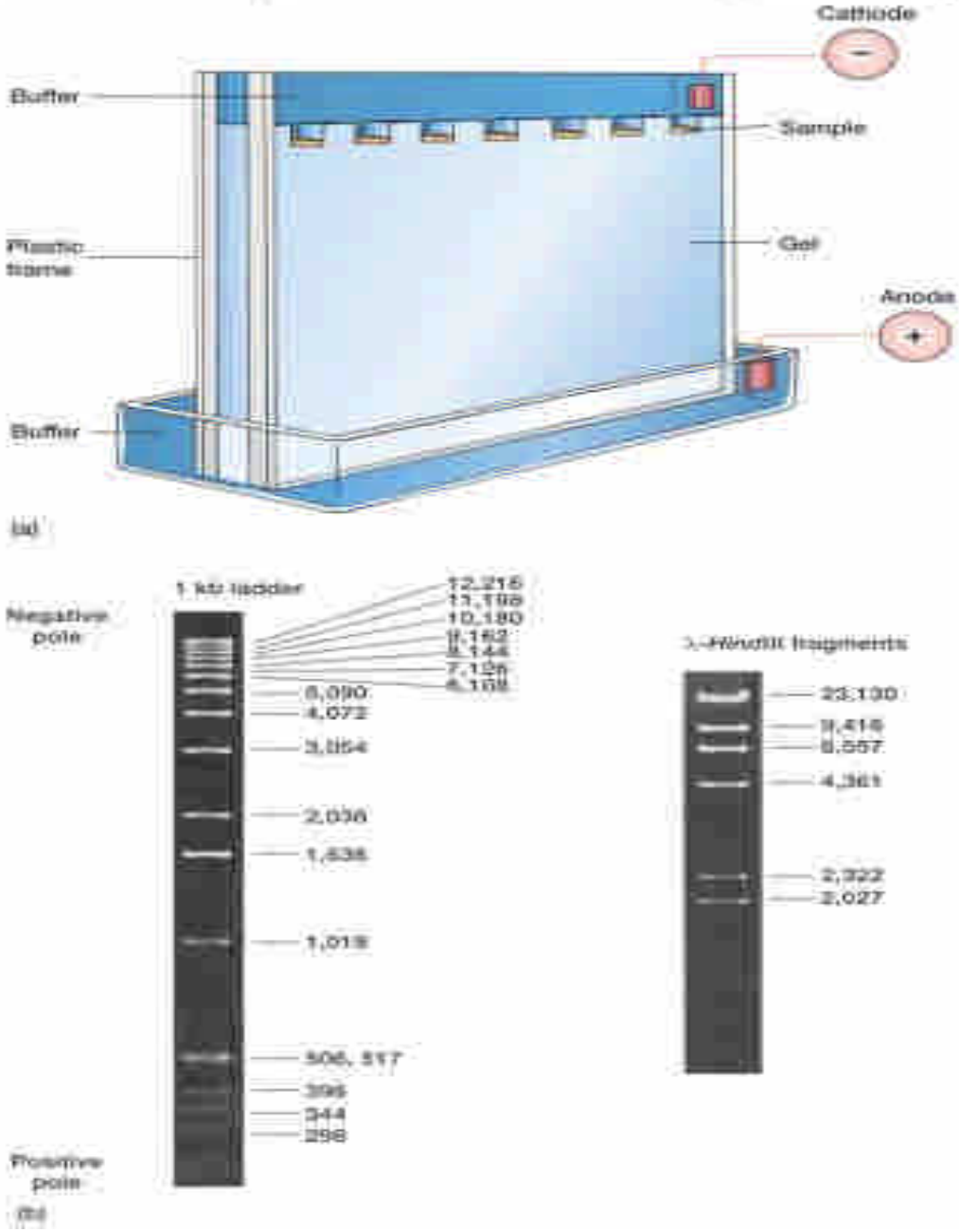
DNA marker.

B: types of enzymes.

ex, restriction enzyme, reverse transcriptase, RNase H, DNA polymerase, DNA ligase….


Cool to 65°C, and
pour into mold
Comb to
make wells
Finished gel
Wells
Microwave
to boil
Mix agarose
and buffer

Copyright © The McGraw-Hill Companies, Inc. Permission required for reproduction or display.



# Recombinant DNA techniques (contd)

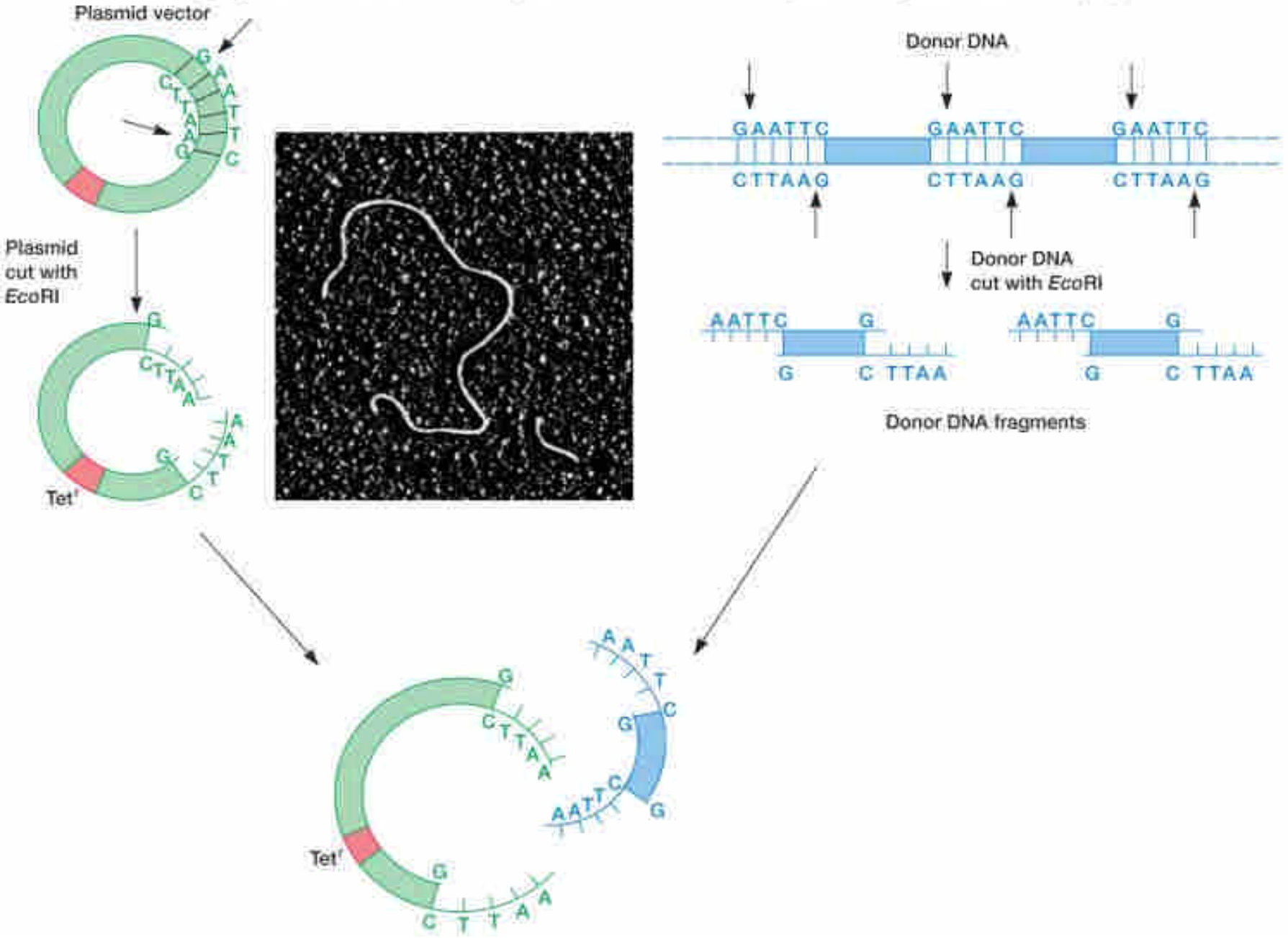
C: types of vector.

1. the cloning vector (複製DNA) and the expression vector (同時表現蛋白質)
2. type of vector

D: types of library to be constructed.

1. looking for genomic structure: genomic library.
2. looking for expression gene: cDNA library.

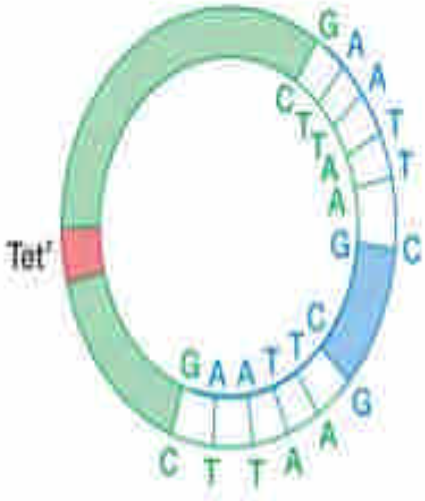
Copyright © The McGraw-Hill Companies, Inc. Permission required for reproduction or display.



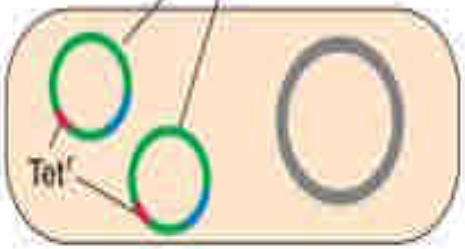
Copyright © The McGraw-Hill Companies, Inc. Permission required for reproduction or display.

Complementary base pairing followed by ligation

Recombinant DNA molecule



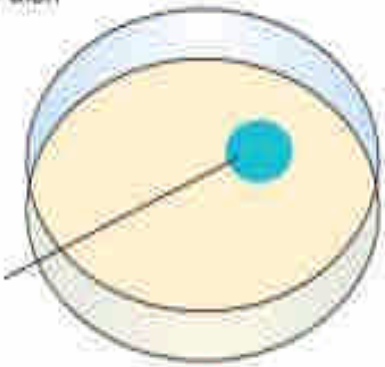

Transform $Ca^{2+}$-treated Tet$^s$ *E. coli*




Transformed cell

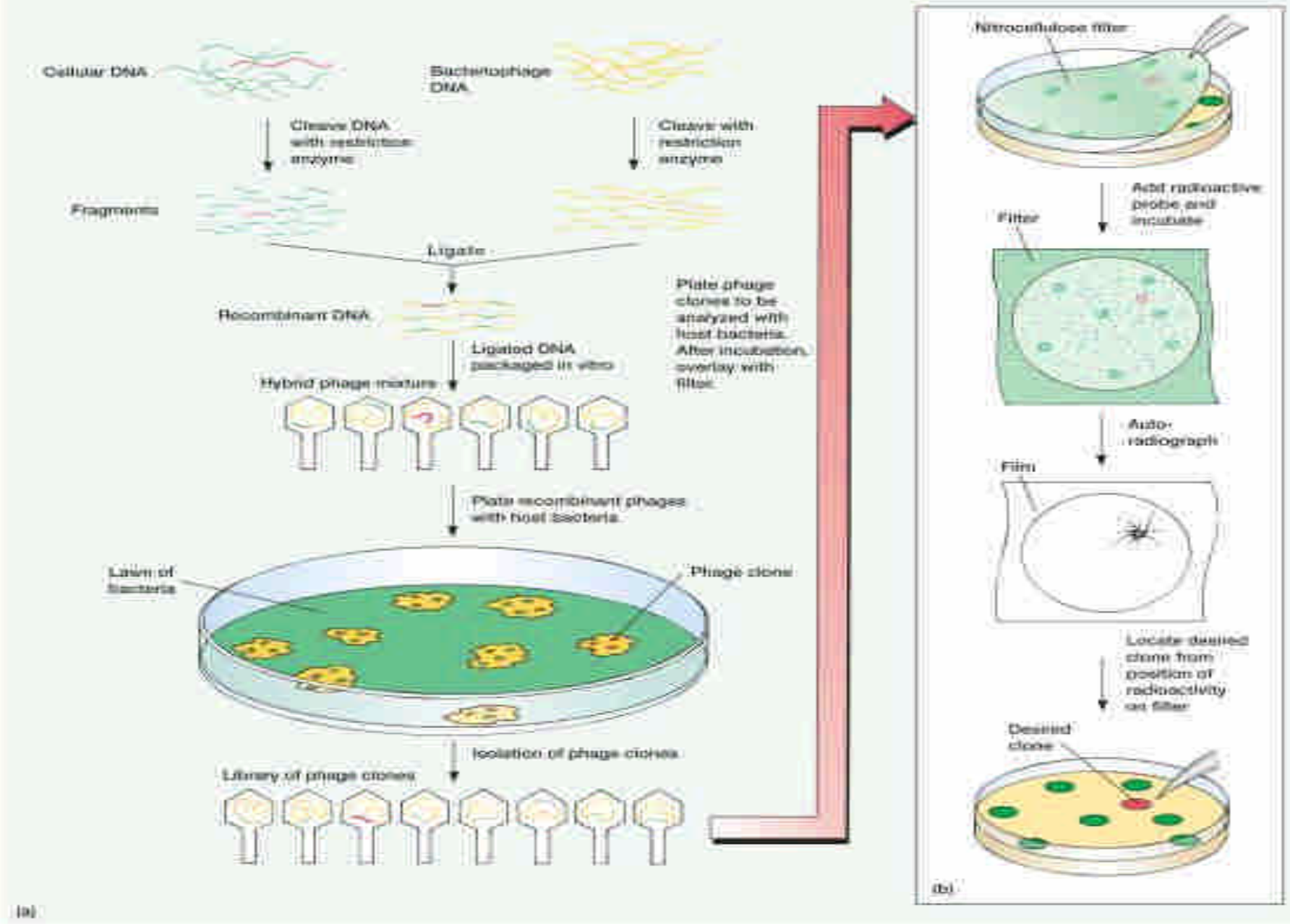
Culture bacteria in medium with tetracycline

Petri dish



Tetracycline-resistant colony from transformed cell

Copyright © The McGraw-Hill Companies, Inc. Permission required for reproduction or display.



# Recombinant DNA techniques (contd)

E: probe preparation.探針

- radiolabel. 32p incorporated by kinase (end-label),
- PCR incorporated methods
- Nonradiolabel (DIG or biotin)
- synthetic DNA.

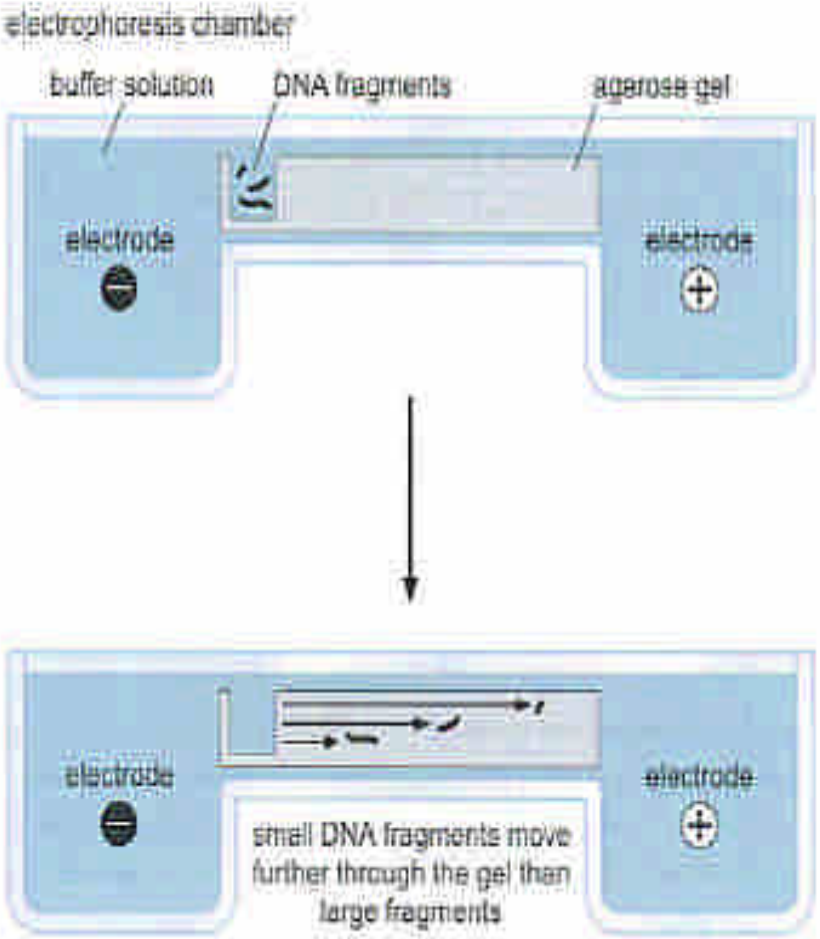
F: blotting and detection.

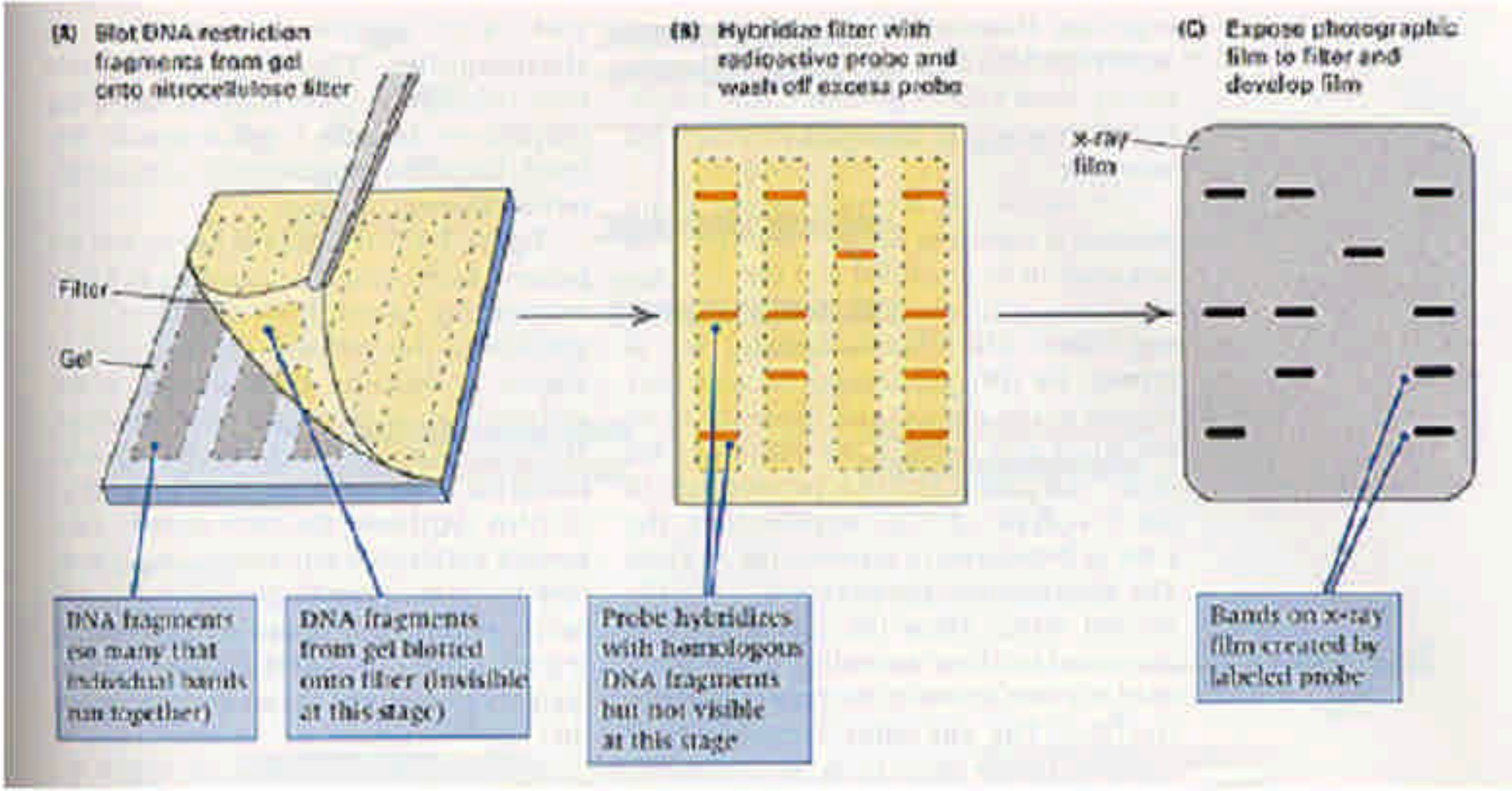
# 墨點技術

- 南方墨點(Southern Blot)、北方墨點(Northern Blot)、西方墨點(Western Blot) – 基因變異檢測技術
  - 先使用電泳法將大分子切開成小分子並予以分離
  - 將小分子轉印(blotting)至濾膜上用標記之探針(probes)與之進行雜交(hybridization)
  - 辨識雜交後之資訊，即可對有興趣的序列進行鑑定或分離

# Gel Electrophoresis for DNA Separation

**FIGURE 20-1 DNA separation by gel electrophoresis.** The figure shows a gel from the side in cross-section. Thus the "well" into which the DNA mixture is loaded onto the gel is indicated at the left, at the head of the gel. That is also the end at which the cathode of the electric field is located, the anode being at the foot of the gel. As a result the DNA fragments, which are negatively charged, move through the gel from the head to the foot. The distance they travel is inversely related to the size of the DNA fragment, as shown. (Source: Adapted from Micklos D.A. and Freyer G.A. 2003. *DNA science: A first course*, 2nd edition, p. 114. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.)





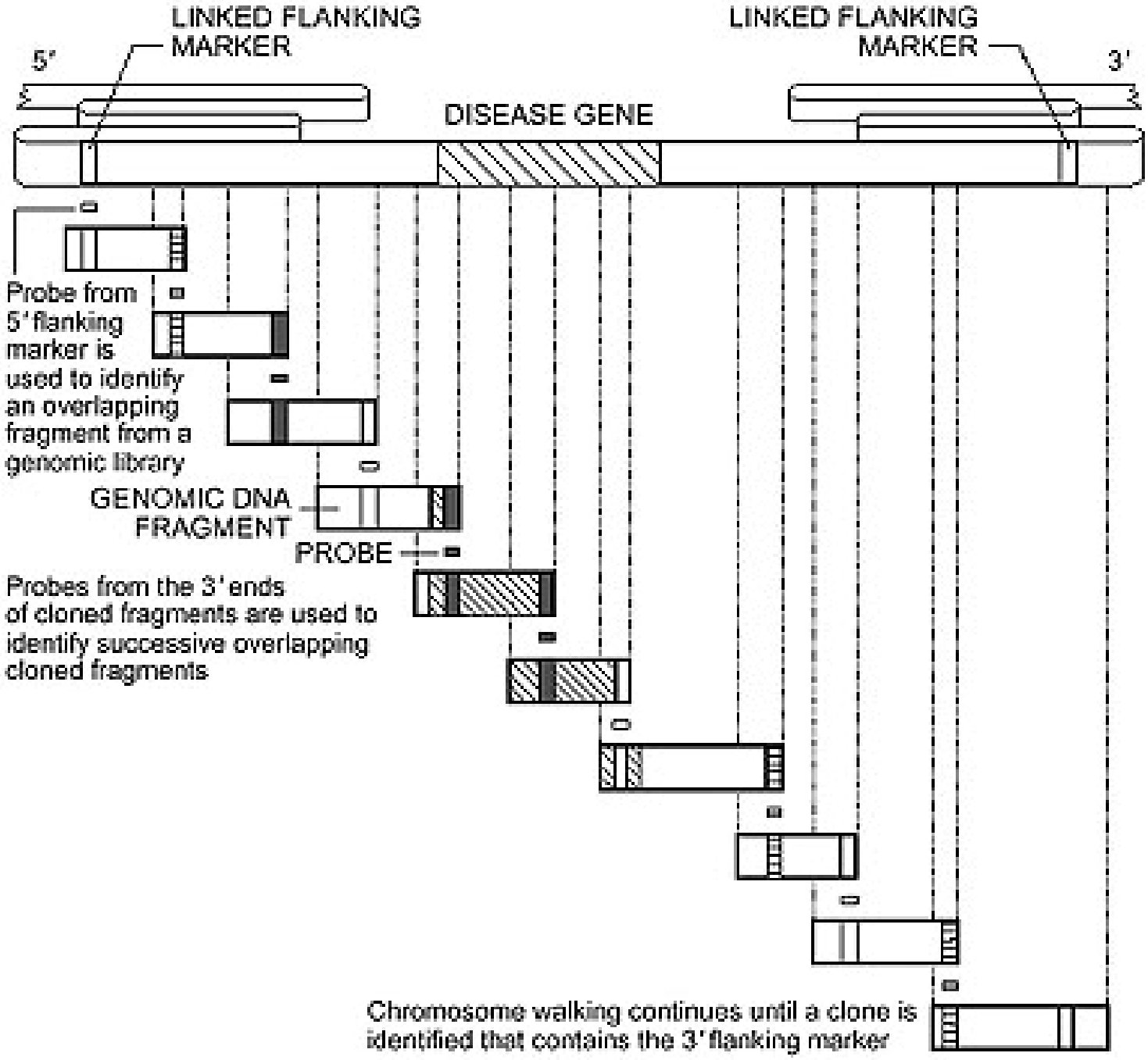

A. 電泳法後，將結果轉錄至濾膜上

B. 使用探針在膜上進行雜交

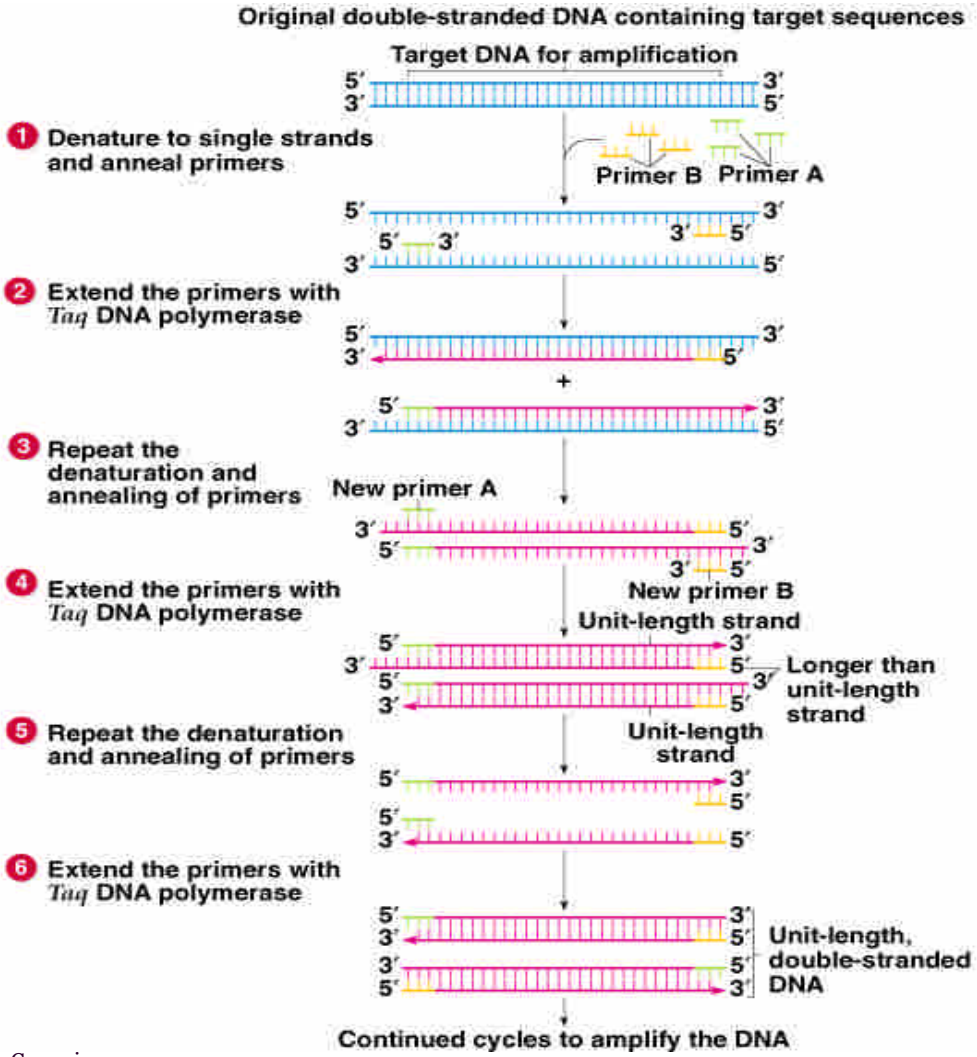
C. 辨識雜交結果，並對有興趣部份進行鑑定或分離

# Chromosome Walking

- 每次定序一小段; 利用重疊區域將小段匯整爲大段; 直到整個染色體被定序爲止.

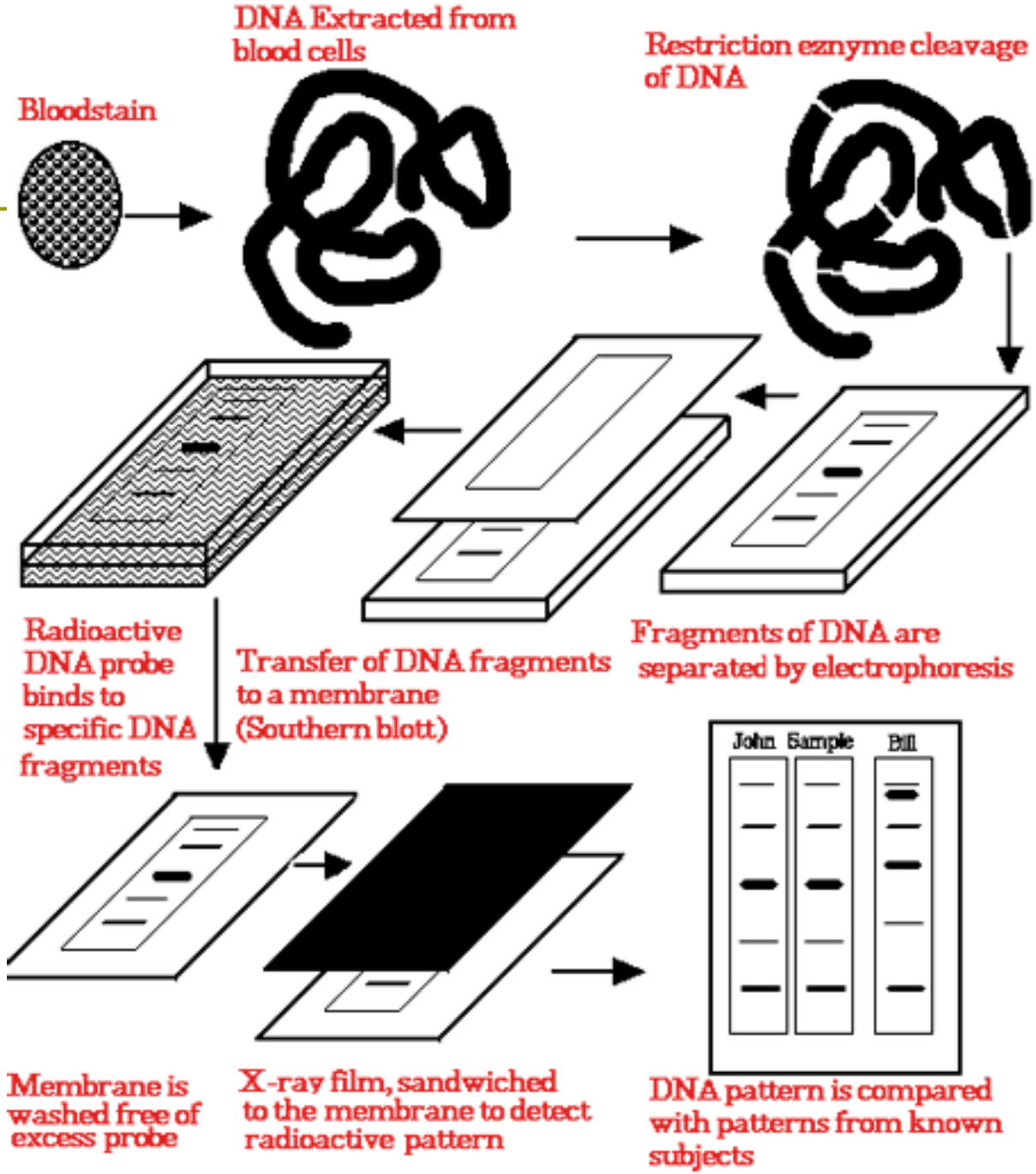

LINKED FLANKING MARKER
5′
LINKED FLANKING MARKER
3′
DISEASE GENE
Probe from 5′ flanking marker is used to identify an overlapping fragment from a genomic library
GENOMIC DNA FRAGMENT
PROBE
Probes from the 3′ ends of cloned fragments are used to identify successive overlapping cloned fragments
Chromosome walking continues until a clone is identified that contains the 3′ flanking marker

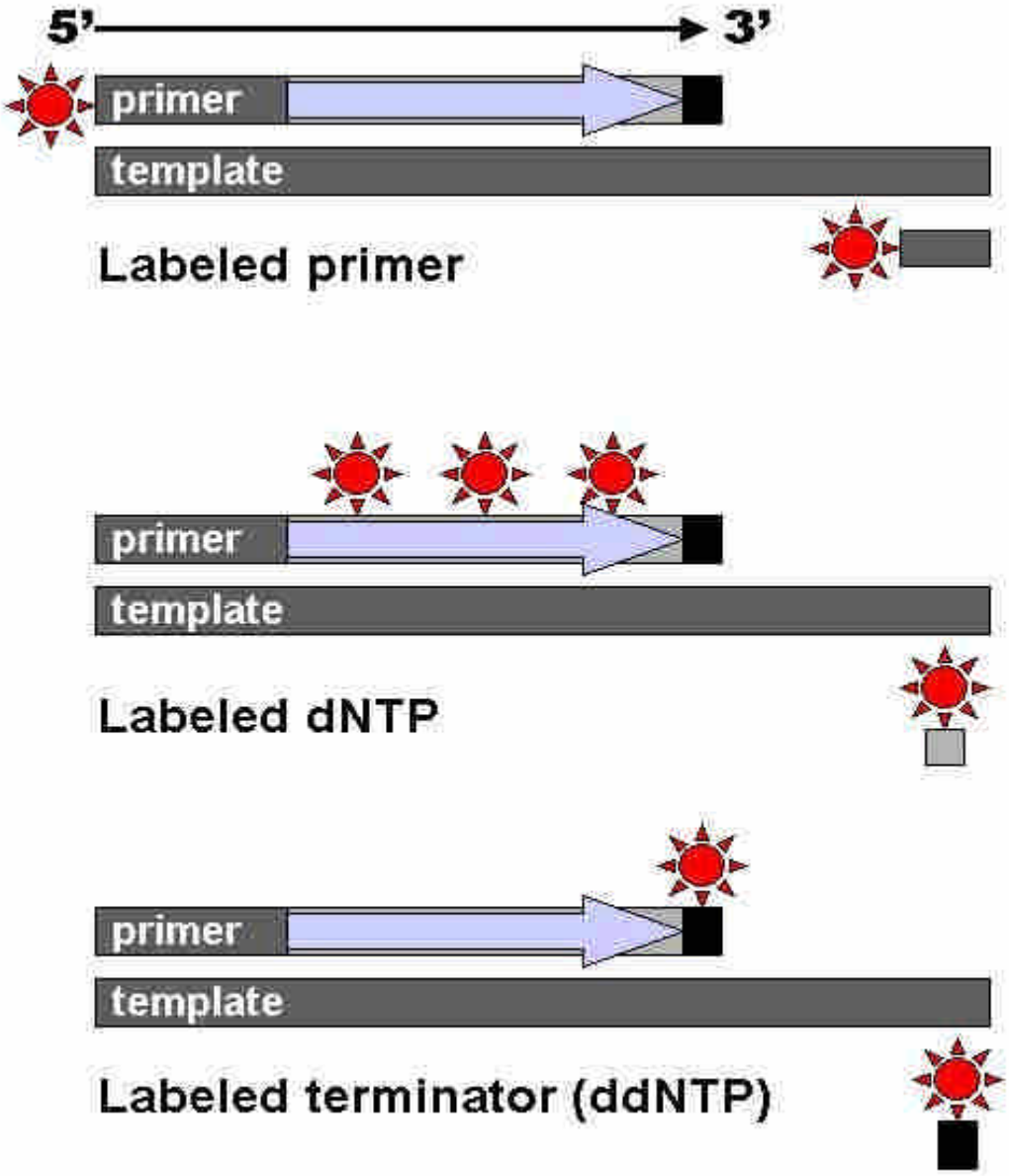
# The polymerase chain reaction (PCR) for selective amplification of DNA sequences




Peter J. Russell, *iGenetics*: Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.

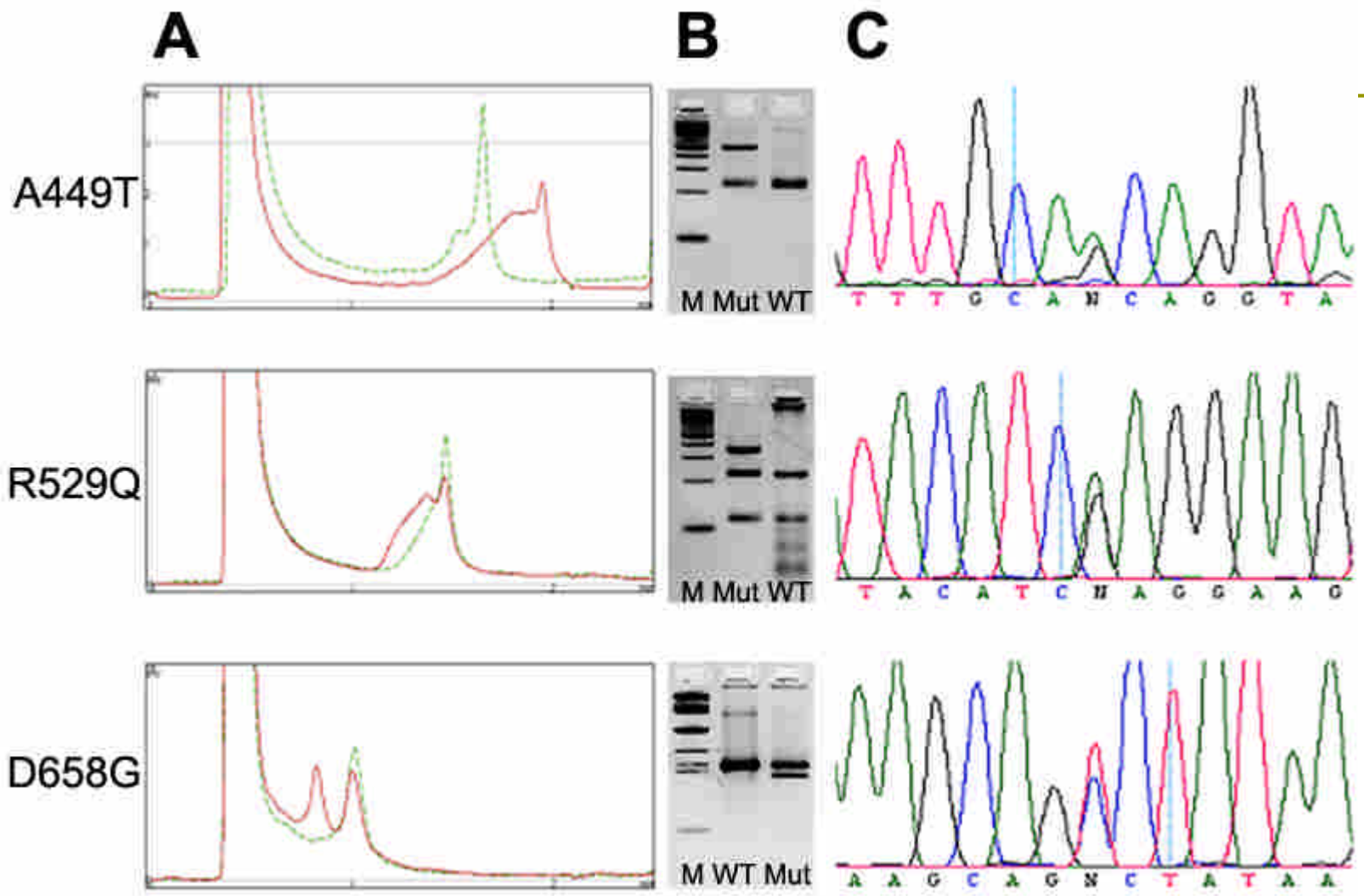
# Restriction Fragment Length Polymorphism (RFLP)





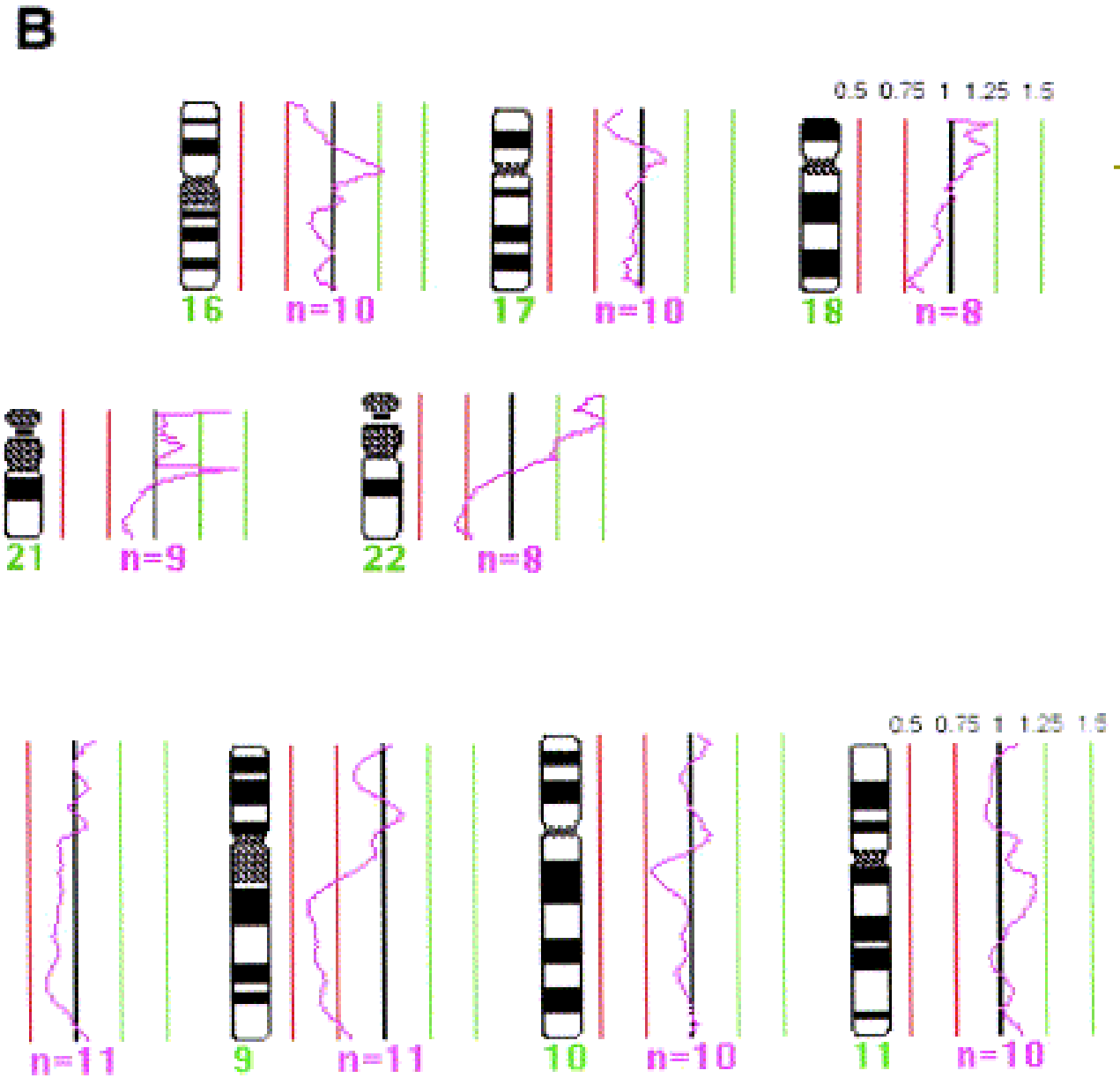

# DNA sequence

## Sanger method


A
B
C
A449T
R529Q
D658G
M Mut WT
M Mut WT
M WT Mut

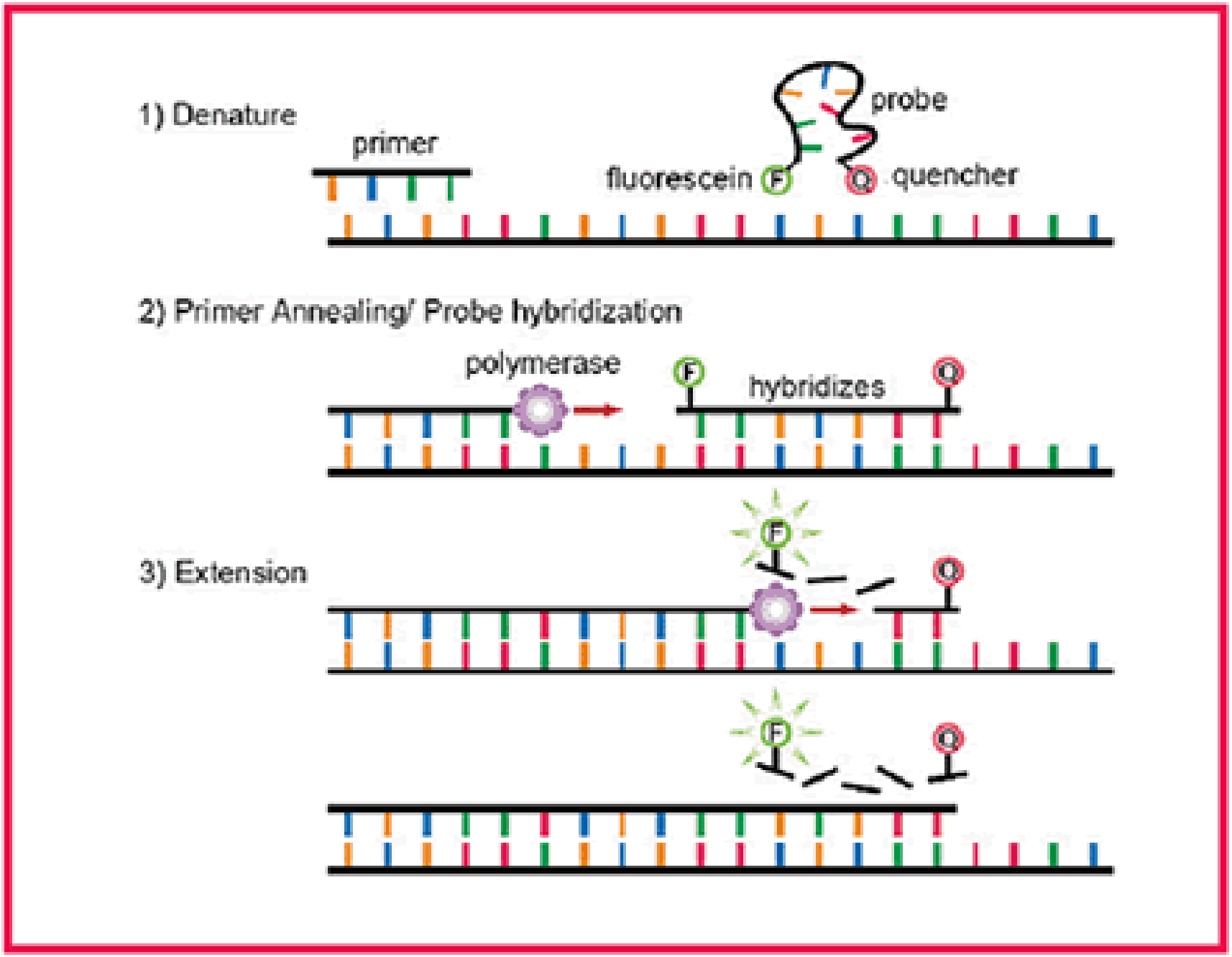
# Comparative genome hybridization (CGH)

- 是細胞分子基因診斷方法, 用以探討癌變基因的變異. 以 DNA 的增減變異做分類, 可以透露出突變的特性.
- 將對照細胞的DNA放大後染成紅色，再將欲檢測細胞的DNA放大後染成綠色，再視綠色/紅色的比率線與標準中央線的偏離率(0.75-1.25)作出染色體是monosomy(偏左過0.75) or trisomy(偏右過1.25)的診斷。

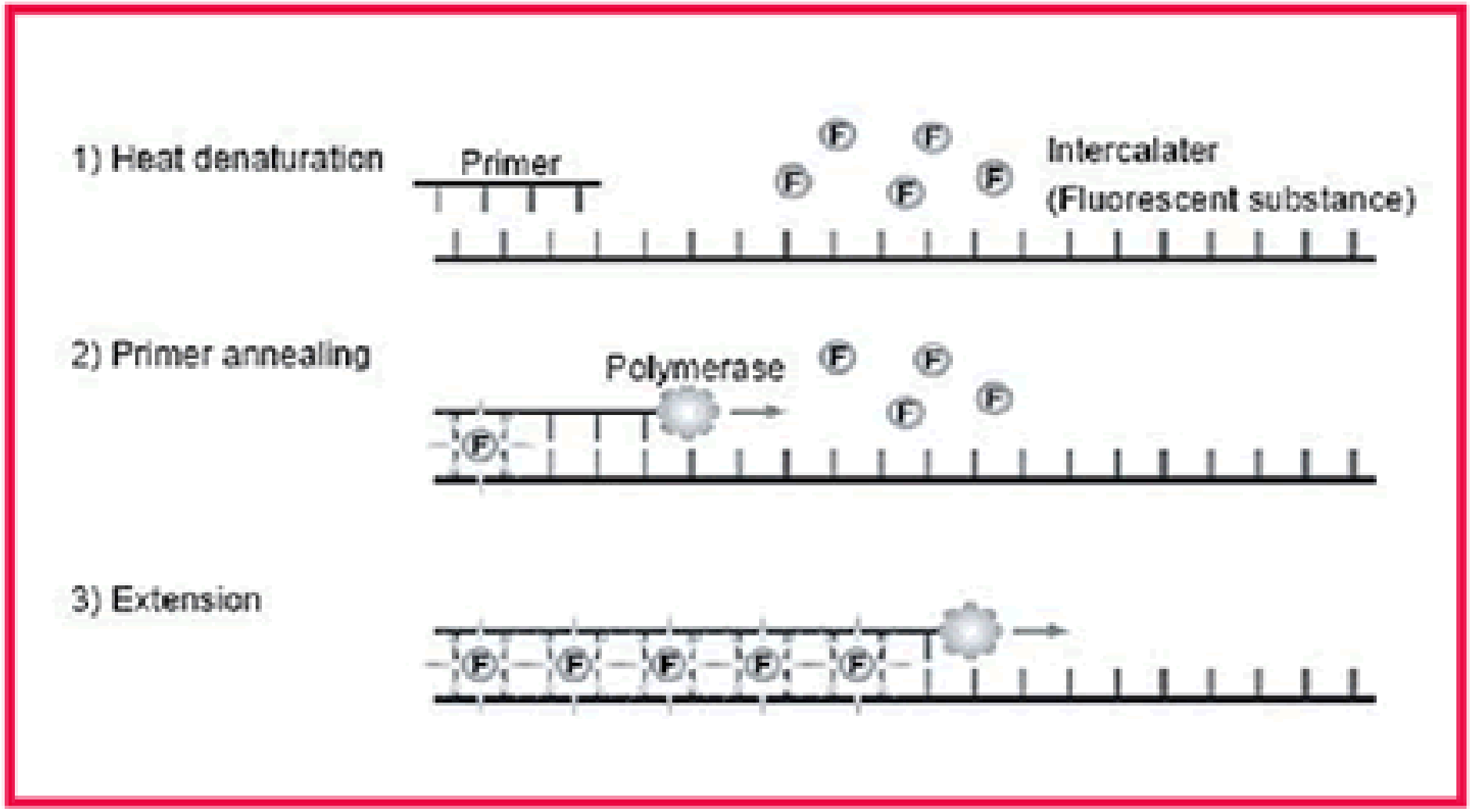

B
0.5 0.75 1 1.25 1.5
16
n=10
17
n=10
18
n=8
21
n=9
22
n=8
C
0.5 0.75 1 1.25 1.5
8
n=11
9
n=11
10
n=10
11
n=10

# Gene expression analysis

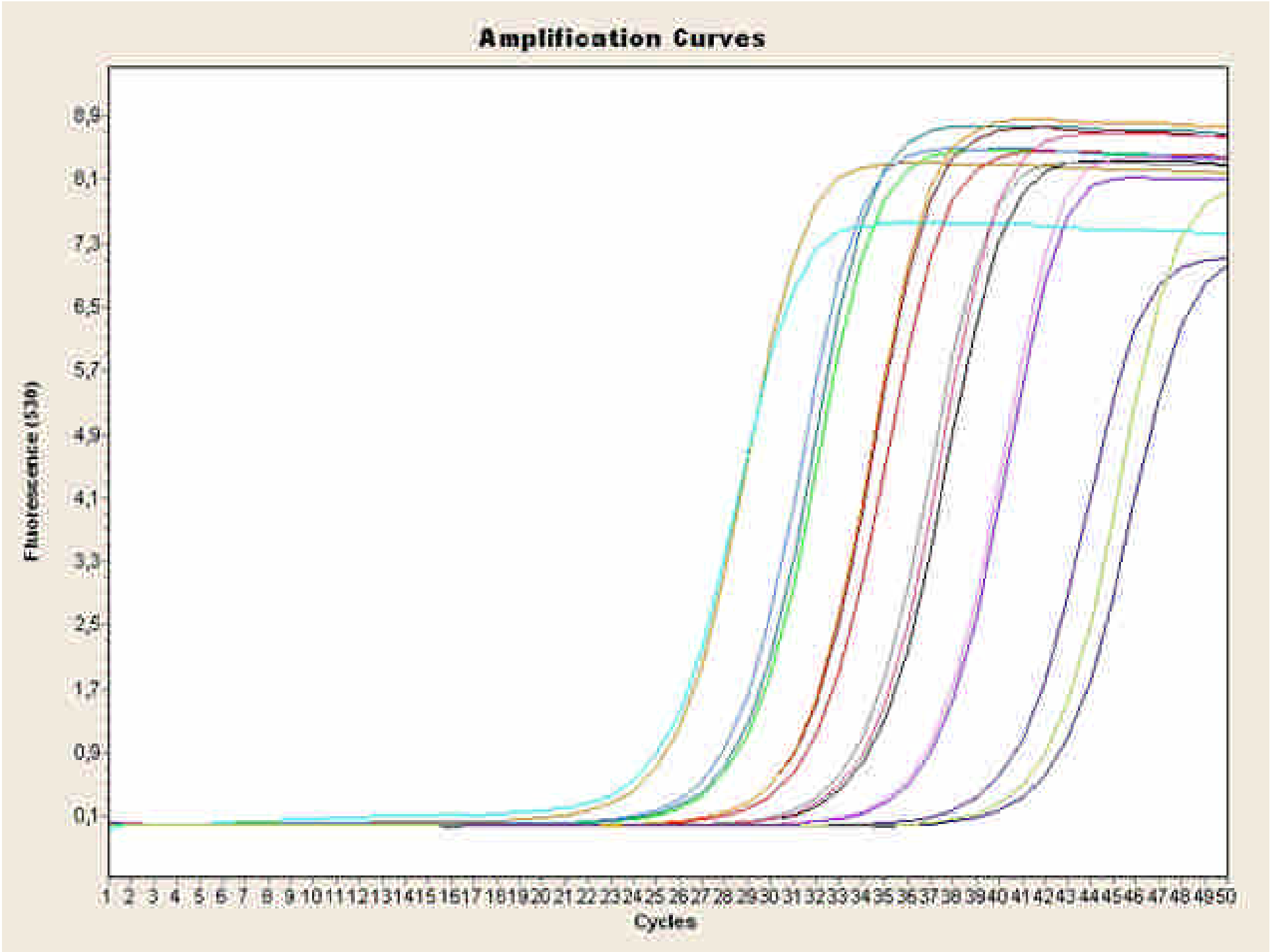
- Northern blotting: 找出某RNA
- RNA interference:破壞某mRNA
- Reverse transcription PCR: 少量RNA便可測出
- Quantitaitve real-time PCR: 可及時定量RNA
- Genetic reporters:  利用enzyme
- Promoter bashing: 看promoter哪一段會被repressor抑制
- Electrophoretic mobility shift assay (EMSA): 看transcription factor由細胞質跑到細胞核
- Chromatin immunoprecipation (ChIP)
- Protein-DNA interaction arrays (ChIP-Chips)
- Microarray to assay gene expression



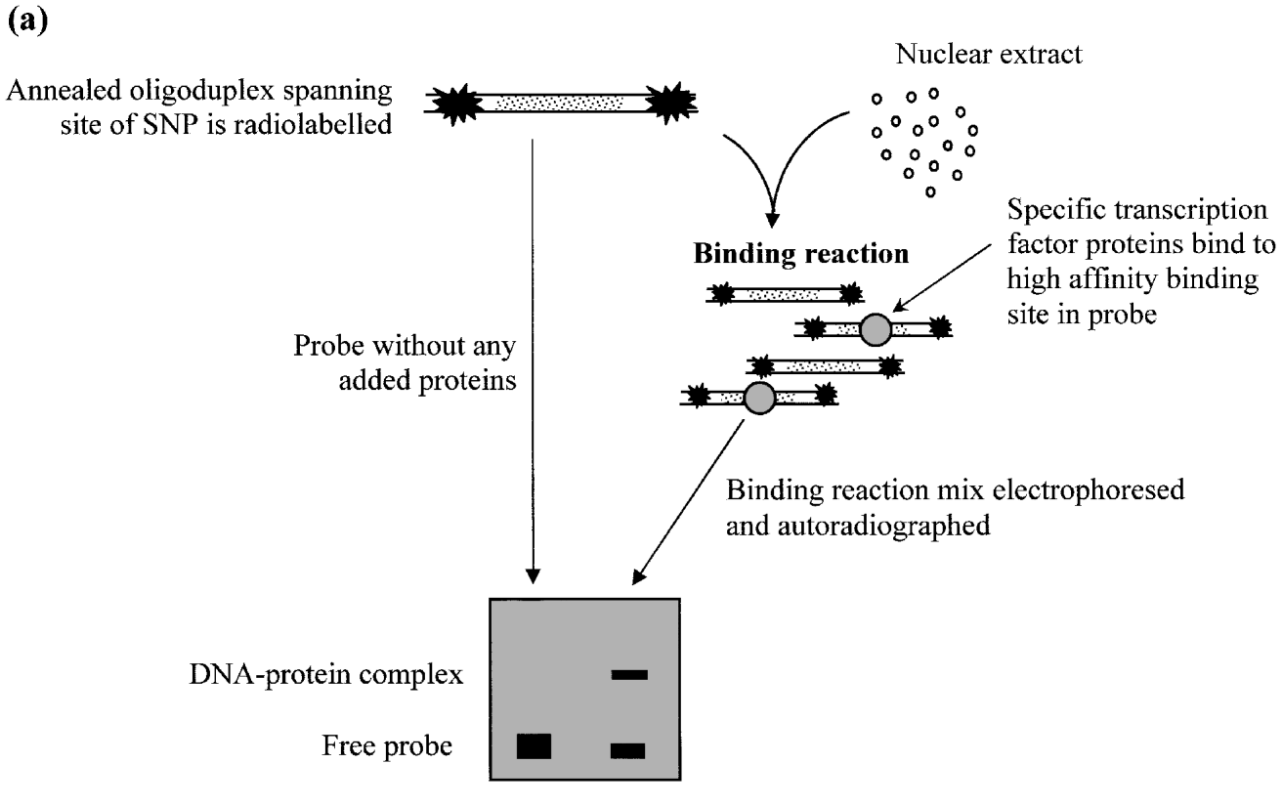

TaqMan® Probe Method

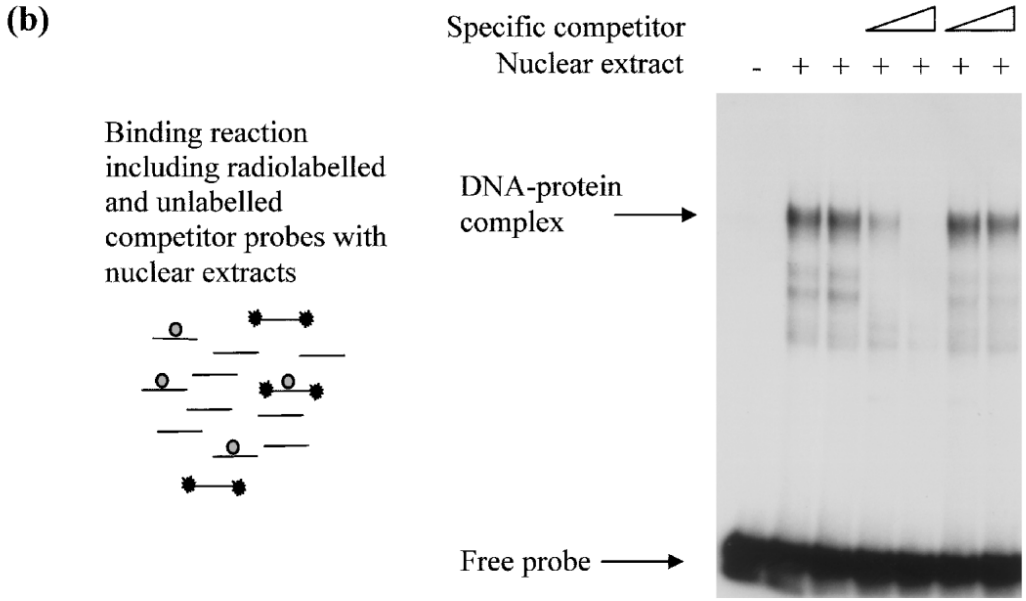



SYBR® Green I Method


Amplification Curves
Fluorescence (530)
8,9
8,1
7,3
6,5
5,7
4,9
4,1
3,3
2,5
1,7
0,9
0,1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
Cycles

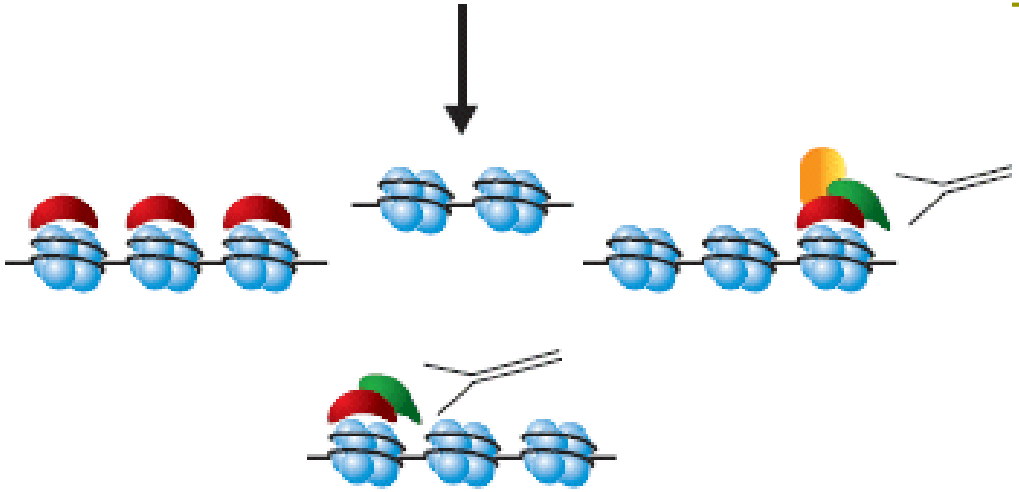
# Electrophoretic mobility shift assay (EMSA)



Crosslink DNA and proteins (optional) and isolate chromatin

Sonicate or digest chromatin

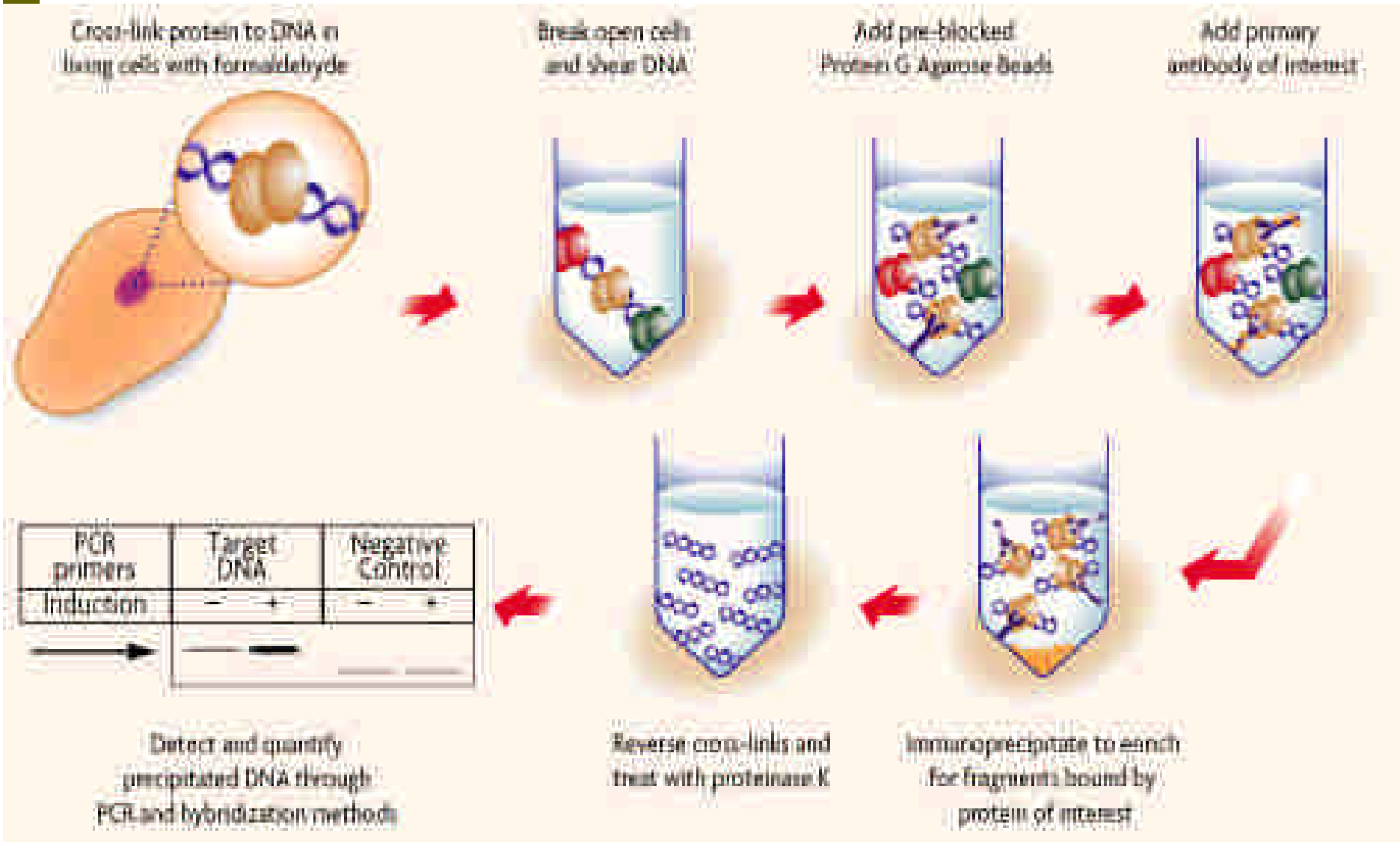


Immunoprecipitate, reverse crosslinking, purify DNA

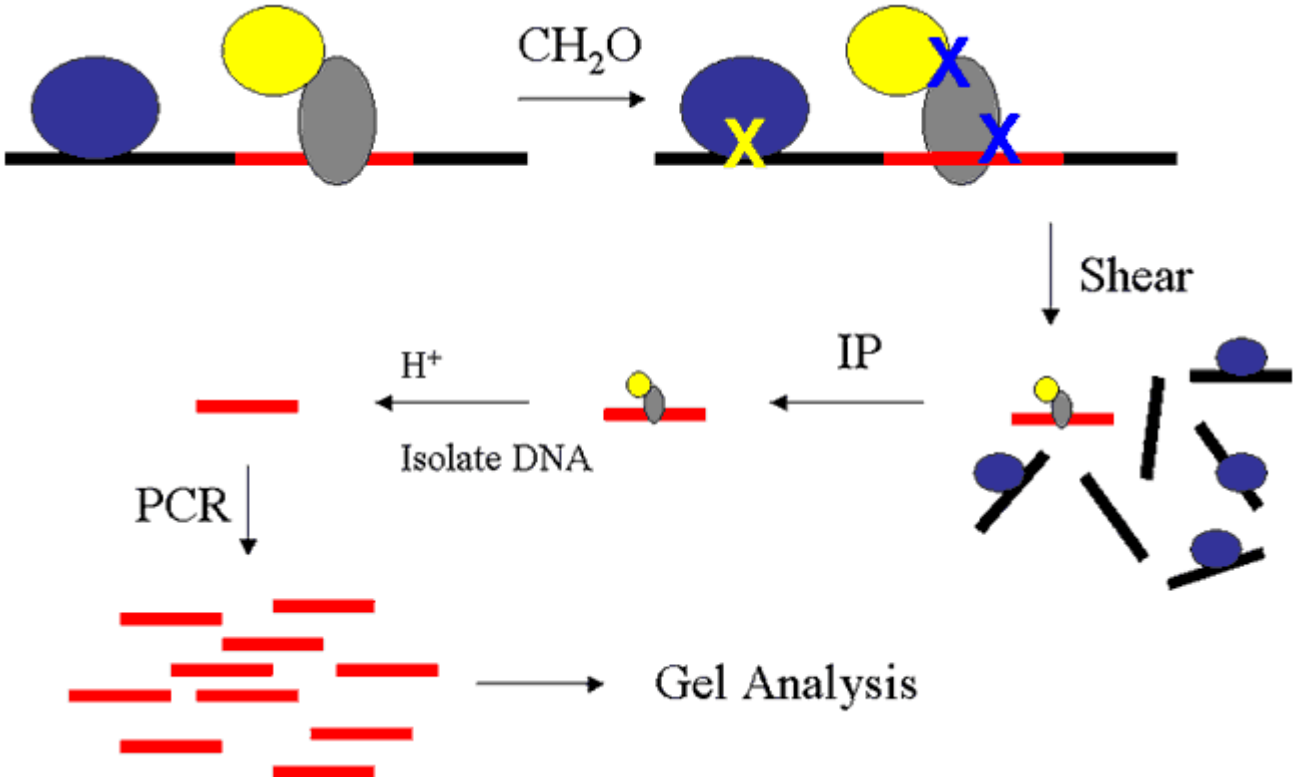
PCR amplify target sequences (or detect by hybridsation)


Lanes

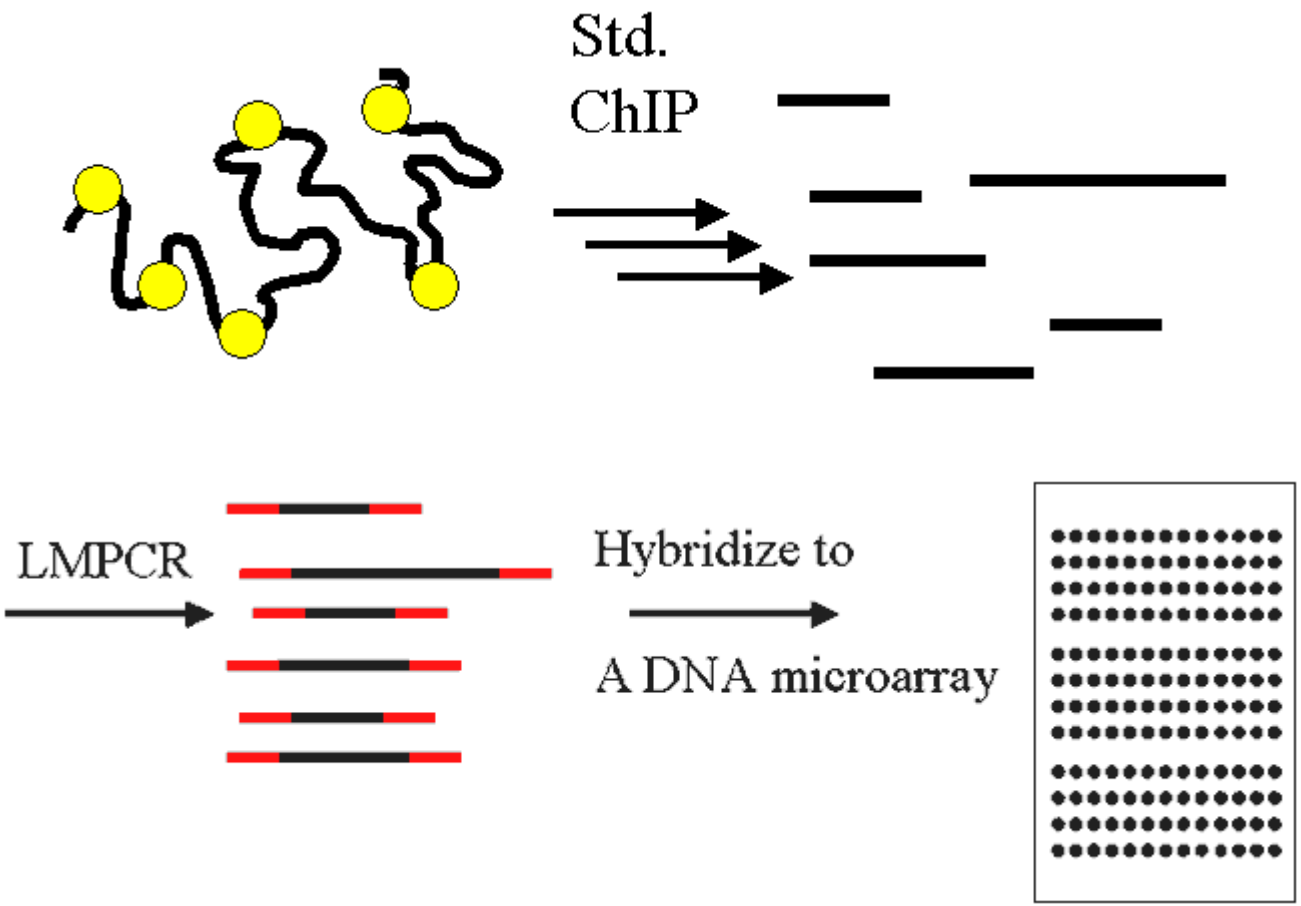
1. No input control
2. Input control
3. Chromatin IP from knockout background
4. Chromatin IP of target antigen


Cross-link protein to DNA in living cells with formaldehyde
Break open cells and shear DNA
Add pre-blocked Protein G Agarose Beads
Add primary antibody of interest
PCR primers
Target DNA
Negative Control
Induction
Detect and quantify precipitated DNA through PCR and hybridization methods
Reverse cross-links and treat with proteinase K
Immunoprecipitate to enrich for fragments bound by protein of interest

# Chromatin immunoprecipation (ChIP)



# Protein-DNA interaction arrays (ChIP-Chips)



# 生物晶片原理

- 將數千甚至數萬個點(spot)-單股DNA，別名探針(probe)，以高密度方式植在約拇指大小之晶片，利用生物結合特性做到同時大量偵測反應效果。
  - 探針來源：寡核甘酸(oligonucleotide)或已經存在於基因庫中的互補核甘酸cDNA
  - 晶片材質：玻璃片、尼龍薄膜
  - 除DNA外，亦可將蛋白質(proteins)、抗原(antigen)、抗體(antibody)放在晶片上做不同之偵測及應用

# 生物晶片種類–依特性分類

- (一)基因晶片(Gene chip, DNA microarray)：利用共軛互補的核酸爲探針，整齊排列晶片之上。使用其互補序列之核酸雜交結合，藉此進行樣品檢驗或環境檢測。另外依晶片上之探針種類不同，基因晶片尚可細分爲下列兩種：
  - 寡核酸陣列(oligonucleotides microarray)
  - 互補核酸陣列(cDNA microarray)

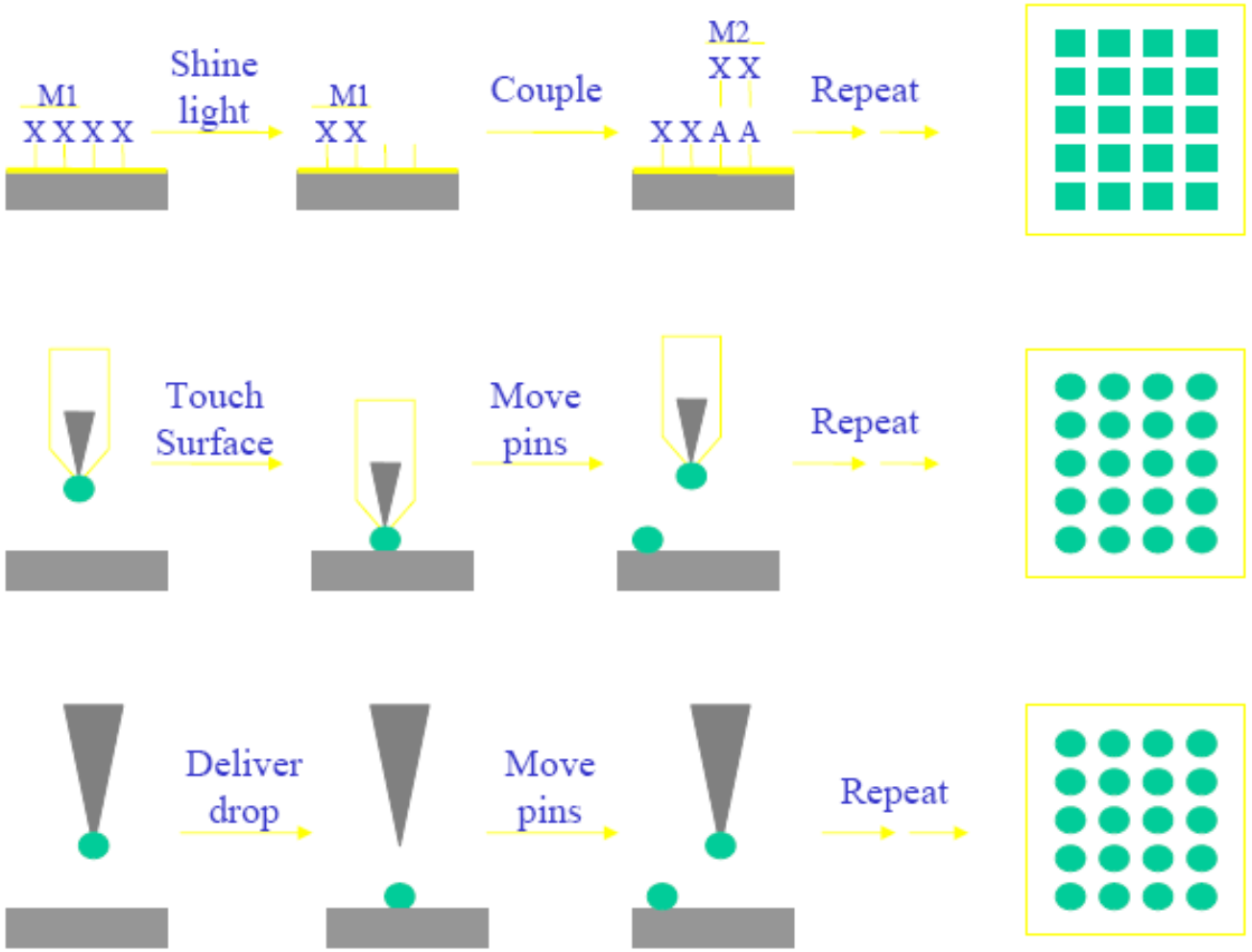
# 生物晶片種類–依特性分類

- (二)晶片實驗室(Lab-on-a-chip )：整合若干微管道及微反應於一塊晶片上以完成各種樣品處理、反應或分析檢測，功能類似一個小型實驗室之縮影。依功能尚可細分成下列兩種：
  - 聚合酶連鎖反應晶片(PCR chip)
  - 毛細管電泳晶片(capillarylectrophoresis chip)
  - PCR + CE

# 生物晶片種類–依特性分類

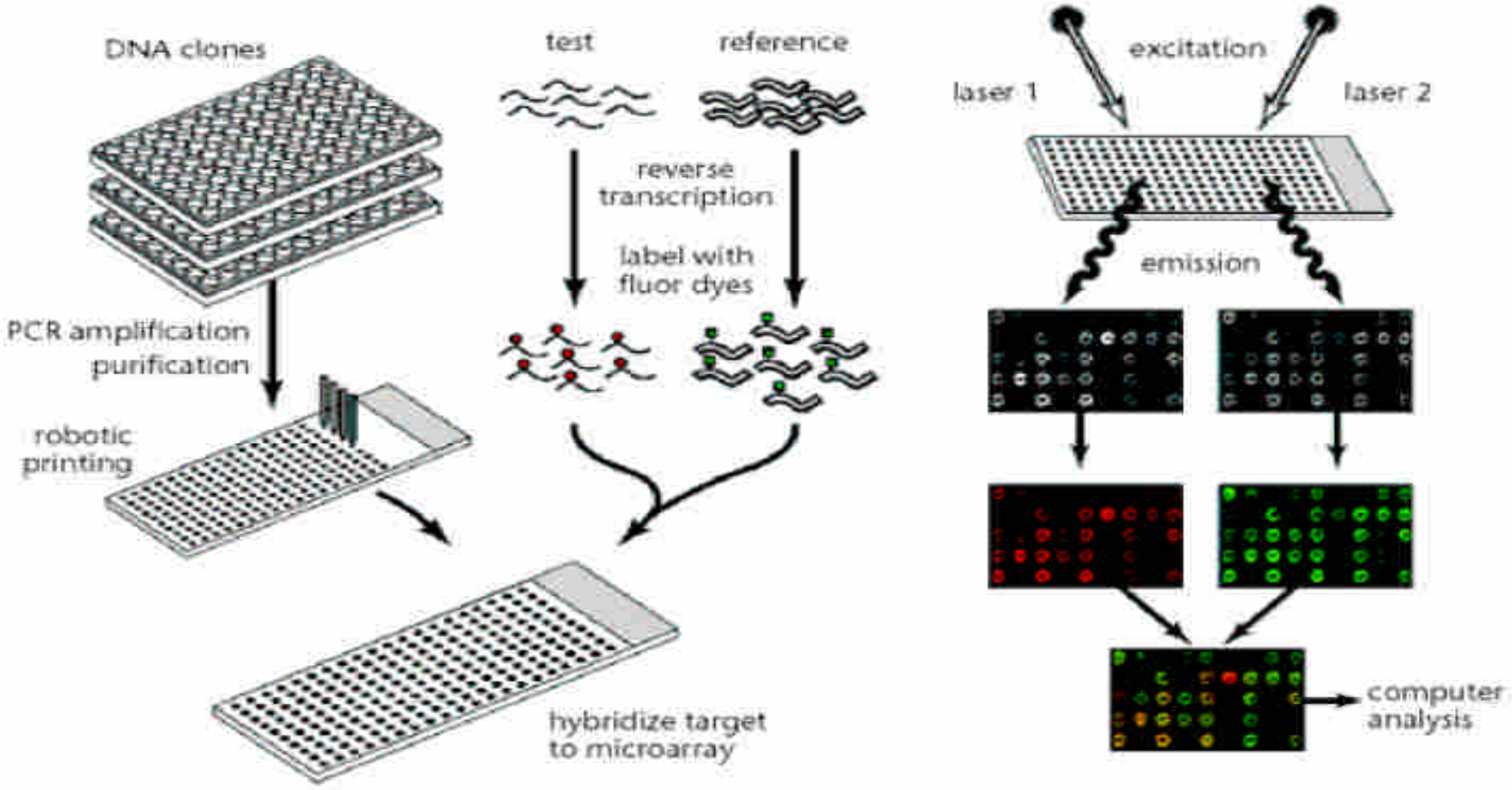
- (三)蛋白質晶片：以蛋白質爲生物探針，整齊的排列在晶片上，進行抗原-抗體免疫反應，用以檢測蛋白質。其尚待克服之技術瓶頸如下：
  - 有效提升結合於晶片上之抗體或結合物之穩定性
  - 有效鍵結具正確方向及足夠數量之抗體或結合物於晶片上
  - 研發足夠數量可資辨識不同生命物質的擷取抗體或晶片結合物
  - 有效提升晶片的偵測靈敏度
  - 依不同用途、材質、訊號傳導、生物相容性等等上有諸多問題待解決

# 生物晶片實例及其運作流程綱要



# 生物晶片實例一

- Spotted DNA arrays (設計製造)



# Protein analysis

- Western blotting: 找出某protein
- Antibody production:
- Immunoprecipation: 看protein-protein interaction
- Far Western blotting: 用Western blotting看protein-protein interaction
- Fluorescent proteins
- Two-Hybrid Screening: 看protein-protein interaction
- Proteomics: 2D electrophoresis等電點