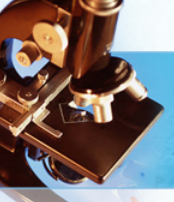
教育部高中生物科學資優生培育計畫-高雄區

- DNA萃取純化與電泳分析
- 重組DNA技術與基因選殖
- 聚合酶連鎖反應(Polymerase Chain Reaction, PCR)
- DNA定序(DNA sequencing)
- 基因定點突變 (Site-directed mutagenesis)技術
- DNA交互配對(雜交)反應(DNA hybridization)



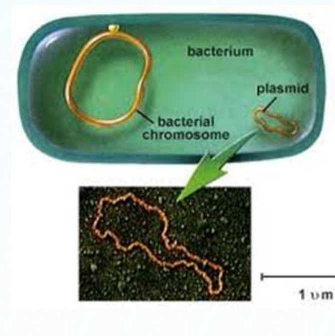
授課教師：楊文仁
任職單位：高雄大學生物科技研究所
日　　期：2011年3月5日

細菌質體DNA

- 質體(plasmid)是細菌染色體以外的遺傳物質，由雙股環狀DNA組成。細菌所帶質體的大小及數目不定，通常只有數千個鹽基對(bp)。
- 質體DNA具有自行複製的能力，可以在細菌間互相轉移，而將外來的基因轉移到其他宿主細胞中表現。
- 在生物技術的應用方面，質體DNA常被用來做為載體 (vector) 以選殖特定的DNA以及表現特定的蛋白質之用。因此，質體DNA的製備是分子生物學的一項基本且重要的技術。

質體DNA製備的原理及步驟

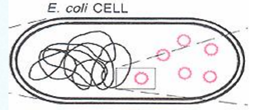
1. 培養並收穫細菌。
2. 破壞細菌的細胞壁與外膜。
3. 破壞細胞膜使細菌裂解。
4. 移除染色體DNA。
5. 移除蛋白質與RNA。
6. 純化質體DNA。



細菌質體DNA萃取

- 萃取質體DNA的方法很多，最常用的是鹼性溶解法(alkaline lysis) 及煮沸法(boiling method) 。
- 鹼性溶解法(alkaline lysis)
  - 原理是利用鹼處理質體DNA和染色體DNA，使兩者雙股打開呈單股狀態，再加入酸中和鹼使得單股DNA回復成為雙股DNA。
  - 細菌以NaOH及SDS分解，並使蛋白質及DNA變性，再以酸中和。
  - 質體DNA分子在中和後恢復原態，但細菌染色體DNA則無法完全復原而與SDS-K^+ 形成複合物，可用離心沉澱加以去除。
  - 上清液中所含的質體則可以酒精或異丙醇將其沉澱。

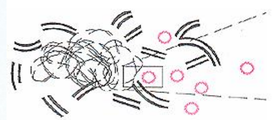
sodium dodecyl sulfate (SDS;十二烷基硫酸鈉)



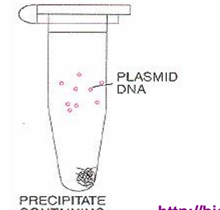
shows the plasmid DNA and chromosomal DNA

提高滲透壓使細胞裂解

addition of NaOH causes denaturation of plasmid DNA and chromosomal DNA



Acid addition neutralizes the base and in turn renatures the DNA and plasmid



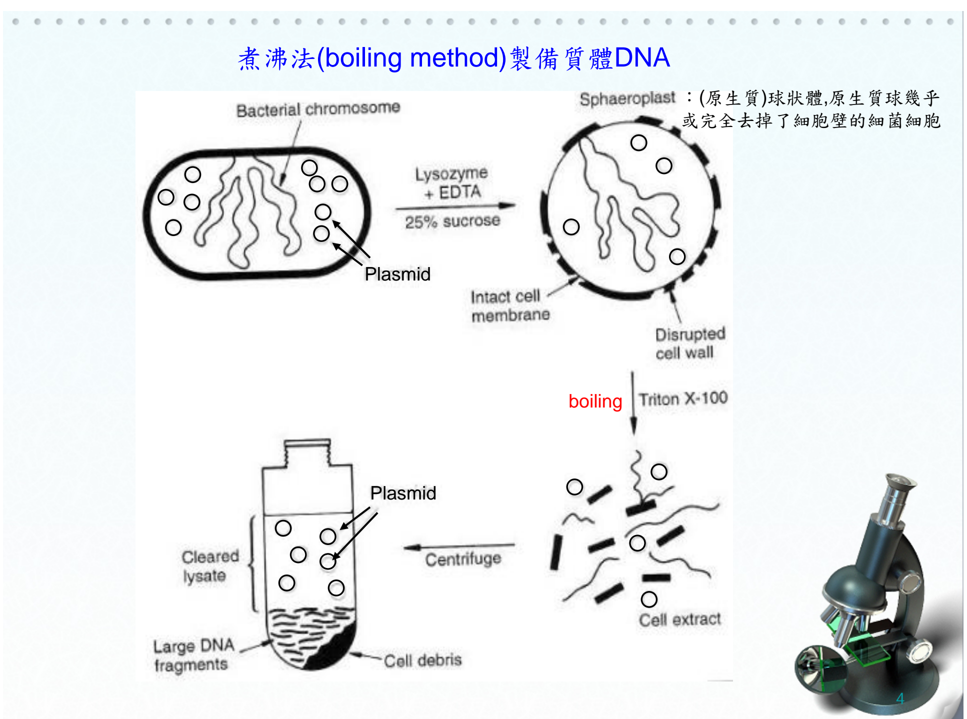
PRECIPITATE CONTAINING CHROMOSOMAL DNA

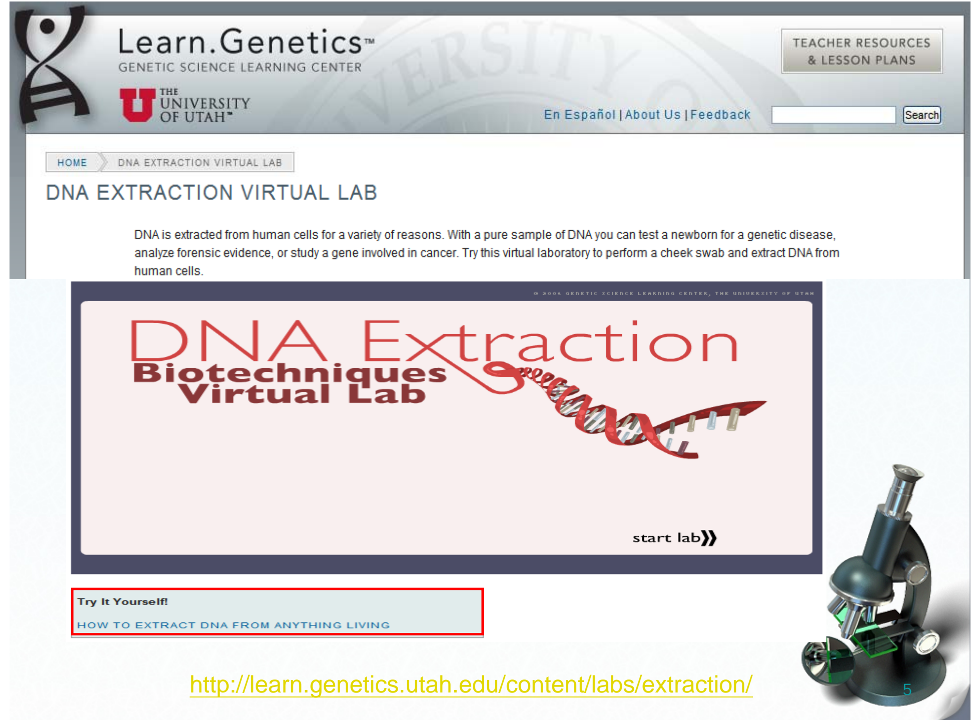
The chromosomal DNA caught within the SDS/ lipids is pellet down in cetrifuge and the plasmid DNA is found in the lysate.

http://bioinfo2010.wordpress.com/

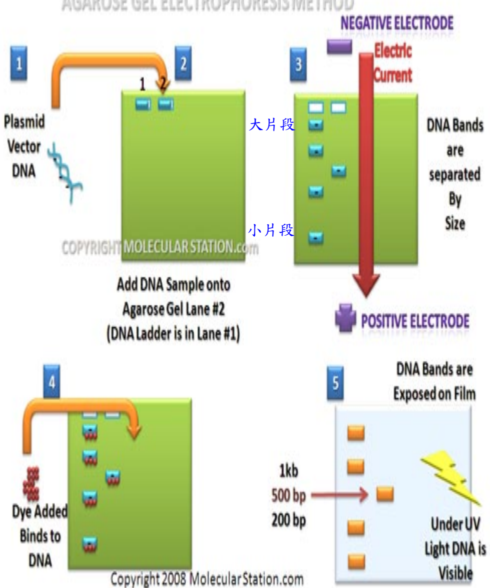
煮沸法(boiling method)製備質體DNA

Sphaeroplast ：(原生質)球狀體,原生質球幾乎或完全去掉了細胞壁的細菌細胞

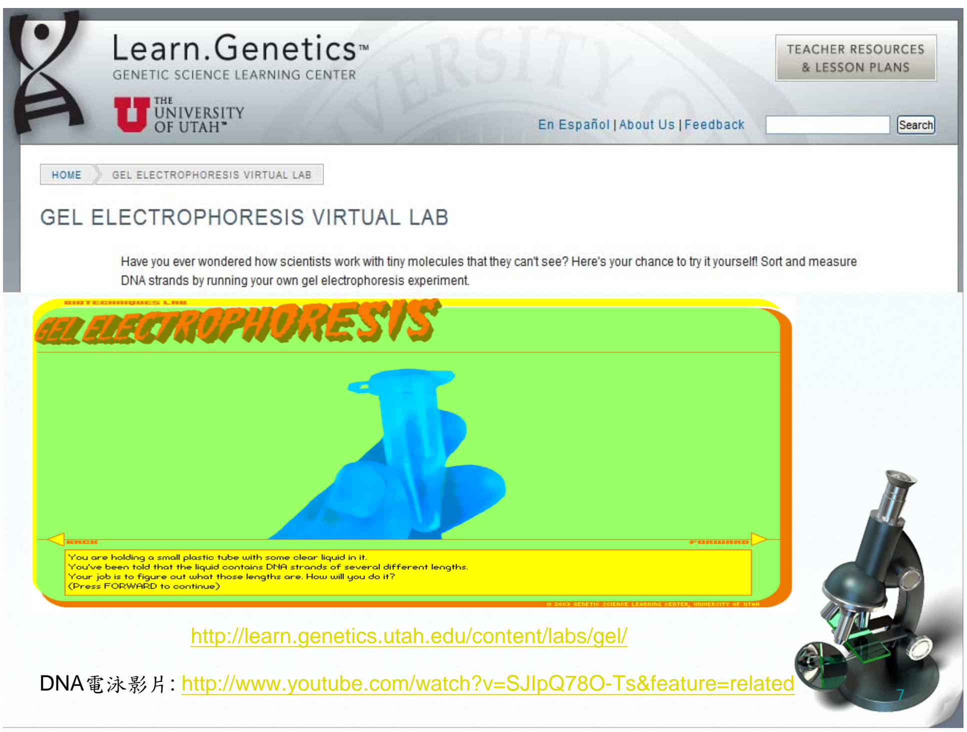




- 萃取的質體，以不同限制酵素作用，經電泳分離DNA片段後，比較各DNA片段之分子量大小，用以檢測所分離的質體DNA之正確性。
- 洋菜膠電泳是把 agarose 加熱溶解後再冷凝，洋菜膠會以氫鍵形成凝膠。而經限制酶切過的 DNA 片段，在電場影響下會在凝膠中移動，帶負電荷的 DNA 分子會向正極方向移動。
- 移動速度依分子大小而定，分子愈大移動性愈慢。而凝膠中 agarose 的濃度決定了空隙的大小，特定大小的 DNA 片段在不同濃度的膠中移動速率均不同，故每次電泳均需以 DNA marker 做為比較。
- 核酸在膠體中可經溴化乙錠 ethidium bromide (EtBr) 染色， EtBr 會嵌入核酸鹼基中，以紫外線照射，則核酸吸收紫外線波長的光線，再經 EtBr 放出可見波長的光線，藉以觀察核酸的位置。



http://www.molecularstation.com/agarose-gel-electrophoresis/



二〇一〇年國際生物奧林匹亞競賽
國手選拔初賽

Q:下列那種處理最不會影響DNA片段在電泳膠體內之泳動速率？

(A) 將DNA片段上所帶的負電加以中和

(B) 增加DNA片段的長度

(C) 將DNA片段的序列改變

(D) 將DNA片段上的胞嘧啶加以甲基化

(E) 將DNA片段縮短

解答:

重組DNA技術(Recombinant DNA technology)
= 基因工程;遺傳工程(Genetic engineering)

Key element of biotechnoloy：use recombinant DNA methods to move a gene from any organism to any other organism.

Proc. Nat. Acad. Sci. USA
Vol. 70, No. 11, pp. 3240-3244, November 1973

Construction of Biologically Functional Bacterial Plasmids *In Vitro*

(R factor/restriction enzyme/transformation/endonuclease/antibiotic resistance)

STANLEY N. COHEN*, ANNIE C. Y. CHANG*, HERBERT W. BOYER†, AND ROBERT B. HELLING†

* Department of Medicine, Stanford University School of Medicine, Stanford, California 94305; and † Department of Microbiology, University of California at San Francisco, San Francisco, Calif. 94122

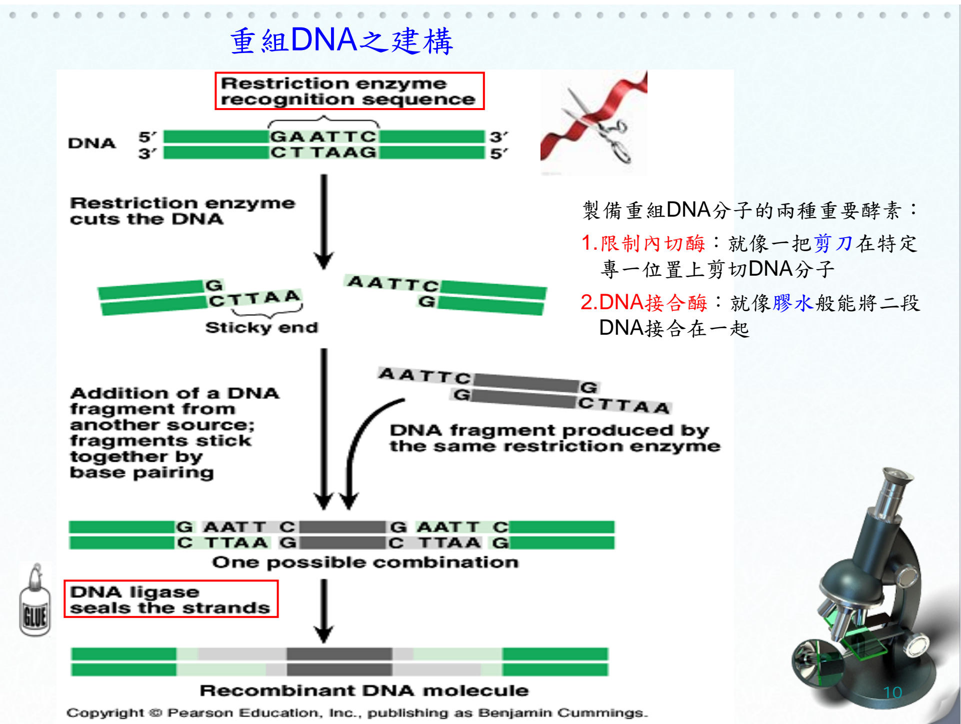
Communicated by Norman Davidson, July 18, 1973

ABSTRACT The construction of new plasmid DNA species by *in vitro* joining of restriction endonuclease-generated fragments of separate plasmids is described. Newly constructed plasmids that are inserted into *Escherichia coli* by transformation are shown to be biologically functional replicons that possess genetic properties and nucleotide base sequences from both of the parent DNA molecules. Functional plasmids can be obtained by reassociation of endonuclease-generated fragments of larger replicons, as well as by joining of plasmid DNA molecules of entirely different origins.

Controlled shearing of antibiotic resistance (R) factor DNA leads to formation of plasmid DNA segments that can be *Eco*RI-generated fragments have been inserted into appropriately-treated *E. coli* by transformation (7) and have been shown to form biologically functional replicons that possess genetic properties and nucleotide base sequences of both parent DNA species.

MATERIALS AND METHODS

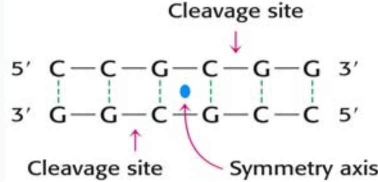
E. coli strain W1485 containing the RSF1010 plasmid, which carries resistance to streptomycin and sulfonamide, was obtained from S. Falkow. Other bacterial strains and R factors and procedures for DNA isolation, electron microscopy, and transformation of *E. coli* by plasmid DNA have been described (1, 7, 8). Purification and use of the *Eco*RI restric-



Restriction enzymes split DNA into specific fragments

📖 Restriction enzymes (*restriction endonucleases, RE*): recognize specific base sequences in double strand DNA and cleave, at specific places, both strands of a duplex containing the recognized sequences.

1. Restriction enzymes are found in prokaryotes. Their biological role is to cleave foreign DNA.
2. The recognized sequence is "***palindromic*** (迴文)". Greek means "running back again".
   e.g. Radar; Do geese see God? 上海自來水來自海上; 花蓮噴水池水噴蓮花



Sac II (from *Streptomyces achromogenes*)

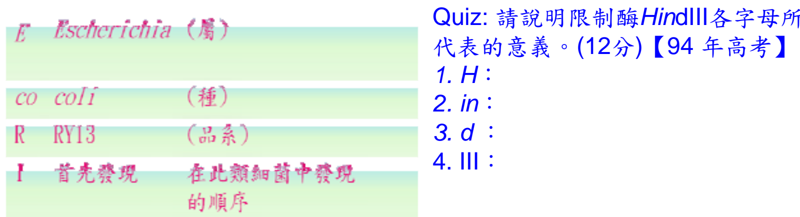
3. The pattern of fragment that DNA be cleaved by several RE can serve as a fingerprint of a DNA molecule (RE map)

限制酶的種類

- 已知的限制酶分為Type I、Type II與 Type III三類。
- Type I與Type III限制酶同時具有endonuclease與methylase的活性。
- Type I限制酶會隨機的切割距離辨識位置~1000 bp的序列，Type III則只會切割距離辨識位置24~26 bp的序列。
- Type II限制酶，則會專一地切割所辨識的核酸序列的位置，一般可辨識雙股DNA上特定的4~8個鹼基，並能在認知序列內的特定位點上切割雙股螺旋DNA。
- 每一種限制酶能辨識一特定的DNA序列並加以切割，但它不會切割細菌自己的 DNA，因為細菌 DNA 上的切割位置已被甲基化，稱為restriction-modification system。
- 不同的細菌中可純化出具不同專一性的限制酵素，可用來做基因剪接時的「剪刀」，使其成為基因或分子遺傳操作上極為有用的工具，目前被廣泛應用於遺傳工程、基因選殖(cloning)和基因圖譜分析(gene mapping) 。

限制酶的命名

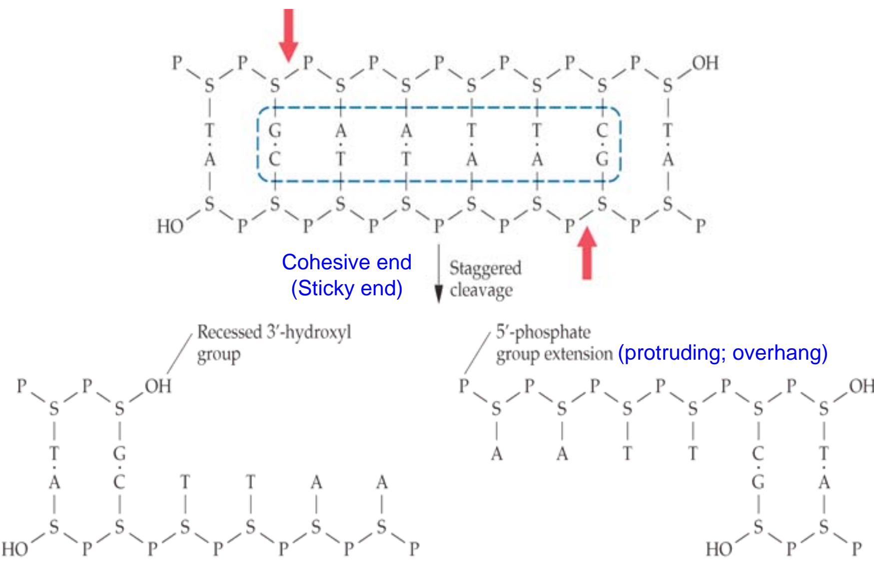
- 限制酶的命名是以該酶來源的原核生物的名稱爲依據，即酶的名稱的第一個大寫字母取自於屬名的第一個字母，第二、三個小寫字母取自於種名的前兩個字母，字母後面的羅馬字則是簡單地表明該種生物中不同限制酶分離的先後順序。
- 限制酶名稱的書寫方式，前三個字母常用斜體或加下橫線表示。例如， 分離自產色鏈黴菌(*Streptomyces achromogenes*)的兩種限制酶被分別命名爲*Sac*I和 *Sac*II。
- 若同一生物種內又分爲不同的血清型或菌株品系，其名稱則放在限制酶名稱的第三個字母之後。
  例如限制酶*Hin*cⅡ和*Hin*dⅢ則是分別來自流感嗜血菌(*Haemophilus influenzae*)的c和d血清型菌株。



Quiz: 請說明限制酶*Hin*dIII各字母所代表的意義。(12分)【94 年高考】
1. *H*：
2. *in*：
3. *d* ：
4. III：

Restriction Endonucleases

Type II restriction endonuclease *Eco*RI cleavage



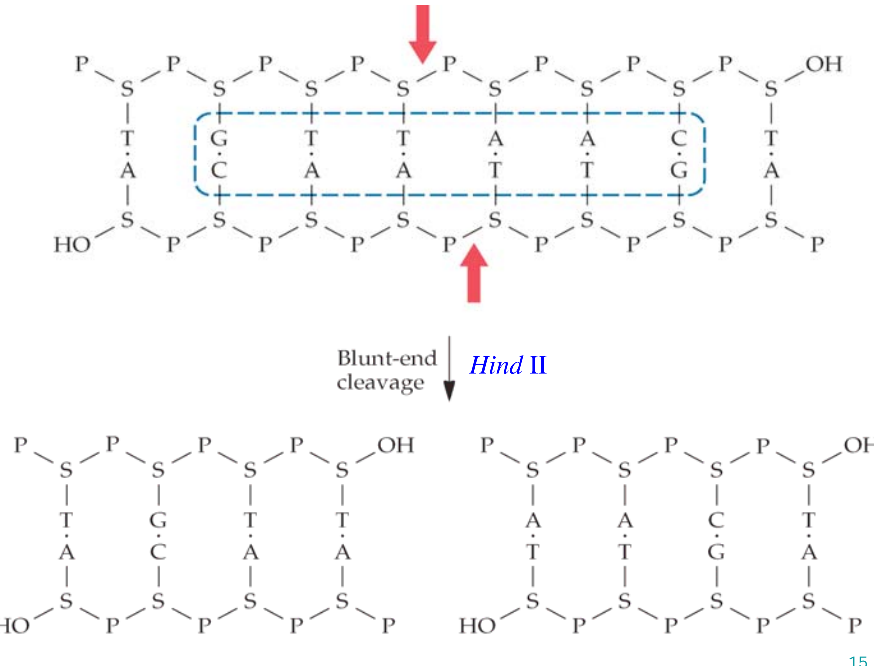
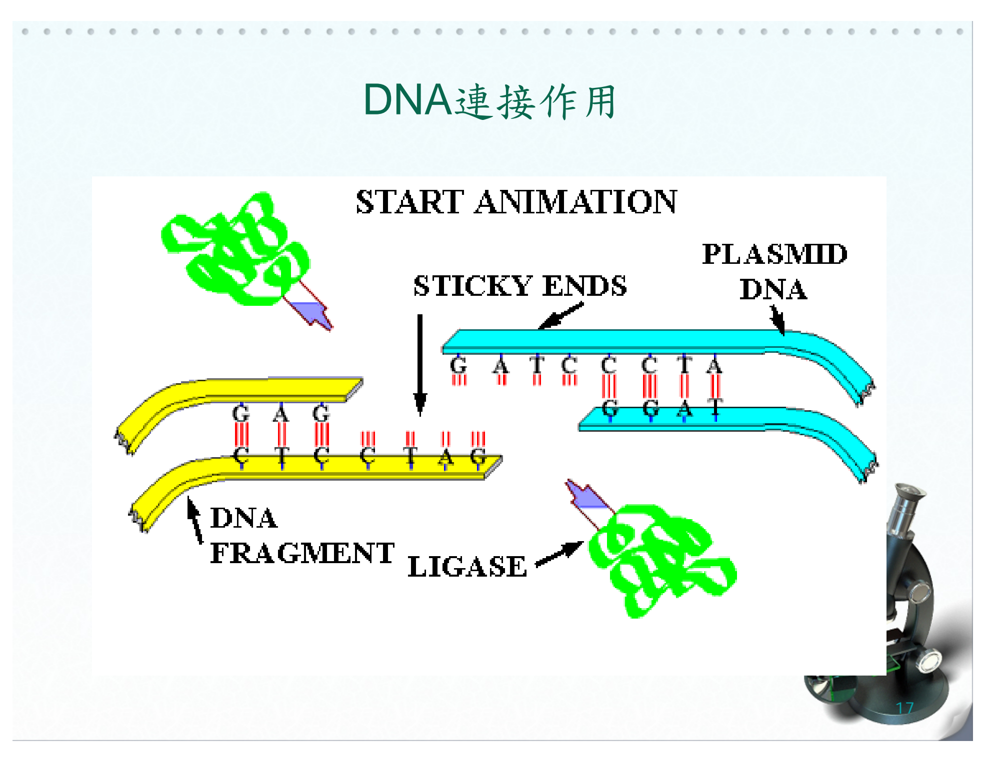


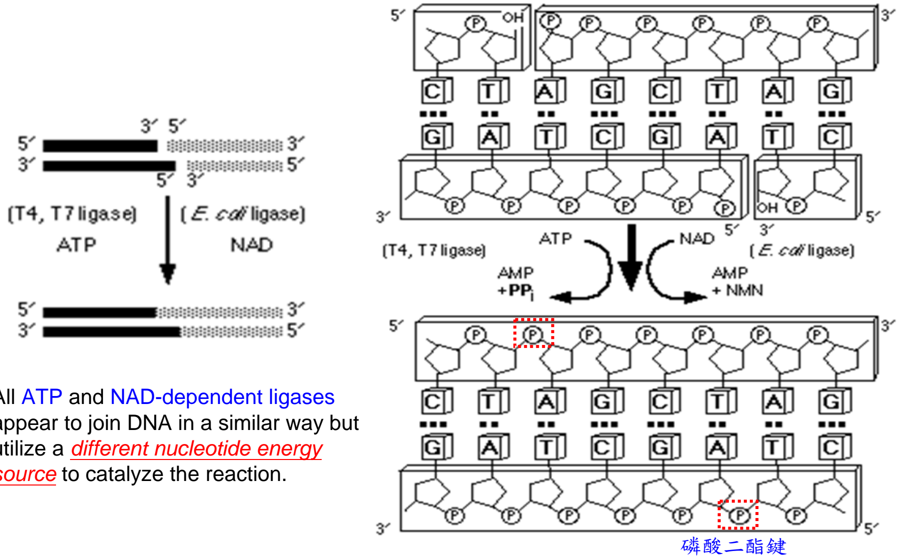
TABLE 3.1 Recognition sequences of some restriction endonucleases

Enzyme	Recognition site	Type of cut end
EcoRI	G↓A–A–T–T–C C–T–T–A–A↑G	5′ phosphate extension
BamHI	G↓G–A–T–C–C C–C–T–A–G↑G	5′ phosphate extension
PstI	C–T–G–C–A↓G G↑A–C–G–T–C	3′ hydroxyl extension
Sau3AI	↓G–A–T–C C–T–A–G↑	5′ phosphate extension
PvuII	C–A–G↓C–T–G G–T–C↑G–A–C	Blunt end
HpaI	G–T–T↓A–A–C C–A–A↑T–T–G	Blunt end
HaeIII	G–G↓C–C C–C↑G–G	Blunt end
NotI	G↓C–G–G–C–C–G–C C–G–C–C–G–G–C↑G	5′ phosphate extension

Arrows denote cleavage sites.



DNA 連接酶作用機制



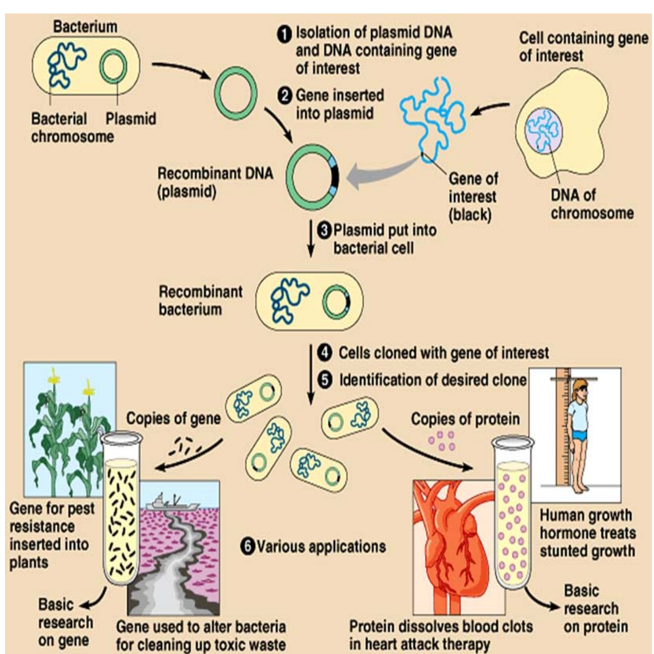
All ATP and NAD-dependent ligases appear to join DNA in a similar way but utilize a *different nucleotide energy source* to catalyze the reaction.

重組DNA影片: http://www.youtube.com/watch?v=x2jUMG2E-ic

基因選殖 (gene cloning)

基因選殖的步驟包括：

- 分離感興趣的DNA(例如胰島素基因)
- 把感興趣的DNA接合到載體
- 把重組的DNA轉形到宿主細胞中
- 篩選含有重組DNA的宿主細胞
- 檢測含有重組DNA或是否產出適當蛋白質產物的宿主細胞



選殖載體 (cloning vector)

選殖載體的條件：

- 具有複製起點，使DNA可以在宿主細胞中進行複製。
- 具有許多單一特殊的限制酶切位，以供選殖DNA片段使用。
- 具有篩選性的標記，可以用來測定選殖重組DNA是否已轉移至細胞中或顯示外來的DNA是否已經插入至載體上。

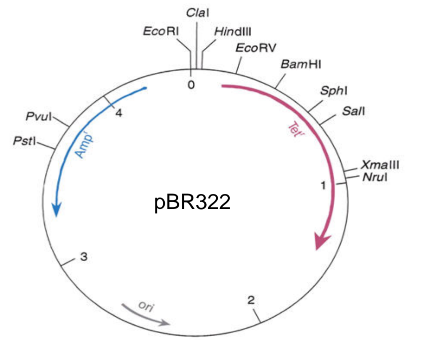
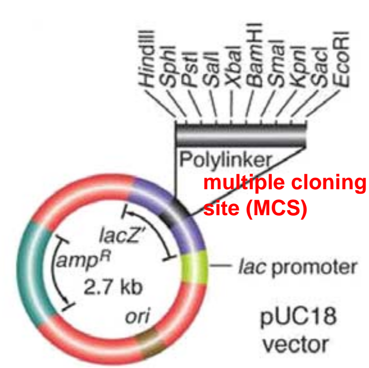
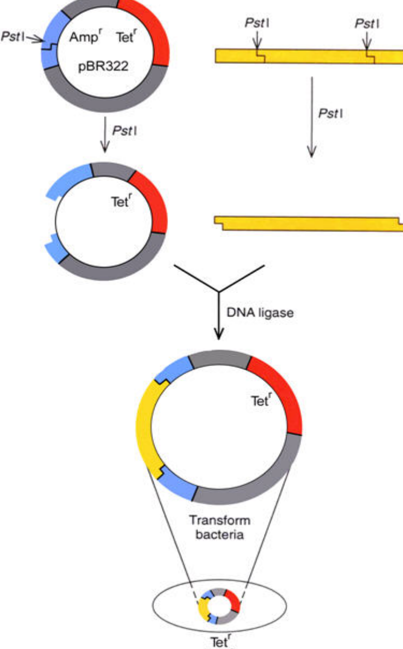


Figure 4.3 The plasmid pBR322, showing the locations of 11 unique restriction sites that can be used to insert foreign DNA. The locations of the two antibiotic resistance genes (Amp^r = ampicillin resistance; Tet^r = tetracycline resistance) and the origin of replication (ori) are also shown. Numbers refer to distances in kilobase pairs (kb) from the EcoRI site.





Clone a foreign DNA into the *PstI* site of pBR322

1. Cut the vector to generate the sticky ends
2. Cut foreign DNA with *PstI* also – compatible ends
3. Combine vector and foreign DNA with DNA ligase to seal sticky ends
4. Now transform the plasmid into *E. coli*

Figure 4.4 Cloning foreign DNA using the *PstI* site of pBR322. Cut both the plasmid and the insert (yellow) with *PstI*, then join them through these sticky ends with DNA ligase. Next, transform bacteria with the recombinant DNA and screen for tetracycline-resistant, ampicillin-sensitive cells. The recombinant plasmid no longer confers ampicillin resistance because the foreign DNA interrupts that resistance gene (blue).

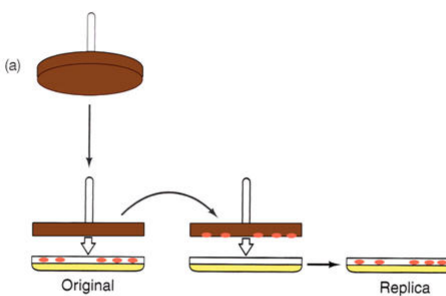
Bacterial Transformation

- Traditional method involves incubating bacterial cells in concentrated calcium salt solution
  - The solution makes the cell membrane leaky, permeable to the plasmid DNA
- Newer method uses high voltage to drive the DNA into the cells in process called electroporation

Screening Transformants

- Transformation produces bacteria with:
  - Religated plasmid
  - Religated insert
  - Recombinants
- Identify the recombinants using the antibiotic resistance (Fig. 4.4)
  - Grow cells with tetracycline so only cells with plasmid grow, not foreign DNA only.
  - Next, grow copies of the original colonies with ampicillin which kills cells with plasmid including foreign DNA.

Screening With Replica Plating



- Replica plating transfers **clone copies** from original **tetracycline** plate to a plate containing **ampicillin.**
- A sterile velvet (天鵝絨) transfer tool can be used to transfer copies of the original colonies
- Desired colonies are those that do **NOT** grow on the new ampicillin plate.

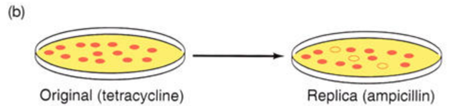


Figure 4.5 Screening bacteria by replica plating. (a) The replica plating process. Touch a velvet-covered circular tool to the surface of the first dish containing colonies of bacteria. Cells from each of these colonies stick to the velvet and can be transferred to the replica plate in the same positions relative to each other. **(b)** Screening for inserts in the pBR322 ampicillin resistance gene by replica plating. The original plate contains tetracycline, so all colonies containing pBR322 will grow. The replica plate contains ampicillin, so colonies bearing pBR322 with inserts in the ampicillin resistance gene will not grow (these colonies are depicted by dotted circles). The corresponding colonies from the original plate can then be picked.

Plasmid cloning影片: http://www.youtube.com/watch?v=acKWdNj936o&NR=1

Vectors for cloning large pieces of DNA

TABLE 3.7 Insert capacities of some commonly used vector systems

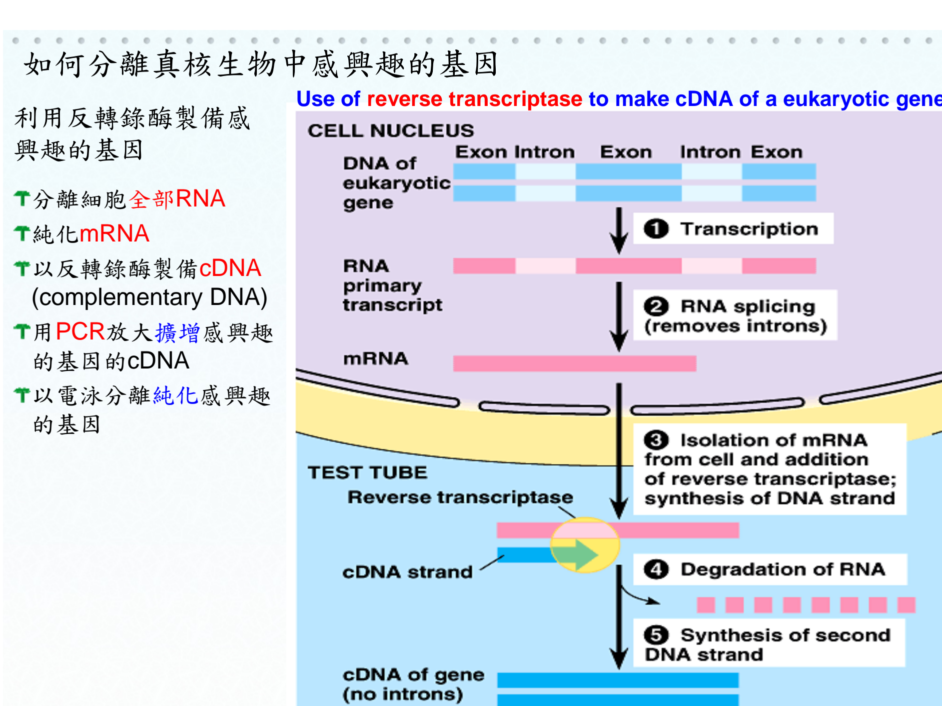
Vector system	Host cell	Insert capacity (kb)
Plasmid	*E. coli*	0.1–10
Bacteriophage λ	λ/*E. coli*	10–20
Cosmid	*E. coli*	35–45
Fosmid	*E. coli*	35–45
Bacteriophage P1	*E. coli*	80–100
BAC	*E. coli*	50–300
P1 bacteriophage-derived artificial chromosome	*E. coli*	100–300
Yeast artificial chromosome	Yeast	100–2,000
Human artificial chromosome	Cultured human cells	>2,000

Fosmids are similar to cosmids but are based on the bacterial F-plasmid. The cloning vector is limited, as a host (usually *E. coli*) can only contain one fosmid molecule. Low copy number offers higher stability than comparable high copy number cosmids.

如何分離真核生物中感興趣的基因

利用反轉錄酶製備感興趣的基因

- 分離細胞全部RNA
- 純化mRNA
- 以反轉錄酶製備cDNA (complementary DNA)
- 用PCR放大擴增感興趣的基因的cDNA
- 以電泳分離純化感興趣的基因



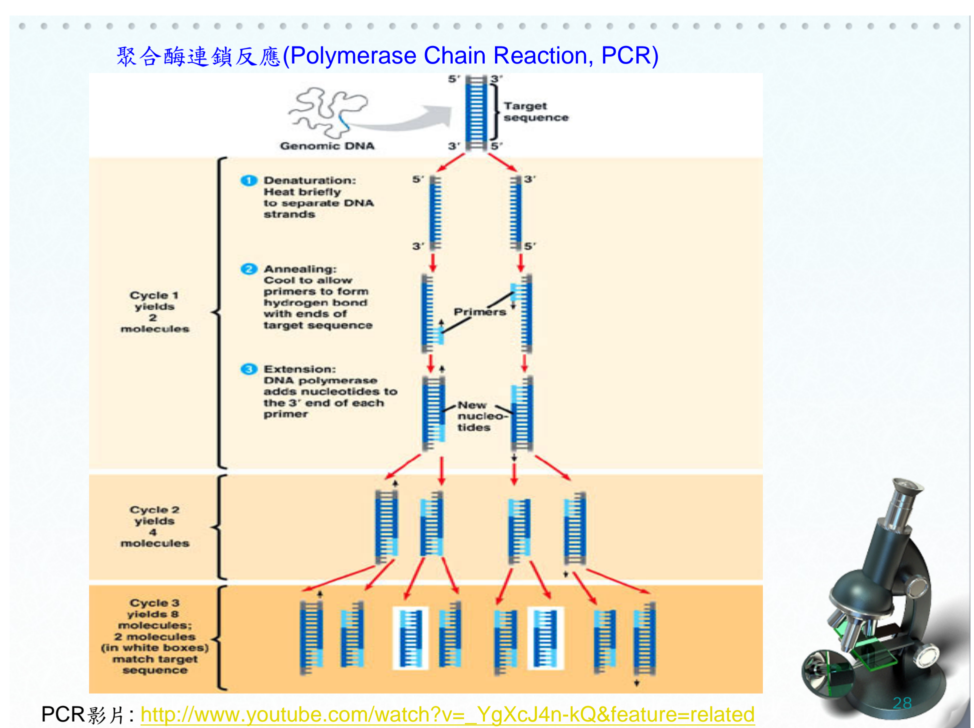
Copyright © Pearson Education, Inc., publishing as Benjamin Cummings.

聚合酶連鎖反應(Polymerase Chain Reaction, PCR)

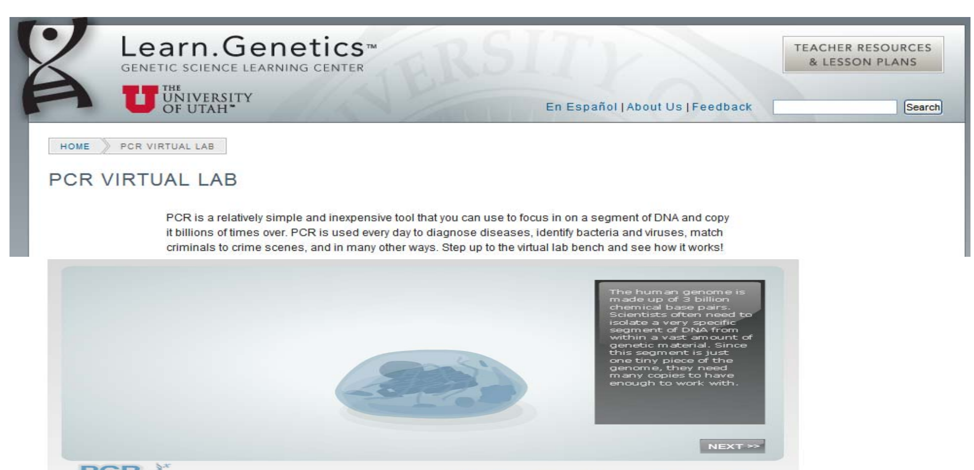
- 由Kary Mullis發明，並於1993年獲得了諾貝爾化學獎。
- PCR能使DNA在試管中大量擴增，其原理是在擬擴增的DNA片段兩端分別設計一個前置引子(forward primer)和反置引子(reverse primer)，使其與已變性的單股目標DNA緩冷配對黏合(annealing)後，利用DNA聚合酶(DNA polymerase)以目標DNA的兩股分別做為模板(template)來合成新的DNA股。
- PCR過程主要分成三大部份:
  1. 變性反應(denaturation):以高溫92℃-95℃使雙股模板DNA分離
  2. 緩冷配對黏合(annealing) :使引子與單股模板DNA做緩冷配對(40℃-52℃)
  3. 延長反應(extension):將溫度調整到DNA聚合酶作用的有效溫度而合成新的DNA股(72℃)
- 在理想的條件下，DNA以幾何級數增加。理論上，一個DNA分子若重複操作PCR 25次，那麼DNA的分子數將會擴增到$2^{25} = 10^{6}$ 個分子。
- 影響PCR DNA合成時精確性的因素
  - 所欲合成的DNA的長度，長度越長出錯機率越高。
  - 循環數(越多時精確度越低)
  - 聚合酶的種類(有校對能力者為佳)
  - Mg^{2+} (0.5-2.5mM)的量等。

聚合酶連鎖反應(Polymerase Chain Reaction, PCR)

- 一般的DNA聚合酶有效作用溫度是37℃，在高溫分離雙股時會破壞DNA聚合酶的活性。然而在耐高溫的細菌(*Thermus aquaticus*)中分離出來的DNA聚合酵素(*Taq* DNA polymerase)在95℃中其活性的半衰期(half life)長達40分鐘，故可供PCR操作使用。
- Taq聚合酶的有效作用溫度為72℃，在這溫度下，每分鐘可合成2000-4000個核苷酸(nucleotides)。由於Taq聚合酵素的發現，使PCR之操作得以自動化。
- Taq聚合酶缺乏3'至5'端外切酵素(exonuclease)的特性，因而在DNA合成時沒有校對(proofreading)的功能，Taq聚合酶合成DNA時，在每一個循環中錯誤配對的頻率可高達1/6000個核苷酸。
- PCR的技術已廣泛地應用在學術，工業和醫學上的研究，例如
  - DNA序列的分析
  - 病原菌的分析
  - 基因定位突變
  - 遺傳病之診斷
  - 基因表現與選殖
  - 水質及食品檢驗

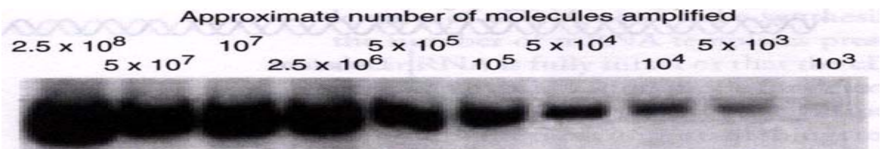


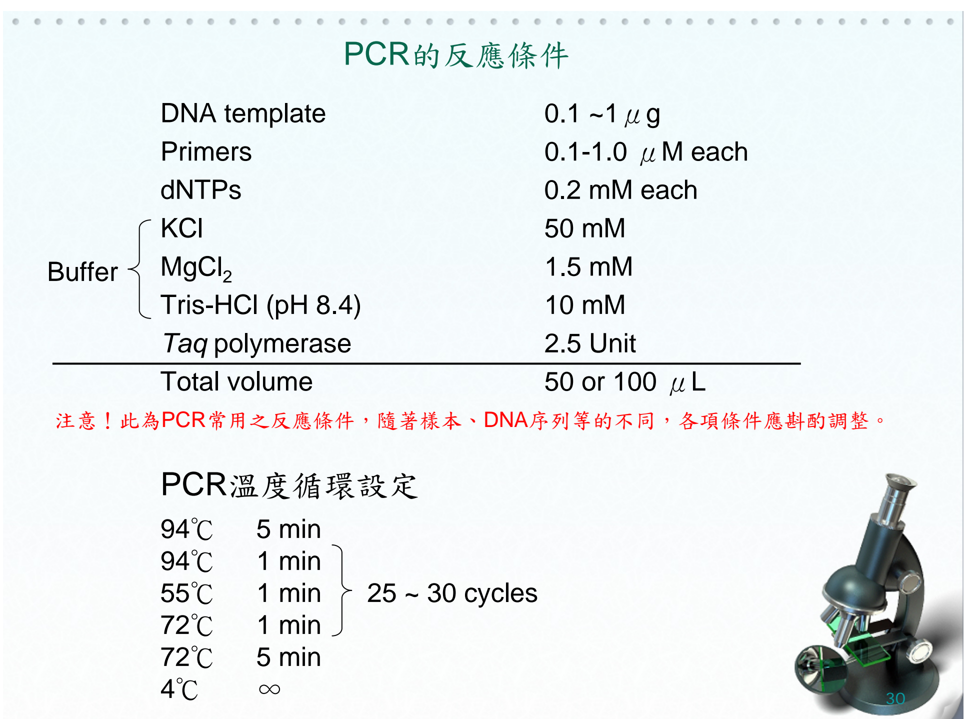
PCR影片: http://www.youtube.com/watch?v=_YgXcJ4n-kQ&feature=related



PCR實驗操作:http://learn.genetics.utah.edu/content/labs/pcr/

利用PCR技術將DNA分子擴增之電泳圖





PCR的反應條件

	DNA template	0.1 ~1 μg
	Primers	0.1-1.0 μM each
	dNTPs	0.2 mM each
Buffer	KCl	50 mM
Buffer	$MgCl_2$	1.5 mM
Buffer	Tris-HCl (pH 8.4)	10 mM
	Taq polymerase	2.5 Unit
	Total volume	50 or 100 μL

注意！此為PCR常用之反應條件，隨著樣本、DNA序列等的不同，各項條件應斟酌調整。

PCR溫度循環設定

94℃	5 min	
94℃	1 min	25 ~ 30 cycles
55℃	1 min	25 ~ 30 cycles
72℃	1 min	25 ~ 30 cycles
72℃	5 min	
4℃	∞	

PCR溫度循環設定

- 起始以高溫將雙股DNA分離
  - 對PCR而言，將雙股DNA完全分開是相當重要的起始步驟，作用不完全將會降低PCR的產量。在GC含量50%以下時，溫度設定在95℃時間1~3分鐘。時間可隨著模版上GC的比例增加逐漸調升。
  - 通常94~95℃進行1~2分鐘即可將兩股DNA分離，如果GC含量過高，可將時間提高至4~5分鐘。或者加入10-15%glycerol、10%DMSO、5% formamide等溶液促使DNA分離，但此等溶液的副作用是會抑制Taq DNA polymerase約50%的活性。
- Primer黏合溫度
  - 一般來說，引子與模版DNA黏合溫度(annealing temperature;Ta)大約是引子分離核酸溫度(melting temperature; Tm)減5 ℃。而這溫度最好落於55-75 ℃之間。如果除了目標產物外，有些非預期的產物出現，則可逐漸提高黏合溫度約1~2℃。
  - 若2 條引子所算出之Ta 相差超過6 ℃以上(可能會影響PCR 的產率)，可以將算出溫度較低的那段引子的3'或5'端加幾個鹼基。

$$T_m(℃) = 2(A+T) + 4(G+C)$$

引子(primer)設計的注意事項：

1. 引子長度通常為18-25個鹼基長。
2. GC含量約佔40-60% 。
3. 最好3'-端的鹼基為G/C。
4. 避免引子本身形成二級結構。
5. 同一反應中的引子序列不可互補，以免造成自相黏合形成primer dimer的情況
6. 避免引子所要黏合的目標形成二級結構
7. 引子黏合溫度盡量不要相差5℃以上。
8. 3'端尤其重要，必須不含有與其他引子互補的序列，並且要與模板完全互補，絕對不可以有mismatch。

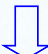
- Design the primer pairs, 15-mer for each, to amplify the gene sequence below with PCR? It needs to indicate the 5'- and 3'-end of each primer.

5'-GCGTTGACGGTATCAAAACGTTAT… …TTTACCTGGTGGGCTGTTCTAATC-3'

Ans:

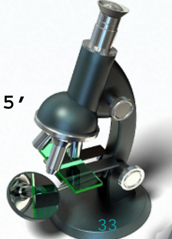
5'-GCGTTGACGGTATCAAAACGTTAT… …TTTACCTGGTGGGCTGTTCTAATC-3'

3'-CGCAACTGCCATAGTTTTGCAATA… …AAATGGACCACCCGACAAGATTAG-5'



5'-GCGTTGACGGTATCAAAACGTTAT… …TTTACCTGGTGGGCTGTTCTAATC-3'

3'-CGCAACTGCCATAGTTTTGCAATA… …AAATGGACCACCCGACAAGATTAG-5'



DNA polymerases的選擇

Thermostable polymerase 種類繁多，一般常使用*Taq* polymerase，但有時針對所合成fragment 的長度及正確性，可考慮其他酵素。

TABLE I

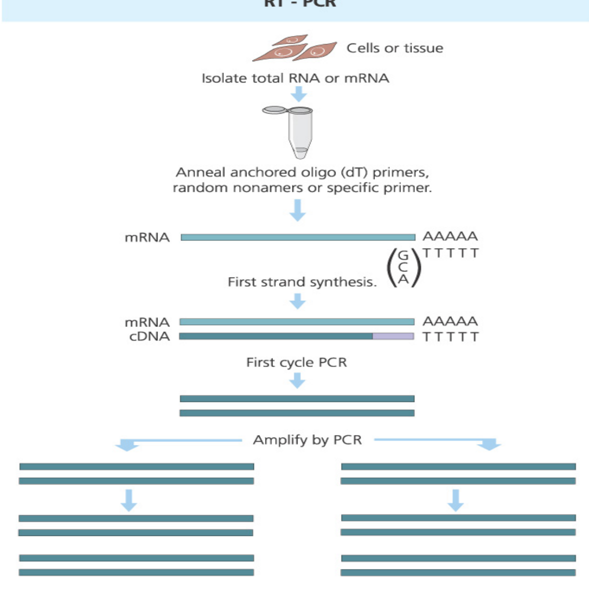
Fidelity Comparison of Thermostable DNA Polymerases Using a *lac*IOZα-Based Fidelity Assay[a]

Thermostable DNA polymerase	Error rate[b]	Percentage (%) of mutated PCR products[c]
* *Pfu* DNA polymerase	1.3×10^{-6}	2.6
Taq DNA polymerase	8.0×10^{-6}	16.0
Vent$_R$® DNA polymerase	2.8×10^{-6}	5.6
Deep Vent$_R$® DNA polymerase	2.7×10^{-6}	5.4
Tfl DNA polymerase	8.3×10^{-6}	16.6
Tbr DNA polymerase	9.5×10^{-6}	19.0
UlTma™ DNA polymerase	55.3×10^{-6}	110.6[d]

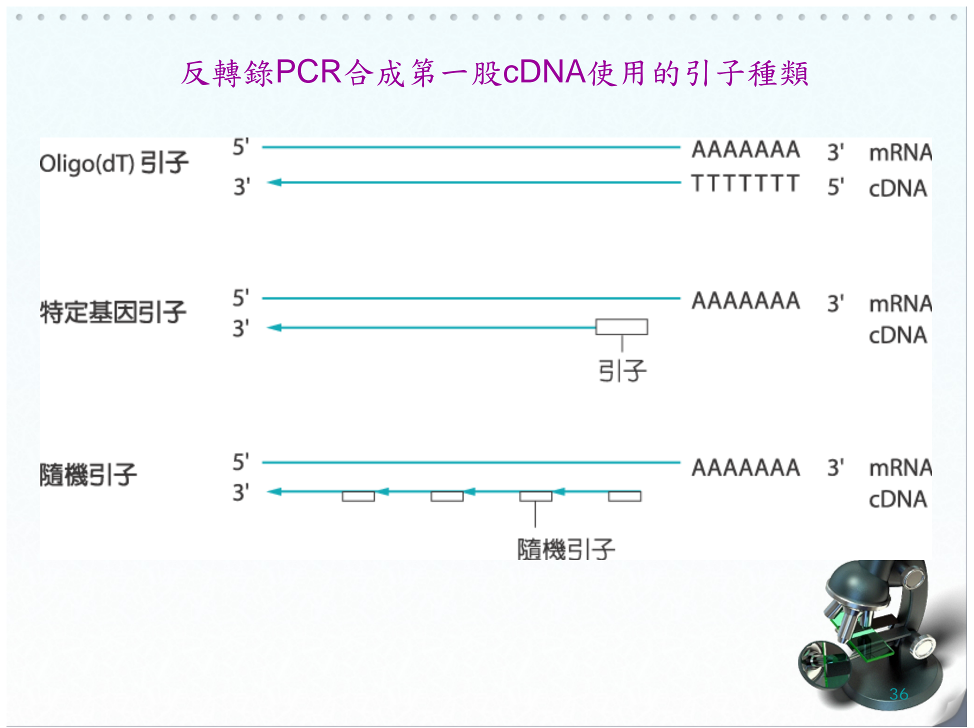
[a] Fidelity is measured using a PCR-based forward mutation assay based on the *lacI* target gene.[1]
[b] The error rate equals mutation frequency per base pair per duplication.
[c] The percentage of mutated PCR products after amplification of a 1-kb target sequence for 20 effective cycles.
[d] Some PCR products will exhibit more than one error.

反(逆)轉錄PCR (Reverse Transcription PCR; RT-PCR)

- 過程:反轉錄酶以RNA為模版合成互補DNA (complementary DNA; cDNA)，接下來再利用PCR的原理來大量擴增這段cDNA。
- RT-PCR是目前在體外 (*in vitro*)觀察基因表現最敏感的方法之一，可以檢測很低拷貝數的RNA。
- RT-PCR廣泛應用於遺傳病的診斷以及檢測RNA含量的定量分析。



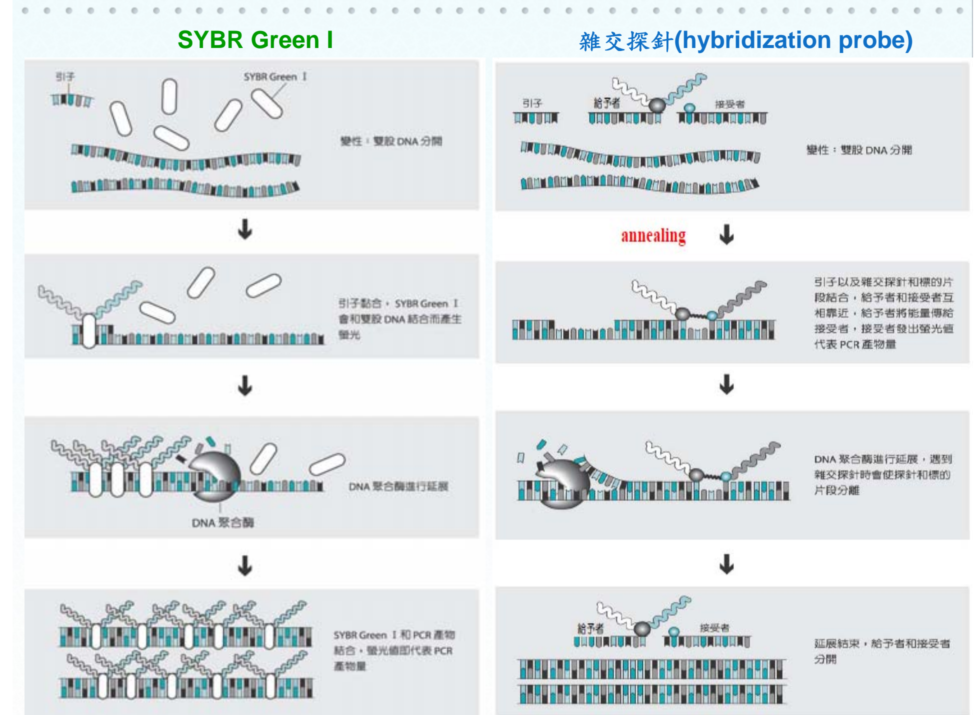
Reverse transcription影片:http://www.youtube.com/watch?v=68zTyjeUsqY



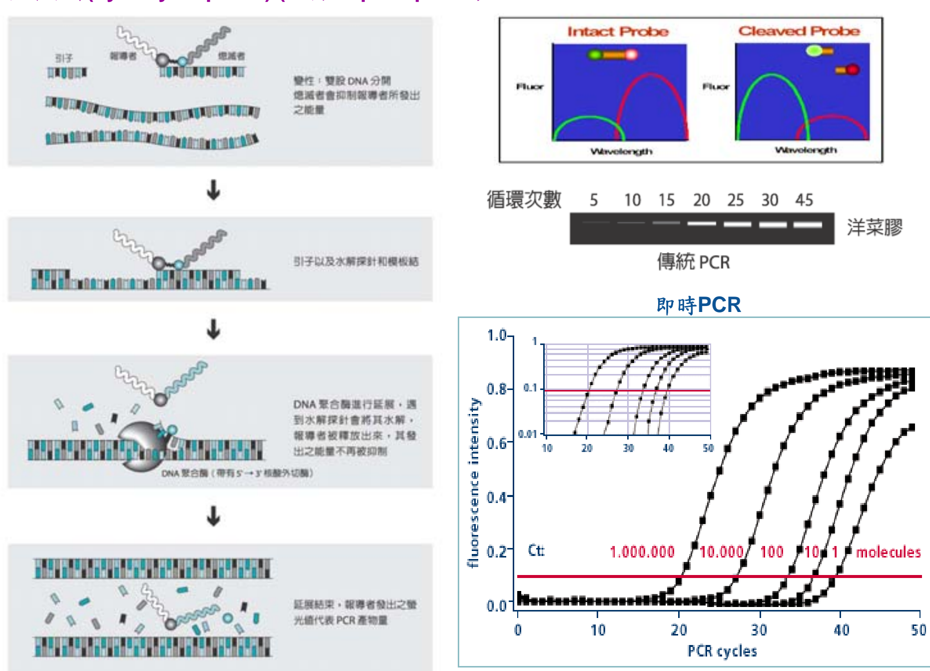
即時聚合酶連鎖反應(Real-time PCR；即時PCR)

- Real-time PCR又稱定量即時聚合酶連鎖反應(Quantitative real time polymerase chain reaction，簡稱 qRT-PCR或q-PCR)，是一種在DNA擴增反應中，以螢光染劑偵測每次PCR循環後產物總量的方法。
- 一般的PCR進行後，反應多已達到飽和，測得的產物為end-point的PCR產物，若要以傳統的PCR做定量分析，則要對同一樣品同時做不同循環的PCR反應，再將產物以電泳分離，費時費工且容易污染。
- 即時PCR的基本原理有兩個要點：
  1. 對PCR反應中的每一個循環的產物能進行即時偵測並記錄下來。
  2. 用於即時偵測PCR產物的螢光染料，標記在一段可以與目標序列進行特異性結合的探針上，並且處於淬滅狀態，只有當探針與模板特異性結合以後才有可能釋放出螢光信號。
- 即時PCR常用螢光探針有三種：
  - DNA結合染劑(SYBR Green I)
  - 雜交探針(hybridization probe)
  - 水解探針(hydrolysis probe，又稱TaqMan probe)

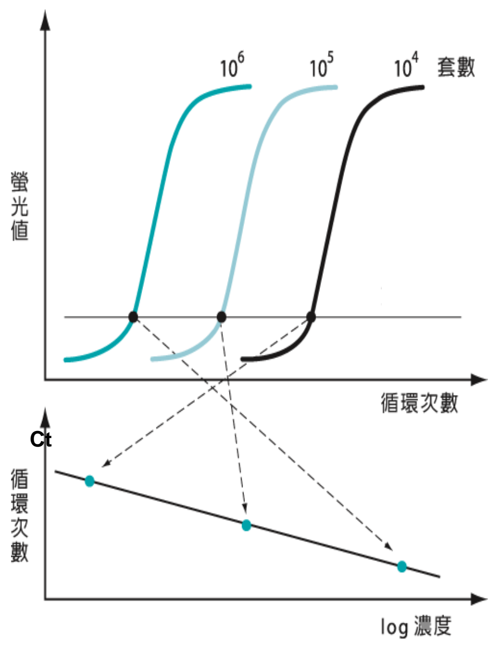
q-PCR Probe 動畫 : http://www.biosearchtech.com/support/videos/real-time-pcr-probe-animation-video.aspx
q-PCR 動畫: http://www.lifetechnologies.com/featured-solutions/pcr/real-time-pcr-animation.html
(因時間因素，請自行上網參閱)



水解探針(hydrolysis probe) (又稱TaqMan probe）



- 每一輪循環中PCR的產出量都以螢光信號被記錄在PCR儀器的光學檢測系統，在某一循環中螢光信號的強度達到預先設定的閾值(Threshold)時，對應此時的循環數稱為Ct 值(Threshold Cycle)。
- 目標DNA起始濃度與其Ct值成反比，目標DNA的起始濃度越高，其螢光值越早達到偵測之閥值，其Ct值越小；反之目標DNA起始濃度越低，其Ct值則越大。
- 根據序列稀釋標準樣品的Ct值及其已知起始濃度，即得一標準曲線 (standard curve)，根據此標準曲線，可以由樣品的Ct值推算其起始濃度，達到定量之目的。



99學年度大學入學考試指定科目【生物科】非選擇題

利用遺傳工程技術，可將不同來源的DNA組合起來，建構出重組DNA。試回答下列問題。

1. 遺傳工程技術利用酵素以切割DNA。請問這種酵素是(a)來自哪一類生物? (1分) (b)其名稱為何? (1分)
2. 利用PCR技術來擴增目標基因時，請問(a)所使用的酵素名稱為何? (1分) (b)在對溫度的敏感性質上，此酵素有何特性? (1分)
3. 能用來接合目標基因的構造稱為載體，請寫出兩種載體的名稱。(2分)
4. 在建構重組DNA過程中，請問(a)能接合目標基因和載體的酵素名稱為何? (1分) (b)哪一類原核生物常被用來複製重組DNA? (1分)

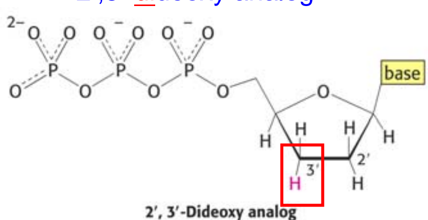
解答:

1. (a) ; (b)
2. (a) ; (b)
3.
4. (a) ; (b)

DNA定序(DNA sequencing)

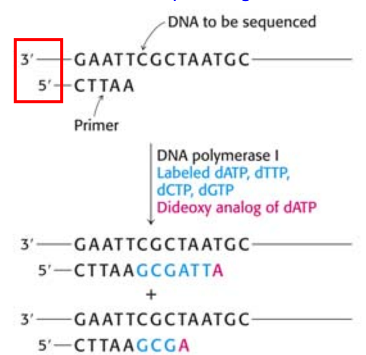
- 在1977年，有兩種DNA定序方法首先被發表出來。
  1. 哈佛大學A. Maxam與W. Gilbert發現一種可以選擇性分解鹼基的化學定序法。
  2. 由F. Sanger發展出來的雙去氧核苷酸定序法，利用雙去氧核苷酸在特定位置上造成DNA鏈合成的終止，進而推導出DNA序列。
- 這兩個方法，DNA都經過標記且利用凝膠電泳分離DNA片段，目前大多數的實驗室都使用Sanger的定序方法。

2',3'-dideoxy analog



Nobel prize in Chemistry (1980)

Strategy of the chain-termination method for sequencing DNA



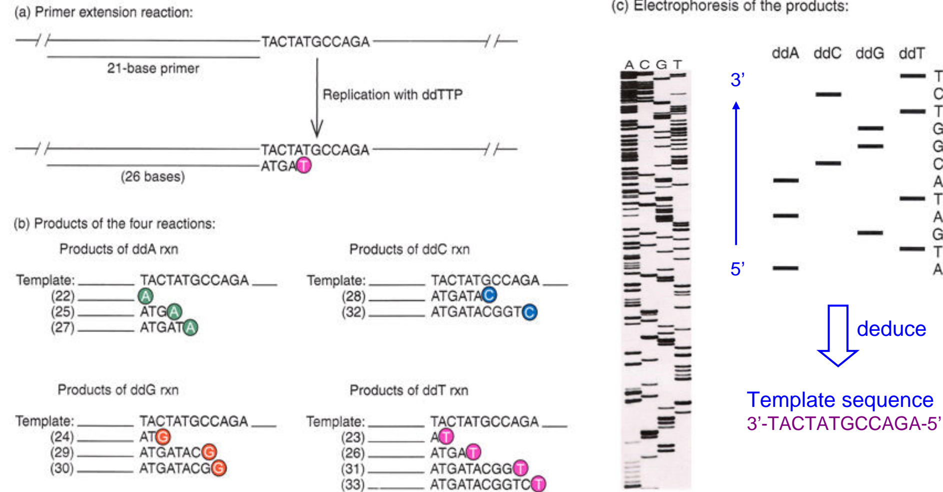
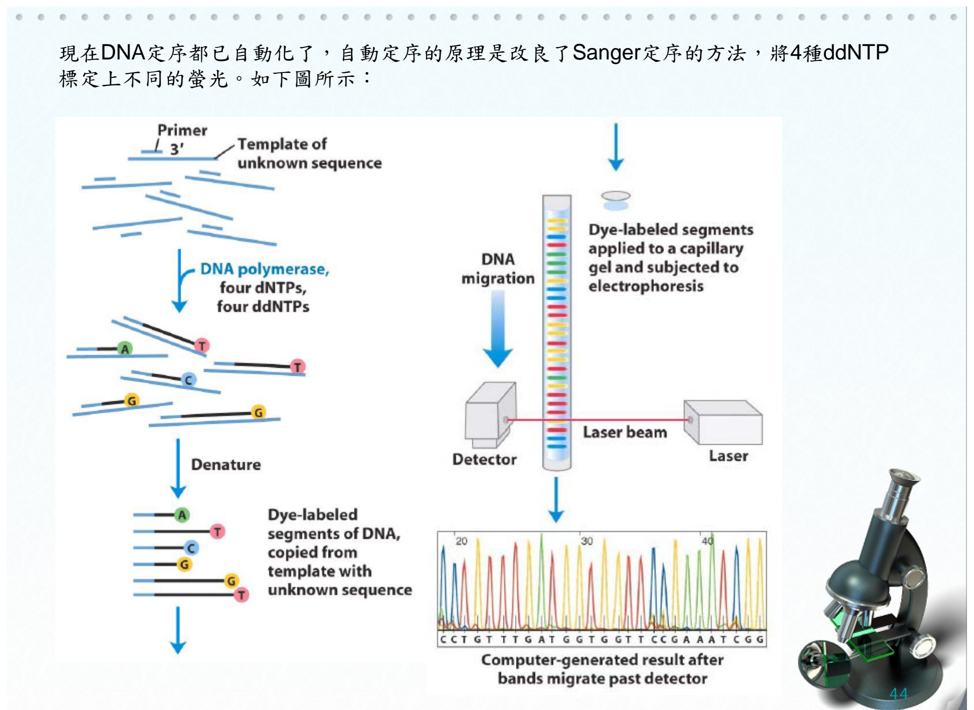


Figure 5.18 The Sanger dideoxy method of DNA sequencing. **(a)** The primer extension (replication) reaction. A primer, 21 nt long in this case, is hybridized to the single-stranded DNA to be sequenced, then mixed with the Klenow fragment of DNA polymerase and dNTPs to allow replication. One dideoxy NTP is included to terminate replication after certain bases; in this case, ddTTP is used, and it has caused termination at the second position where dTTP was called for. **(b)** Products of the four reactions (rxns). In each case, the template strand is shown at the top, with the various products underneath. Each product begins with the 21-nt primer and has one or more nucleotides added to the 3′-end. The last nucleotide is always a dideoxy nucleotide (color) that terminated the chain. The total length of each product is given in parentheses at the left end of the fragment. Thus, fragments ranging from 22 to 33 nt long are produced. **(c)** Electrophoresis of the products. The products of the four reactions are loaded into parallel lanes of a high-resolution electrophoresis gel and electrophoresed to separate them according to size. By starting at the bottom and finding the shortest fragment (22 nt in the A lane), then the next shortest (23 nt in the T lane), and so forth, one can read the sequence of the product DNA. Of course, this is the complement of the template strand.

現在DNA定序都已自動化了，自動定序的原理是改良了Sanger定序的方法，將4種ddNTP標定上不同的螢光。如下圖所示：



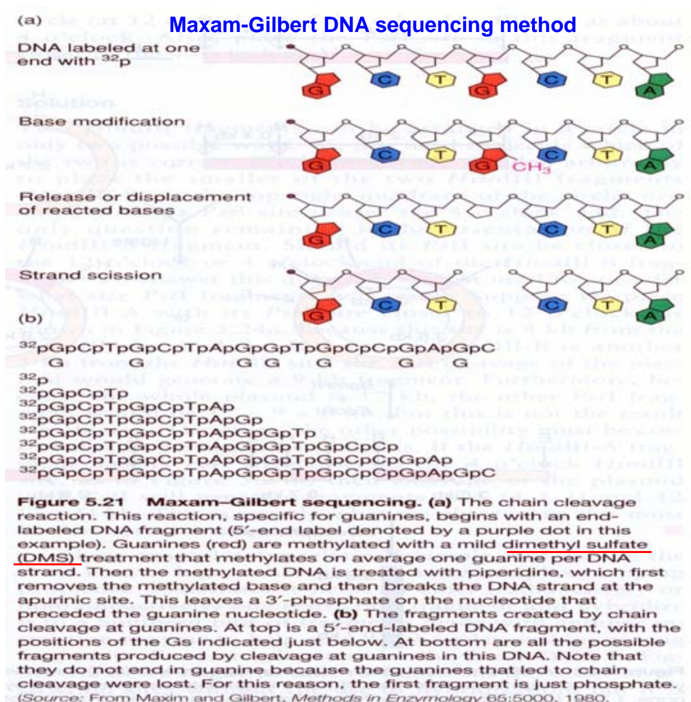
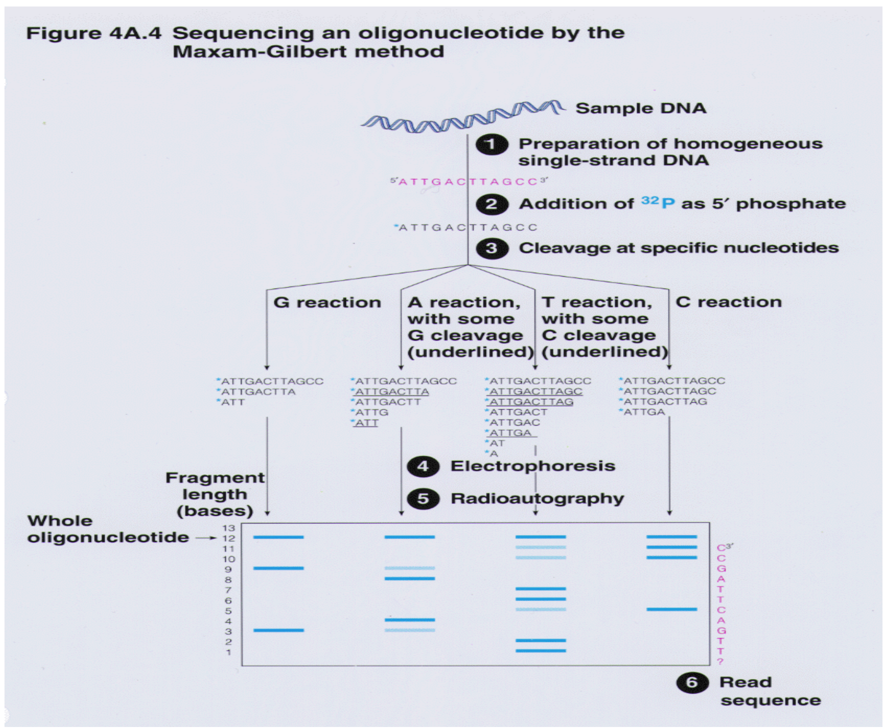


Figure 5.21 Maxam–Gilbert sequencing. (a) The chain cleavage reaction. This reaction, specific for guanines, begins with an end-labeled DNA fragment (5′-end label denoted by a purple dot in this example). Guanines (red) are methylated with a mild dimethyl sulfate (DMS) treatment that methylates on average one guanine per DNA strand. Then the methylated DNA is treated with piperidine, which first removes the methylated base and then breaks the DNA strand at the apurinic site. This leaves a 3′-phosphate on the nucleotide that preceded the guanine nucleotide. **(b)** The fragments created by chain cleavage at guanines. At top is a 5′-end-labeled DNA fragment, with the positions of the Gs indicated just below. At bottom are all the possible fragments produced by cleavage at guanines in this DNA. Note that they do not end in guanine because the guanines that led to chain cleavage were lost. For this reason, the first fragment is just phosphate. (*Source:* From Maxim and Gilbert, *Methods in Enzymology* 65:5000, 1980. Copyright 1980 Academic Press, Orlando, FL. Reprinted with permission.)



Chemicals used for Maxam-Gilbert sequencing method

Base specificity	Chemical used for base alteration	Chemical used for altered base removal	Chemical used for strand cleavage
G	Dimethylsulphate	Piperidine	Piperidine
A+G	Acid	Acid	Piperidine
C+T	Hydrazine	Piperidine	Piperidine
C	Hydrazine + alkali	Piperidine	Piperidine
A>C	Alkali	Piperidine	Piperidine

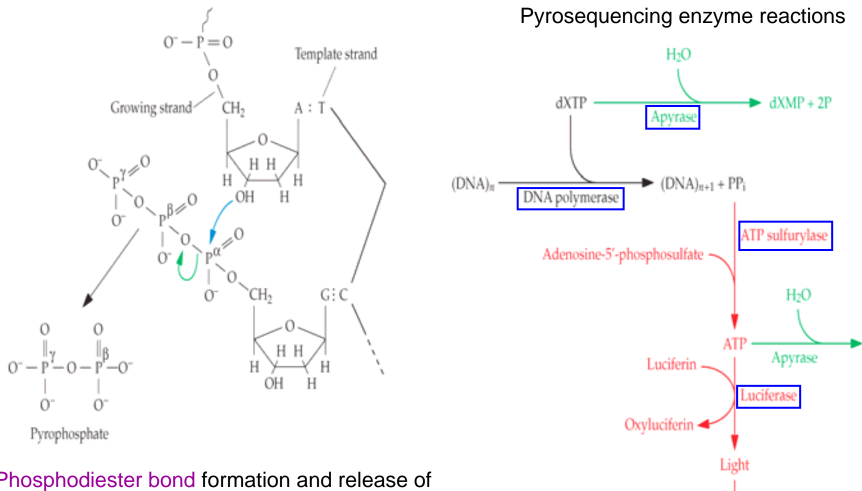
焦磷酸定序(pyrosequencing)技術

- Sanger方法的特點為解讀序列能力較強，每次反應約可得到1,000個核苷酸序列，因此可提供較多的DNA訊息。但其缺點為操作較複雜，不易於一定時間內分析大量檢體。
- 對DNA序列分析的要求通常是解讀的序列越長越好，但在許多分子診斷工作中，往往需在短時間內偵測很多DNA檢體，而且只需短短的幾十個核苷酸序列的資訊，就可以提供正確診斷。
- 例如：在臨床分子診斷領域中，對細菌和其他病原微生物的分子診斷，或在偵測單一核苷酸多型性(single nucleotide polymorphism; SNP)時，只需對最具代表性的十幾到幾十個核苷酸片段進行序列分析即可。
- 在這種情況下，Sanger 方法未必是最合適的DNA序列分析技術。最新發展的焦磷酸定序(pyrosequencing)技術，是目前最適合這些目的的DNA序列分析技術。

- Pyrosequencing技術是新世代的DNA序列分析技術(next generation sequencing technology)，是針對短到中等長度的DNA序列樣品進行高通量、高精確度(99%精確)和再現性佳的分析技術。
- 其原理不同於傳統Sanger方法，係利用幾種生化反應之組合來測定在DNA合成時，過程中會產生的焦磷酸基團(PPi)之特性，進而將PPi轉換成ATP，ATP再促使螢光酶(luciferase)放出生物冷光(bioluminescence)。放出的冷光強度經冷光儀(luminometer)偵測後，轉讀出DNA序列。
- 該技術特點包括：對DNA的序列分析無須進行電泳、DNA片段無須螢光標記(因此無須螢光分子的激發和檢測裝置)、可在96孔盤上進行反應，因此能同時進行多檢體序列分析。
- 但也因反應特性的限制，精準讀序目前只在20-30個核苷酸序列左右。有的研究者經過改良，可使該技術的讀序長度增加一倍以上。

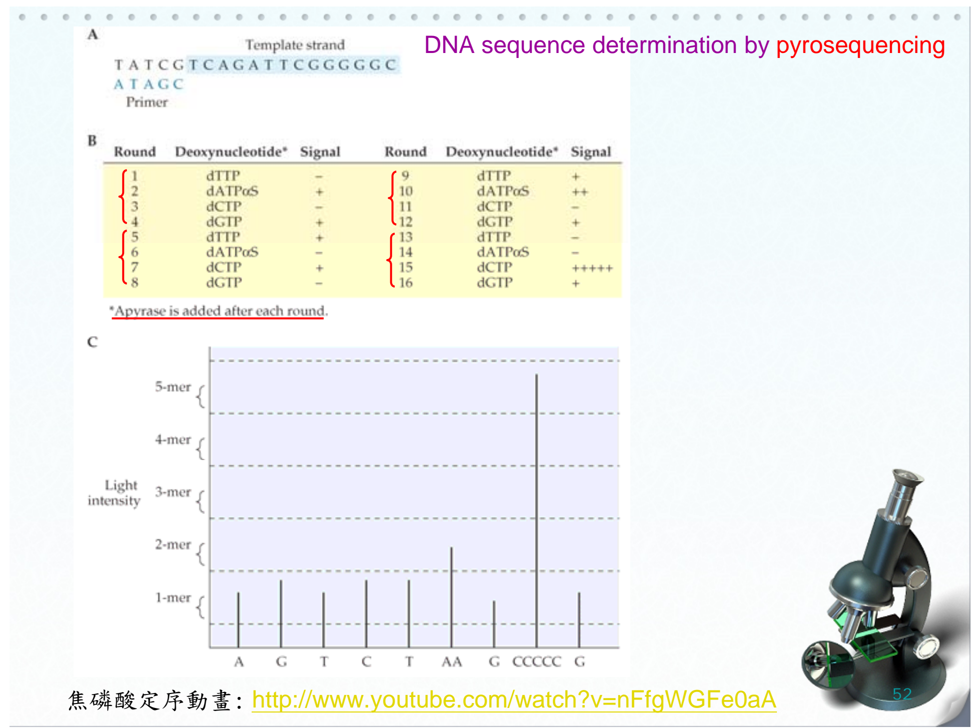
Pyrosequencing技術的基本步驟及原理如下：

1. 一個特異性的定序引子和單鏈DNA模板結合，然後加入酶混合物(包括DNA polymerase、ATP sulfurylase、Luciferase及Apyrase)和受質混合物(包括Adenosine-5'-phosphosulfate (APS) 和Luciferin)。
2. 在反應中加入一種dNTP，若它正好能和DNA模板的下一個鹼基配對，則會在DNA 聚合酶的作用下，添加到引子的3'端，同時釋放出一個分子的焦磷酸(PPi)。
3. 在ATP硫酸化酶的作用下，生成的PPi可以和APS結合形成ATP；在冷光酶的催化下，生成的ATP又可以和冷光素結合形成氧化冷光素，同時產生可見光。通過CCD光學系統即可獲得一個特異的檢測峰，峰值的高低則和相匹配的鹼基數成正比。
4. 反應體系中剩餘的dNTP和殘留的少量ATP在Apyrase的作用下發生降解。
5. 加入另一種dNTP，重覆進行第2~4步驟反應，根據獲得的峰值圖即可讀取準確的DNA序列訊息。



Phosphodiester bond formation and release of pyrophosphate during the incorporation of a nucleotide at the end of a growing DNA strand.

Apyrase is an ATP diphosphohydrolase. It catalyses the removal of the gamma phosphate from ATP and the beta phosphate from ADP. The phosphate from AMP is not removed.

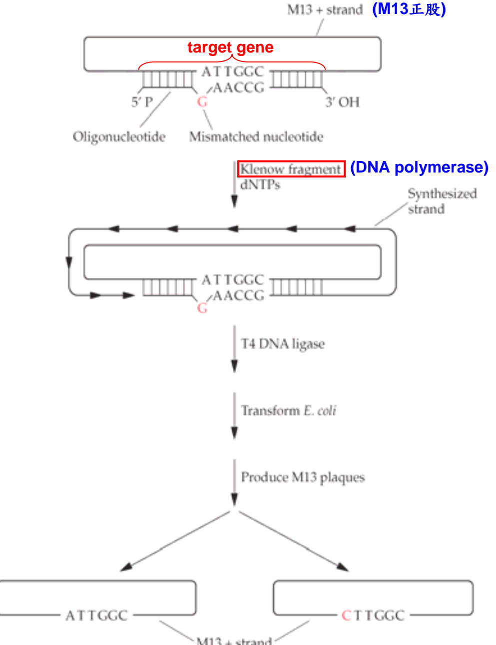


Round	Deoxynucleotide*	Signal	Round	Deoxynucleotide*	Signal
1	dTTP	–	9	dTTP	+
2	dATPαS	+	10	dATPαS	++
3	dCTP	–	11	dCTP	–
4	dGTP	+	12	dGTP	+
5	dTTP	+	13	dTTP	–
6	dATPαS	–	14	dATPαS	–
7	dCTP	+	15	dCTP	+++++
8	dGTP	–	16	dGTP	+

*Apyrase is added after each round.

基因定點突變(site-directed mutagenesis)技術

- 定點突變是加拿大科學家Michael Smith發明，他與PCR發明者Kary Mullis 在1993年共同獲得諾貝爾化學獎。
- 基因突變可經由自發性或經誘導產生。在定點突變法之前，突變株的產生必須經由自然界或用化學等方法讓基因突變，這屬於隨機突變。而突變株必須在生物體上產生改變，才能確定有突變發生。突變位置必須利用分生或化學方法來確定。
- 定點突變是經由設計好的寡核苷酸，在任何一個基因片段上進行隨意或設計好的突變，意即此突變是預先設定好的，所以也有人將它稱為“反向遺傳法”。



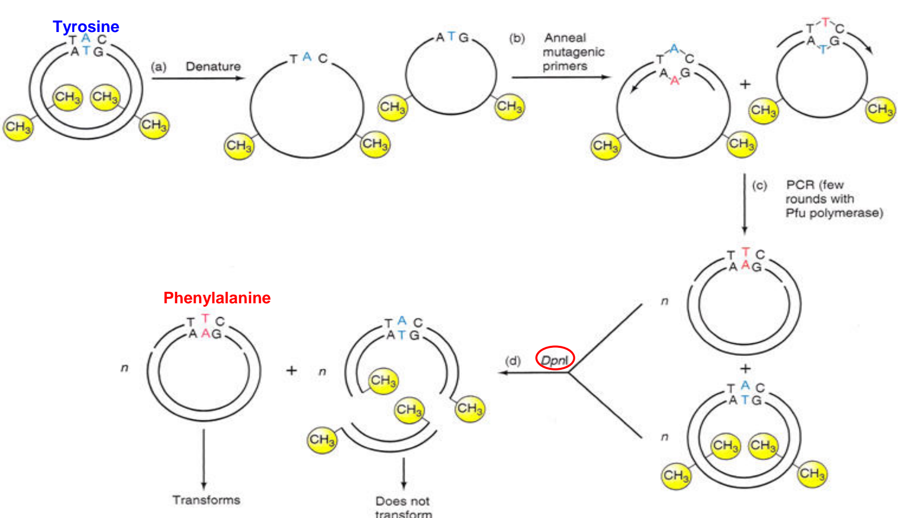
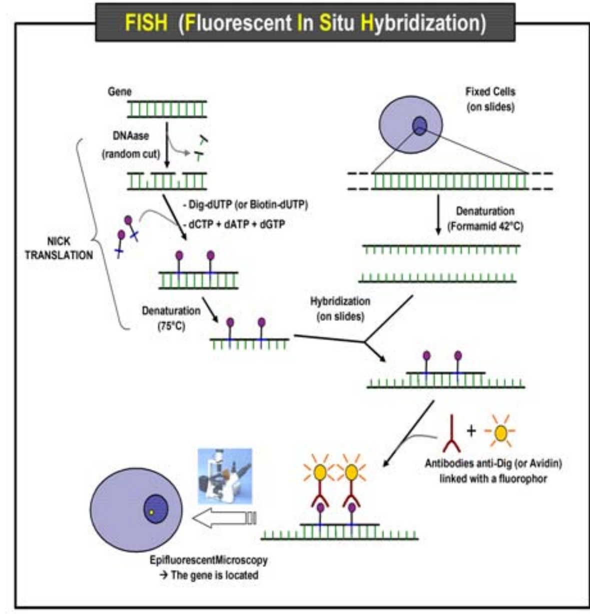
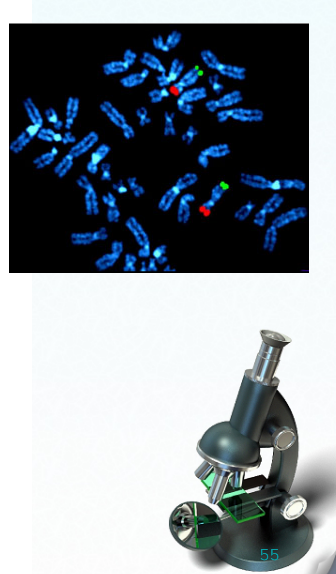


Figure 5.25 PCR-based site-directed mutagenesis. Begin with a plasmid containing a gene with a TAC tyrosine codon that is to be altered to a TTC phenylalanine codon. Thus, the A–T pair (blue) in the original must be changed to a T–A pair. This plasmid was isolated from a normal strain of *E. coli* that methylates the A's of GATC sequences. The methyl groups are indicated in yellow. **(a)** Heat the plasmid to separate its strands. The strands of the original plasmid are intertwined, so they don't completely separate. They are shown here separating completely for simplicity's sake. **(b)** Anneal mutagenic primers that contain the TTC codon, or its reverse complement, GAA. The altered base in each primer is indicated in red. **(c)** Perform a few rounds of PCR (about eight) with the mutagenic primers to amplify the plasmid with its altered codon. Use a faithful, heat-stable DNA polymerase, such as Pfu polymerase, to minimize mistakes in copying the plasmid. **(d)** Treat the DNA in the PCR reaction with *DpnI* to digest the methylated wild-type DNA. Because the PCR product was made in vitro, it is not methylated and is not cut. Finally, transform *E. coli* cells with the treated DNA. In principle, only the mutated DNA survives to transform. Check this by sequencing the plasmid DNA from several clones.

DNA交互配對(雜交)反應(DNA hybridization)





南方墨點法(Southern blot)

- 由英國科學家Edwin Southern發明，因此命名為Southern blot，用以偵測經由膠體電泳分離的樣品中，含有特定序列的DNA片段。
- 類似的技術也被用來偵測經由膠體電泳分離的樣品中，含有特定序列的RNA片段，稱為北方墨點法(Northern blot)。用以偵測蛋白質的方法稱為西方墨點法(Western blot) 。

Southern blot 動畫:
http://www.youtube.com/watch?v=6FjjjATsr50

Northern blot 動畫:
http://www.youtube.com/watch?v=KfHZFyADnNg&feature=related

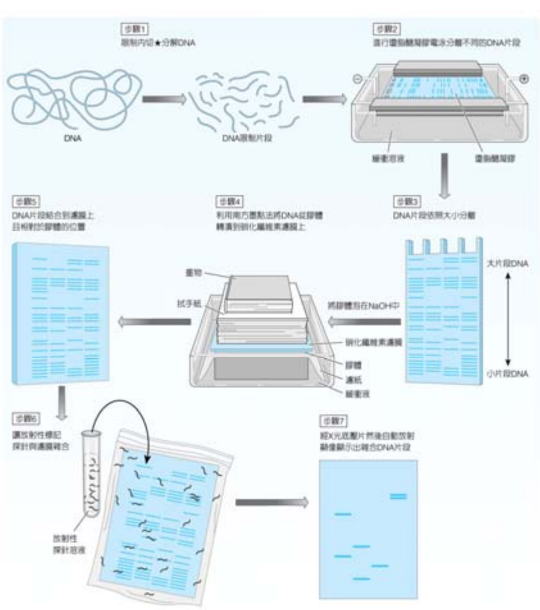
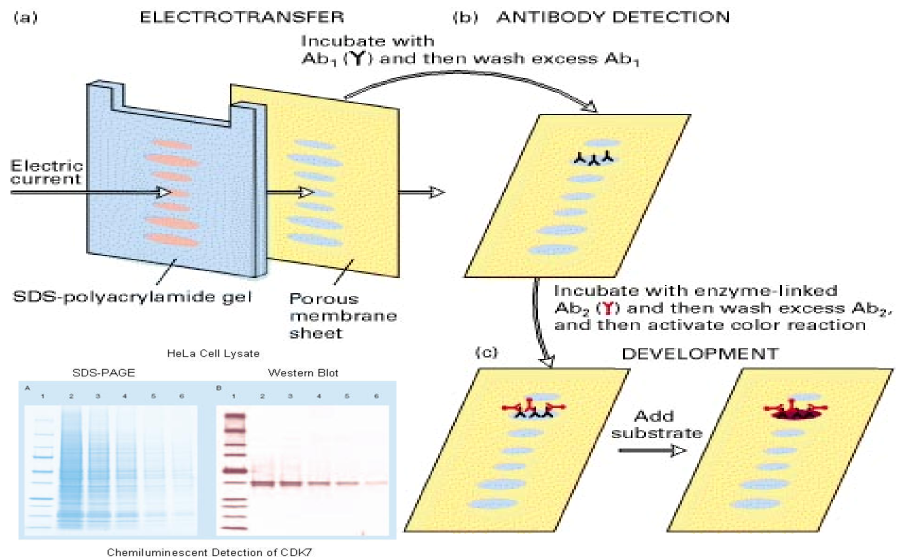


圖3-20
操作南方墨點雜合法的步驟。在南方墨點法中，DNA藉由膠體、濾膜、濾紙與拭手紙疊成「三明治」的方式將位於膠體上的DNA轉移至濾膜上。下方鹽類溶液藉著毛細管作用而向上移動，這也促使DNA由膠體轉移至濾膜上。

西方墨點法(Western blot)



Western blot 動畫:
http://www.youtube.com/watch?v=aGu-NKvKIoA&feature=related