

# 分子生物學應用

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# 主題介紹

I. 即時定量聚合酶連鎖反應 ( Real-time quantitative PCR, RT-qPCR/Q-PCR)

II. 報告基因分析 (Reporter gene assay)

III. 核糖核酸干擾 (RNA interference, RNAi)

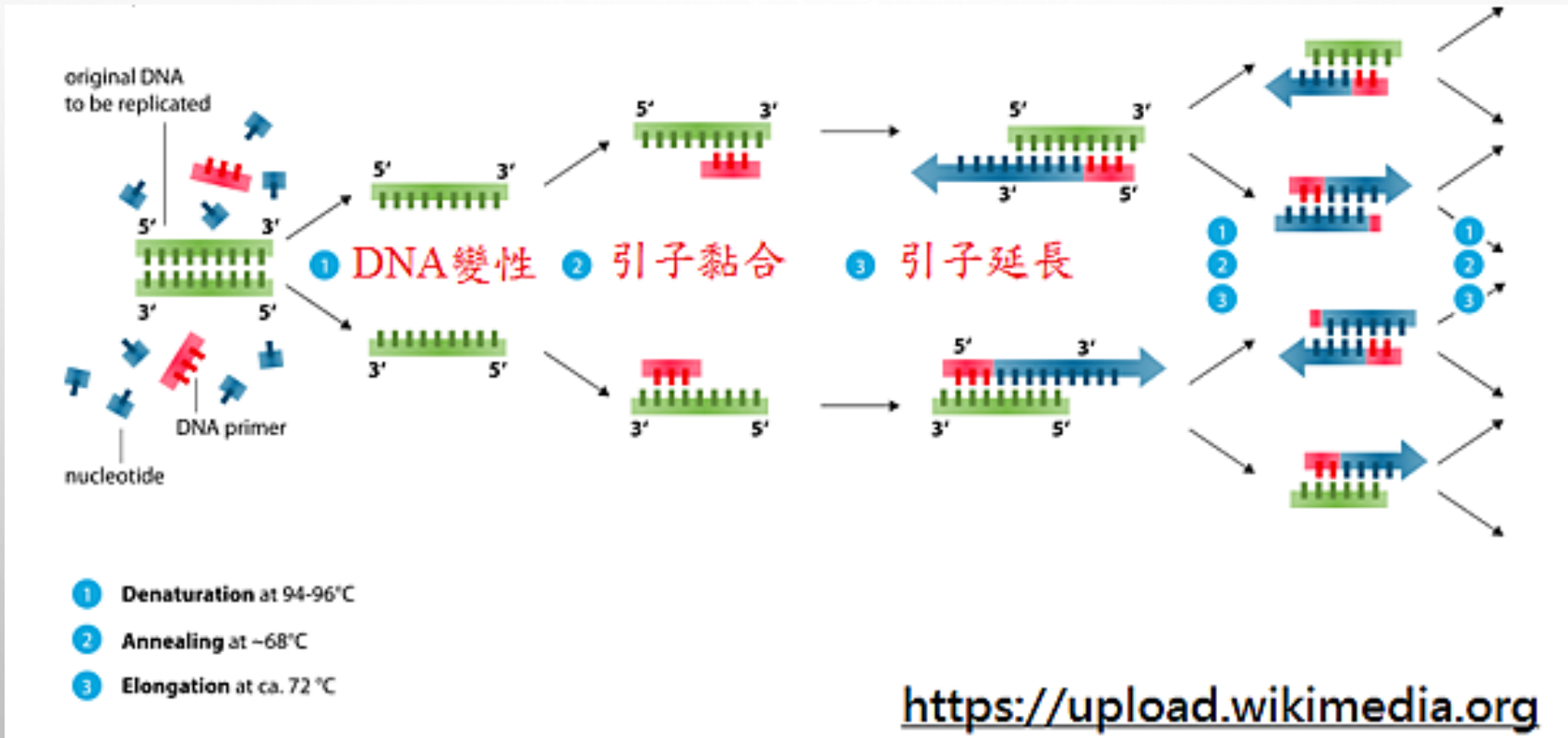
# I. 即時定量聚合酶連鎖反應 (Q-PCR)

# 上課內容

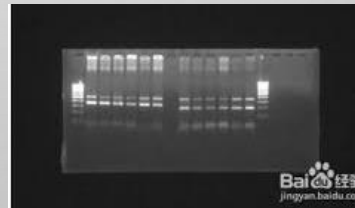
- 聚合酶連鎖反應(PCR)
- Q-PCR原理
- Q-PCR步驟
- Q-PCR反應混合物
- TaqMan Probe(探針)& SYBR Green(染料)
- 使用的儀器/系統
- 如何避免Q-PCR錯誤
- Q-PCR應用



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



- 以電泳確認PCR產物
- 以 $2^n$  (n表示循環次數)的速度在複製DNA



# The Nobel Prize in Chemistry 1993

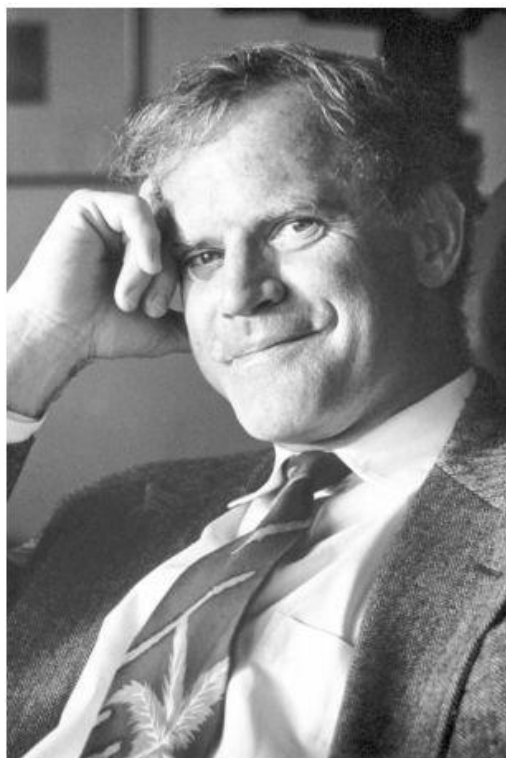


Photo from the Nobel Foundation archive.

**Kary B. Mullis**

Prize share: 1/2



Photo from the Nobel Foundation archive.

**Michael Smith**

Prize share: 1/2

Kary B. Mullis: for his invention of the **polymerase chain reaction (PCR) method.**

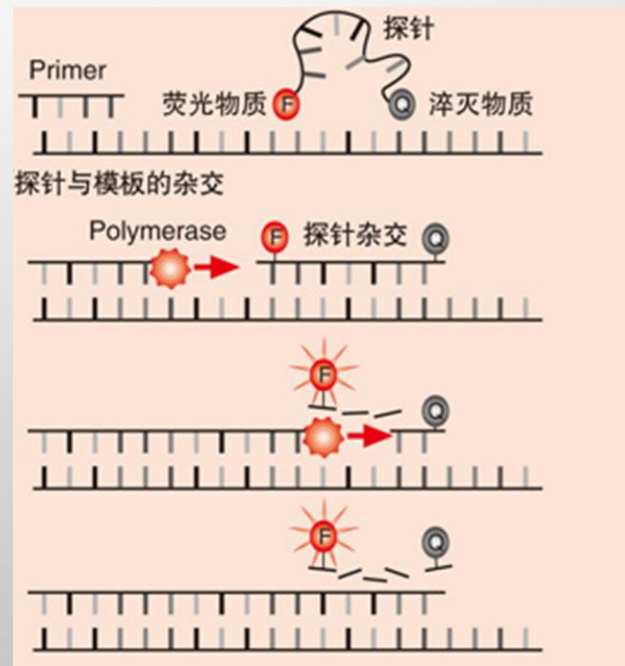
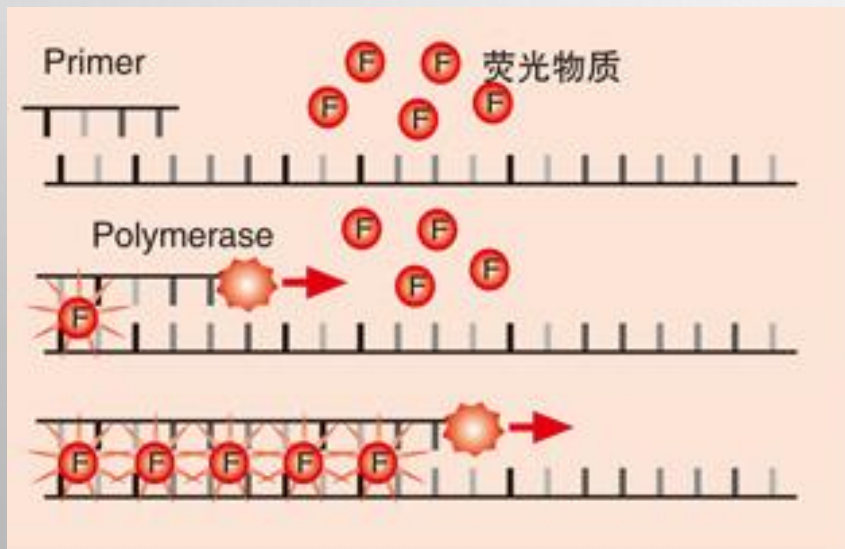
Michael Smith: for his fundamental contributions to the **establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies.**

# Q-PCR 原理

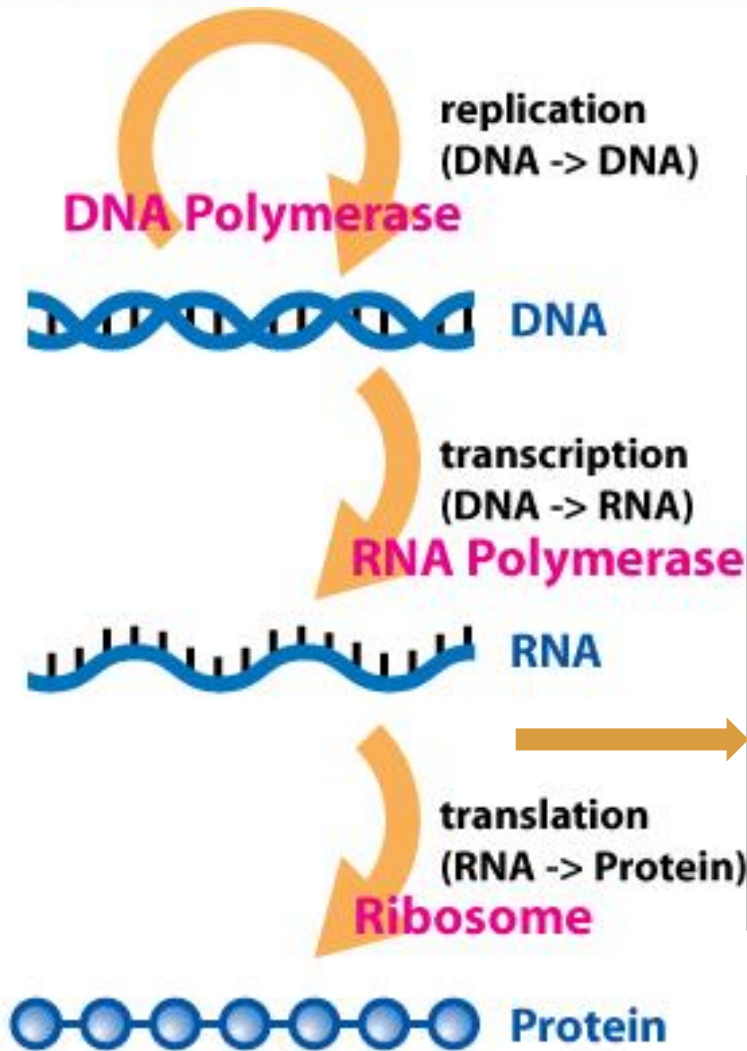
uses **reverse transcription (反轉錄)** to produce a DNA template from an RNA source that can then be amplified.



- 這是一種基於聚合酶鏈反應(PCR) 的技術。
- 將反應放入機器中，該機器使用相機或檢測器**實時監測**觀察並螢光反應檢。
- 檢測分子產生的螢光，隨著反應的進行，該分子增加。
- 可以定量相對少量的PCR產物。

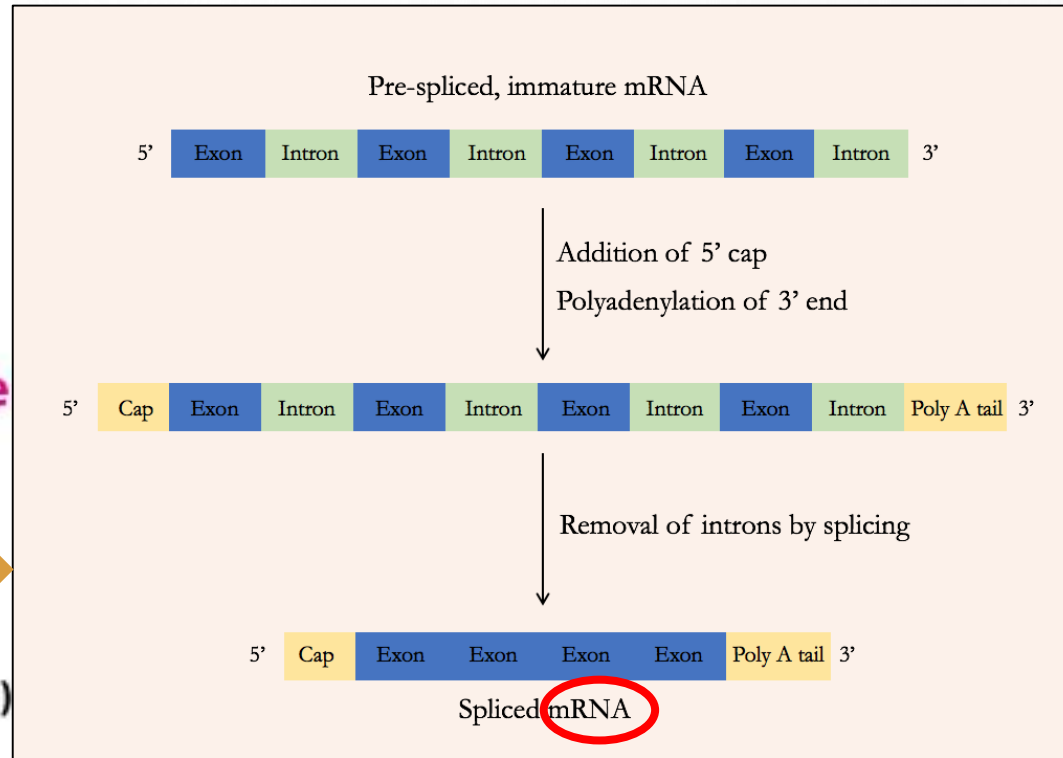


# 中心法則



<https://zh.wikipedia.org>

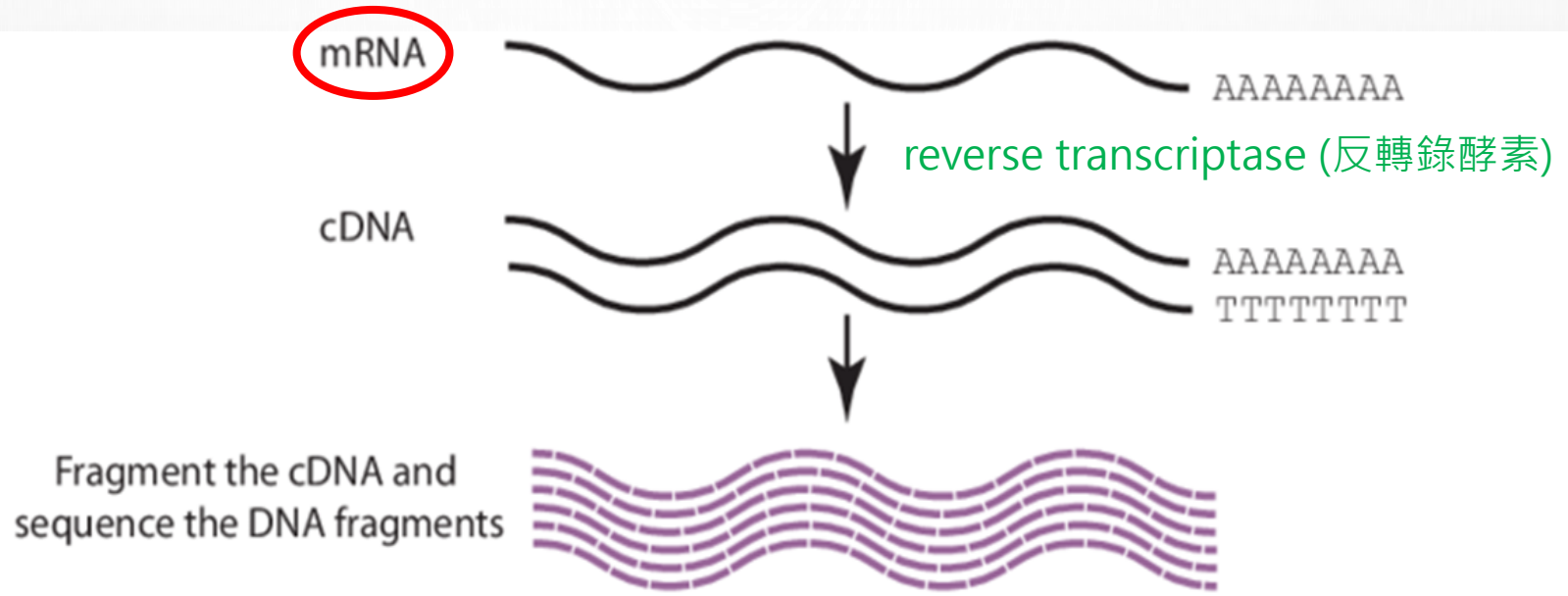
## 成熟RNA修飾



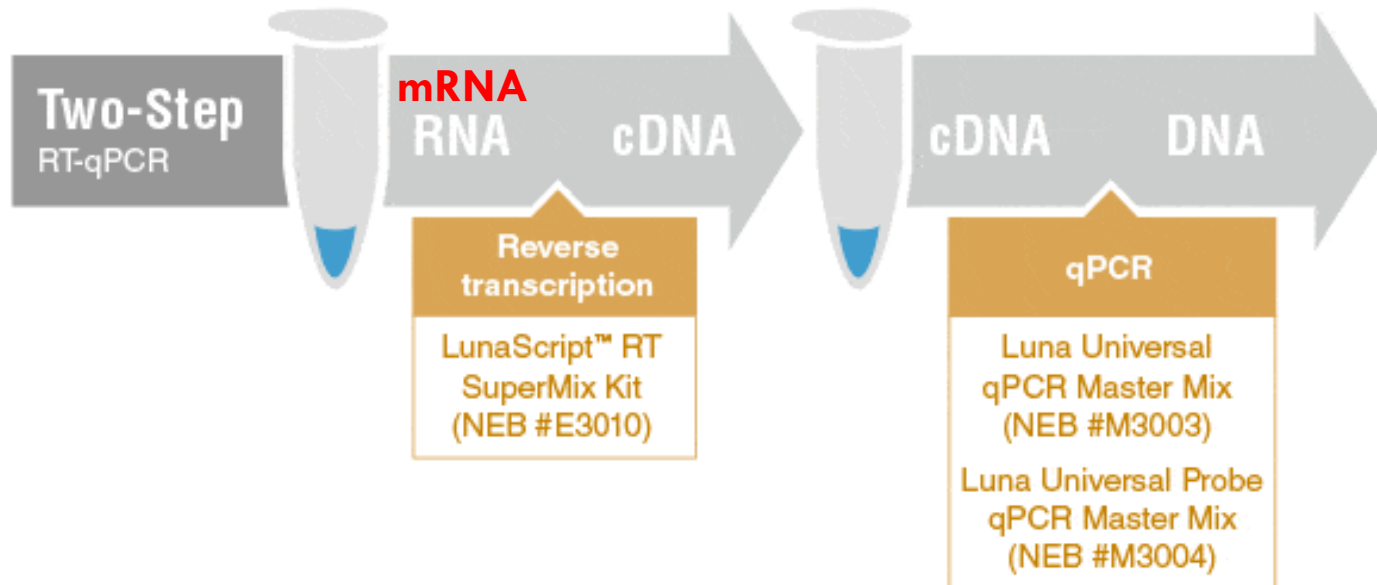
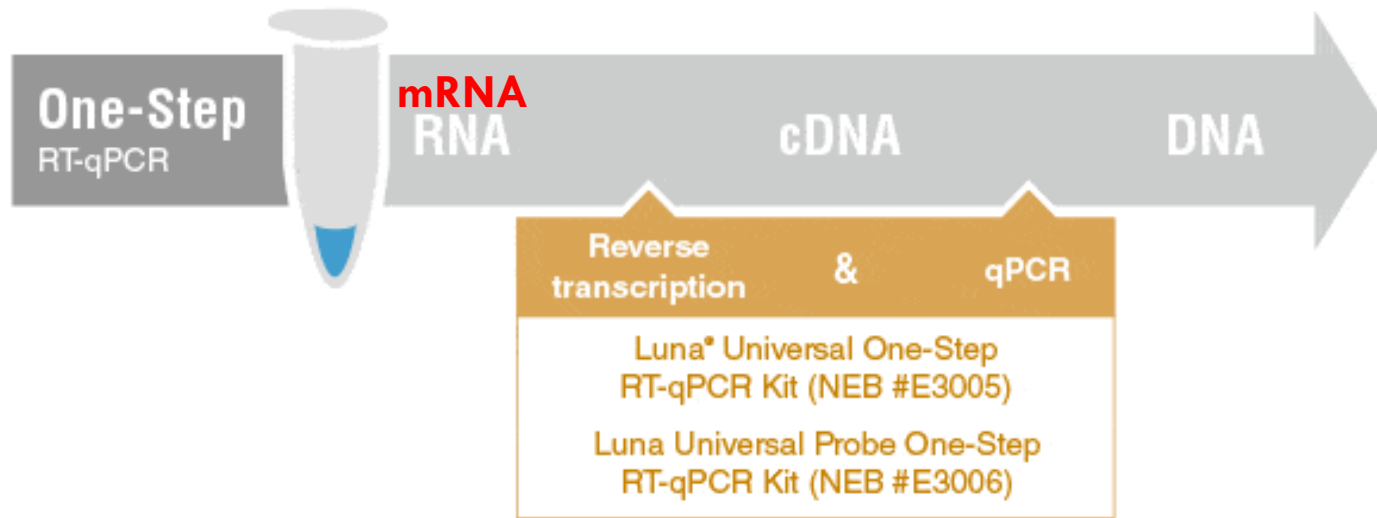
<https://commons.wikimedia.org/>



# 信使RNA (mRNA) 反轉錄成為互補 DNA (cDNA) 的流程



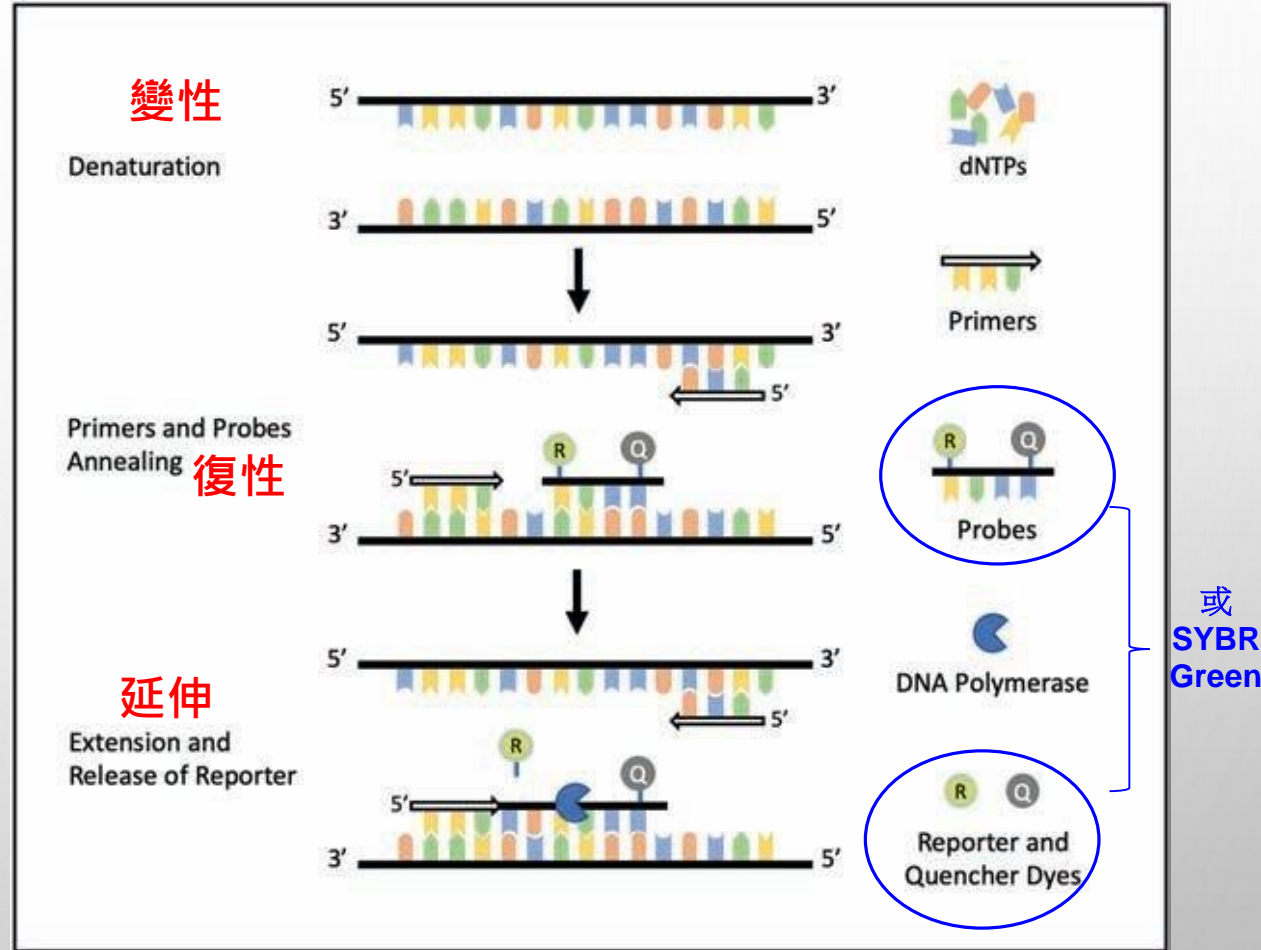
# Q-PCR步驟



**a. 變性**-高溫用於將雙鏈DNA「熔化」成單鏈，並鬆動單鏈DNA中的二級結構。通常使用DNA聚合酶可以承受的最高溫度（通常為95°C）。如果範本GC含量高，可以增加變性時間。

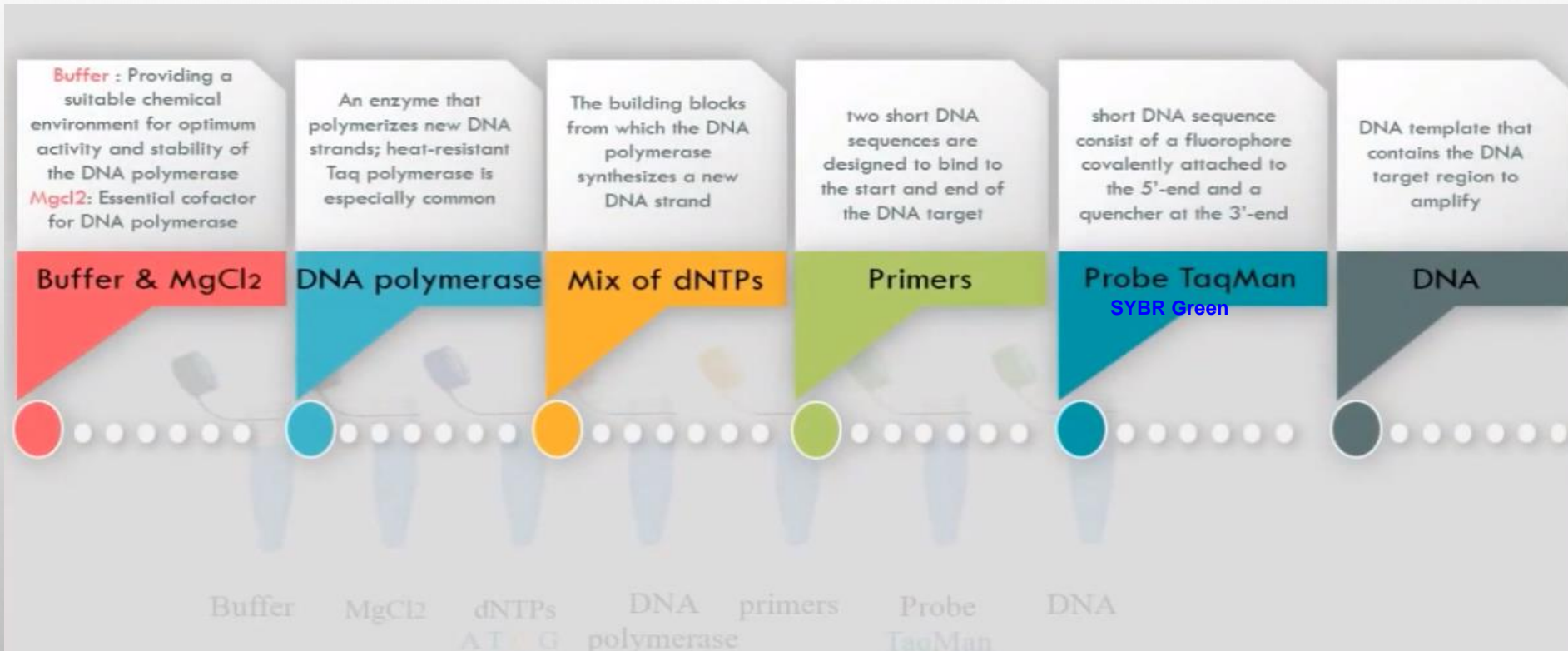
**b. 復性**-在復性過程中，互補序列有機會雜交，因此使用基於引物的計算熔融溫度（ $T_m$ ）的適當溫度（低於引物 $T_m$ 的5°C）。

**c. 延伸**-在70-72°C時，DNA聚合酶的活性是最佳的，引物延伸以高達每秒100個鹼基的速率發生。當即時螢光定量PCR中的擴增子很小時，該步驟通常與使用60°C作為溫度的復性步驟相結合。





# Q-PCR反應混合物

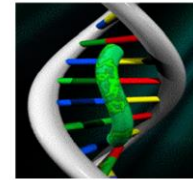


## TaqMan Probe(探針)& SYBR Green(染料)

- 每個Q-PCR 都包含一個螢光分子（例如 TAQMAN® 探針或 SYBR® GREEN染料），用於監測 PCR 產物的積累。
- 隨著標靶基因擴增數量的增加，螢光團發出的螢光量也隨之增加。
- 為使用Q-PCR 進行基因表達研究而開發的兩種類型的化學物質是：

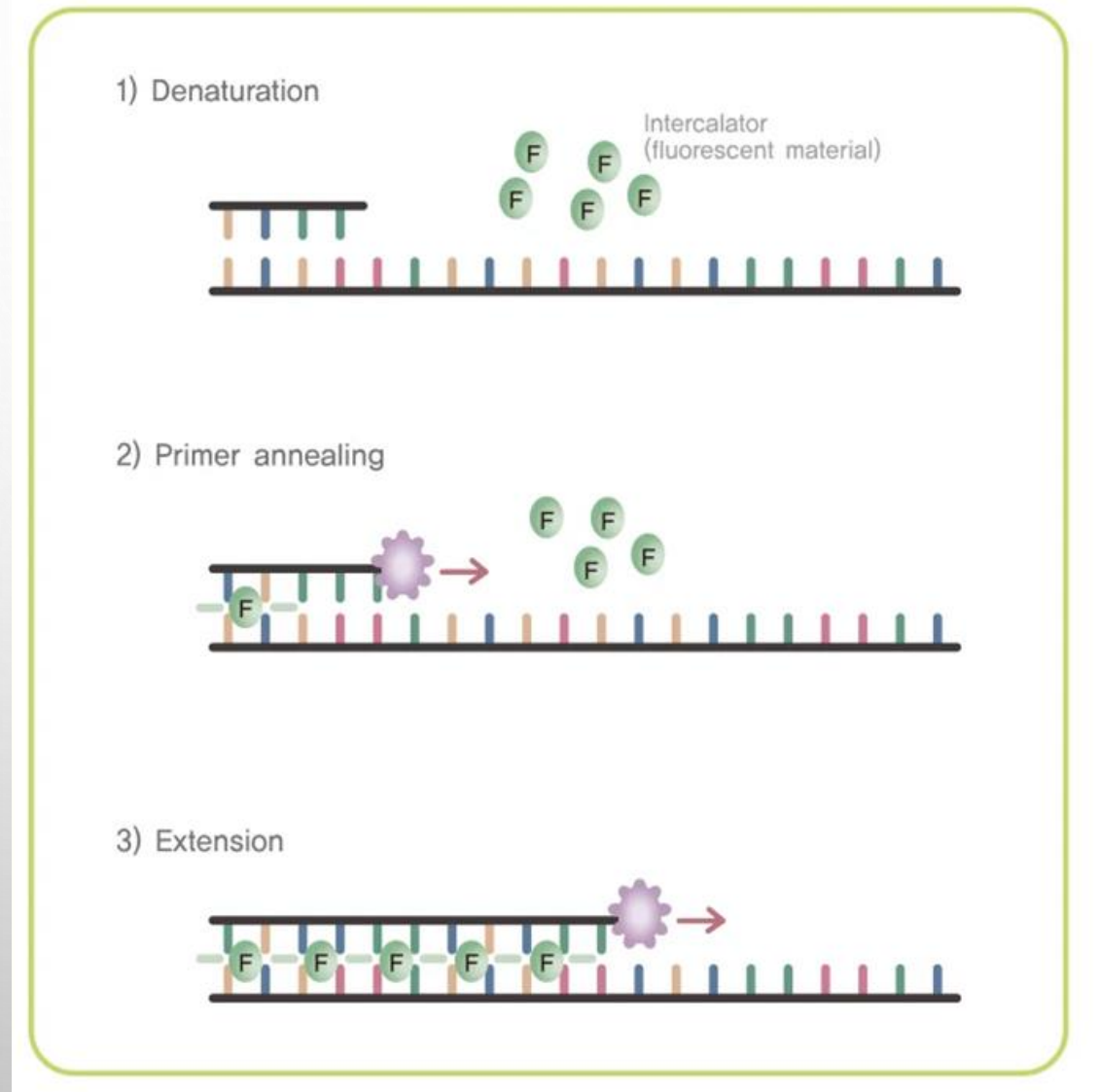
(1) SYBR Green 染料

(2) TaqMan Probe 探針

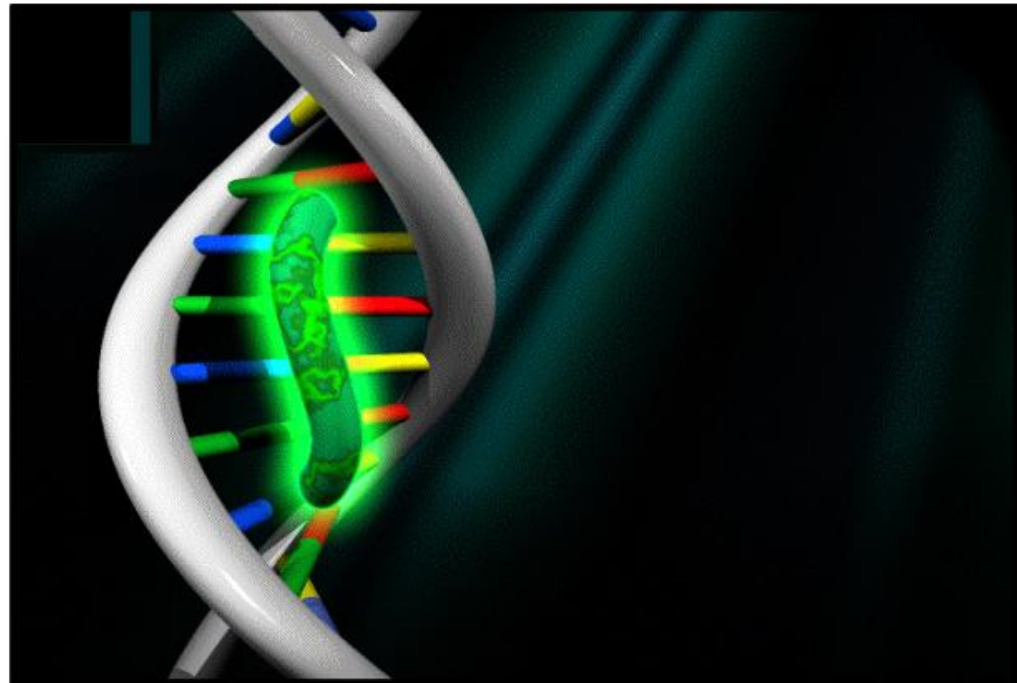
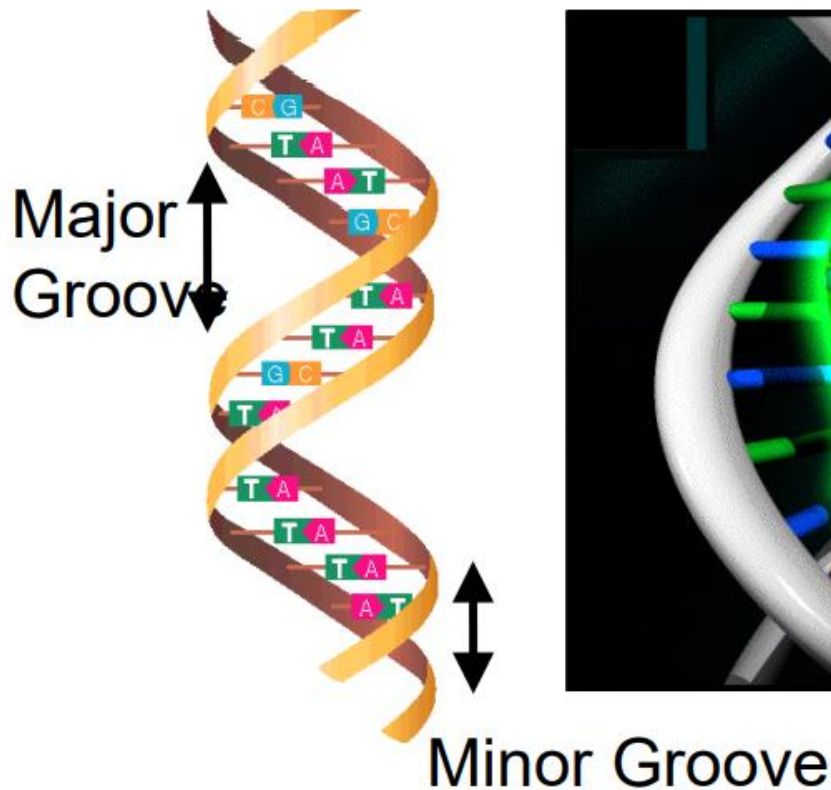


# (1) SYBR GREEN 染料

- 這是一種染料，當它在DNA的微小凹槽處**非特異性結合**時，會發出突出的螢光信號。
- 也可以使用其他螢光染料，如**溴化乙錠(EtBr)**或**吡啶橙(Acridine Orange)**，但**SYBR Green**更適合用於其**更高**的信號強度。
- SYBR Green比TaqMan Probe更受歡迎，因為它可以提供有關每個擴增周期的資訊以及有關TaqMan Probe無法獲得的熔化溫度的資訊。
- 然而，與TaqMan Probe相比，其缺點是**缺乏特異性**。



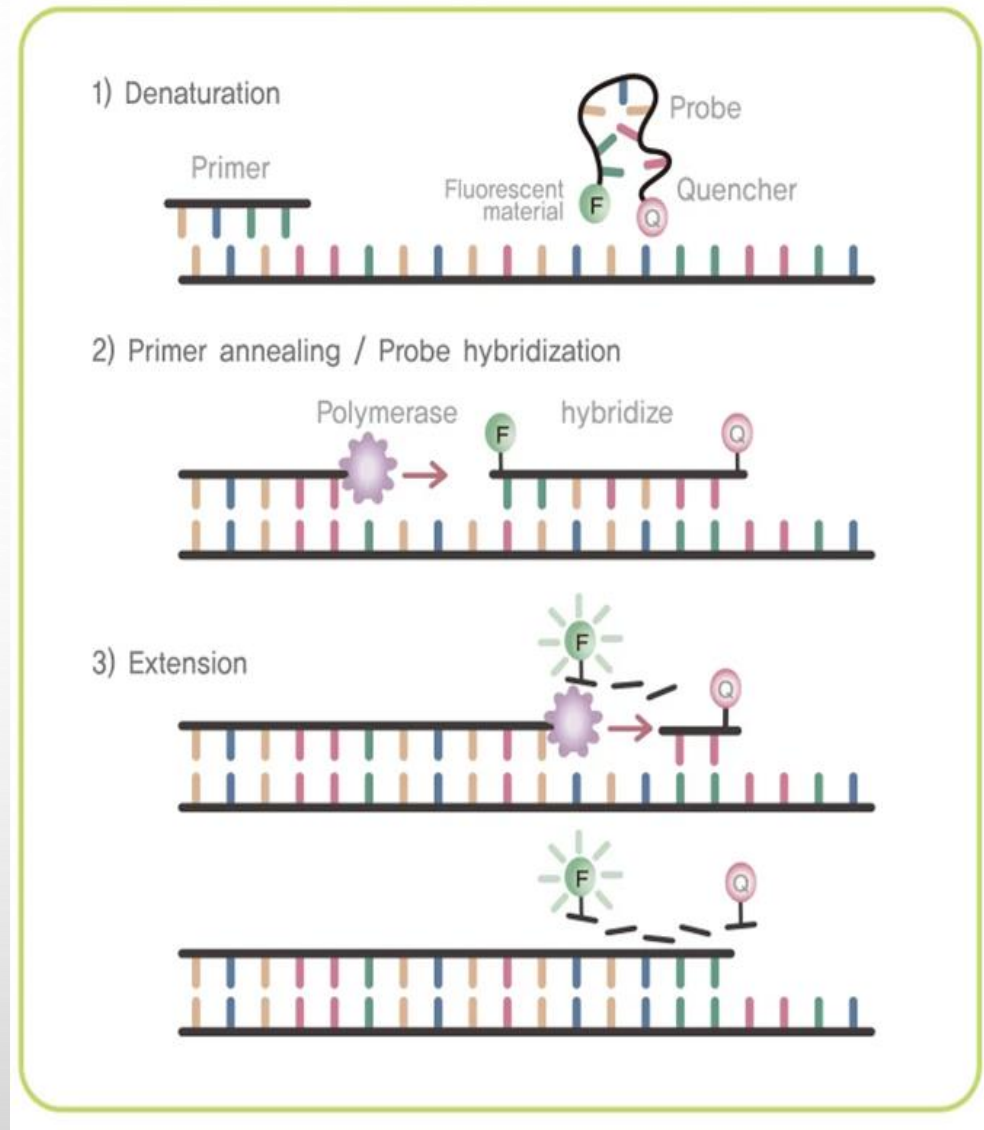
- A 'minor groove'-binding molecule specific to the minor groove of double-stranded DNA
- Fluoresces at an increased intensity when bound





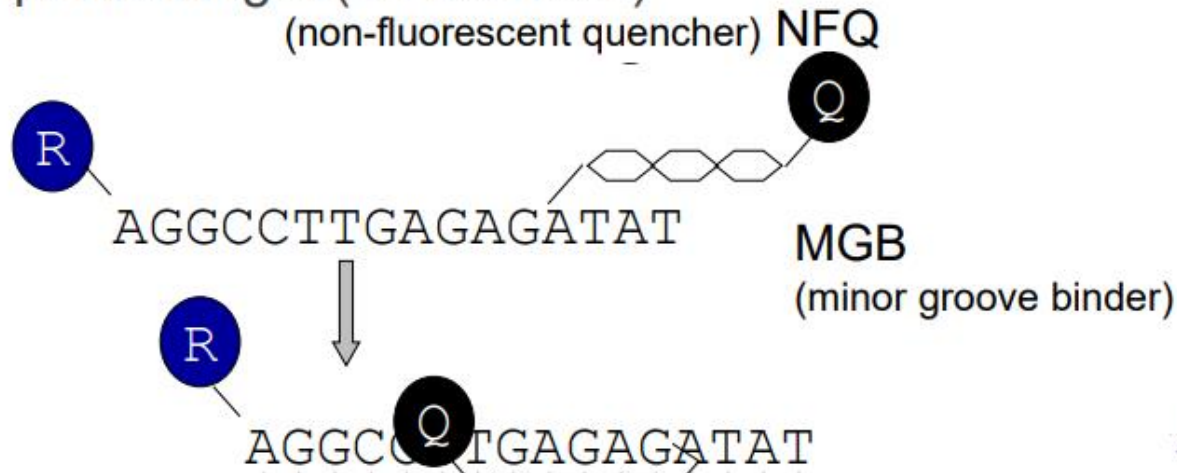
## (2) TaqMan Probe 探針

- 它是一種水解探針，帶有染料，通常在其5端有螢光素 ( FAM )，並且在寡核苷酸的3端附著一個淬滅劑 ( non-fluorescent quencher )。
- 在正常情況下，探針保持盤繞在自身上，使螢光染料靠近淬滅劑，從而抑制或淬滅染料的螢光信號。
- 當聚合酶在延伸階段開始合成新的DNA鏈時，它通過5'末端核酸酶活性導致探針降解，螢光素與淬滅劑分離，從而產生螢光信號。
- 隨著該過程的繼續，在每個迴圈中，信號分子的數量增加，導致螢光的增加，這與靶標的擴增呈正相關。



# TaqMan<sup>®</sup> Probe: TaqMan<sup>®</sup> MGB/NFQ Probes

- Minor Groove Binder (MGB)
  - Small molecule that fits snugly into minor groove of duplex DNA
  - Stabilizes probe annealing
- Non-fluorescent Quencher (NFQ)
  - “Dark” quencher acts as energy transfer acceptor that doesn’t emit a detectable fluorescent signal
  - MGB probe design uses a special algorithm in Primer Express<sup>®</sup> Software
- Shorter probe length (13-25-mers)



life  
technology

ThermoFisher  
SCIENTIFIC

GCTACACAGTCCGGA ACTCTCTATAGCATCACAC

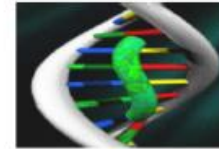


<https://www.youtube.com/watch?v=ob3teCrpgxY>

## TaqMan<sup>®</sup> Probe



## SYBR<sup>®</sup> Green 1 Dye



### Specificity

- Highly specific
- Probe Hybridization

- Less specific

### Sensitivity

- Very High

- Very High

### Flexibility

- Multiplex PCR
- SNP detection
- +/- application

- No Probe is required
- Screening tool

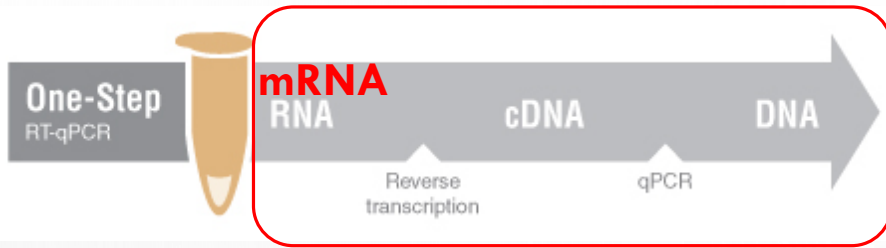
### Optimization

- Ready to use 20x primer/probe mix - no need to optimize
- Gold standard for MAQC
- PCR efficiency 100% ±10%

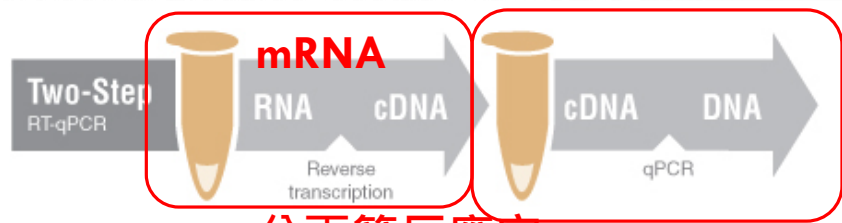
- Need to optimize PCR program
- Need to check primer-dimer info
- Need to check PCR efficiency



一管全部反應完



Reaction mixture	
Master mix	Volume per one reaction
2X SYBR Green RT-PCR reaction mix	12.5 $\mu$ L
Forward primer (10 $\mu$ M)	1 $\mu$ L
Reverse primer (10 $\mu$ M)	1 $\mu$ L
Nuclease-free H <sub>2</sub> O	9 $\mu$ L
RNA template (1 pg to 100 ng total RNA)	1 $\mu$ L
iScript reverse transcriptase for one-step RT-PCR	0.5 $\mu$ L
Final reactions volume	25 $\mu$ L



分兩管反應完

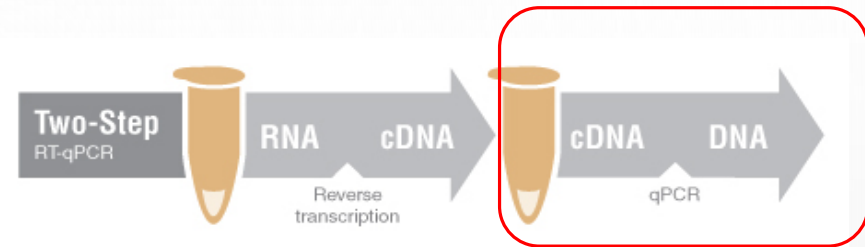
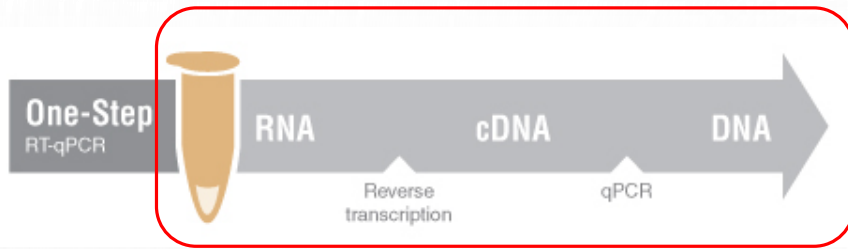


1<sup>st</sup>

Reagents for reverse transcription	Volume ( $\mu$ L)
Random primers	3
dNTP mix 10 mM	3
ddH <sub>2</sub> O	36
5X First strand buffer	12
DTT 0.1 M	6
RNase OUT 40 U/ $\mu$ L	3
RT enzyme	1
Total volume	64

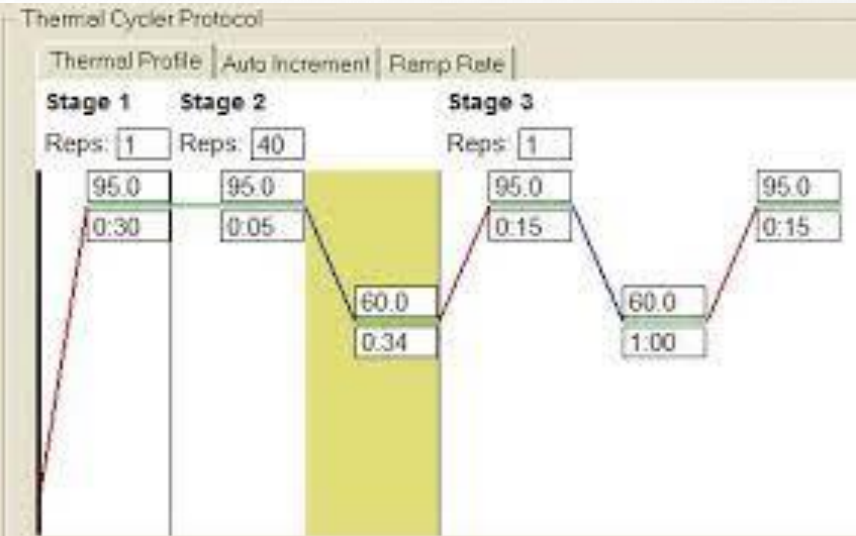
2<sup>nd</sup>

Reagents for PCR	Volume ( $\mu$ L)
10X PCR buffer with MgCl <sub>2</sub>	2.5
dNTP mix 10 mM	0.5
Fwd primer 10 $\mu$ M	2
Rev primer 10 $\mu$ M	2
Taq DNA polimerase (5U/ $\mu$ L)	0.5
Biotinylated dUTP	0.6
Autoclaved ddH <sub>2</sub> O	16.9
Total volume	25



### Protocol

Process	Duration/temperature
cDNA synthesis	20 min at 50°C
iScript reverse transcriptase inactivation	4 min at 95°C
PCR cycling and detection (standard PCR cycle)	
Denaturation	10 sec at 95°C
Annealing/extension	30 sec at 61°C (data collection step)
	Repeated for (30 to 45 cycles)
	1 min at 95°C
Melt curve analysis (optional)	1 min at 55°C
	10 sec at 55°C (80 cycles, increasing each by 0.5°C each cycle)



# 常用哪種儀器/系統

## (1) ABI系統

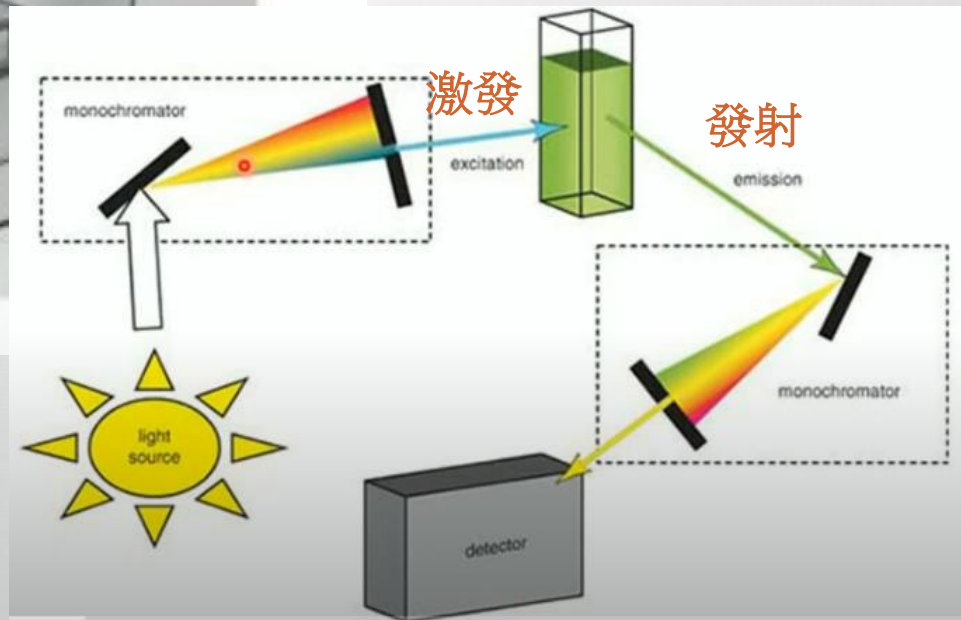
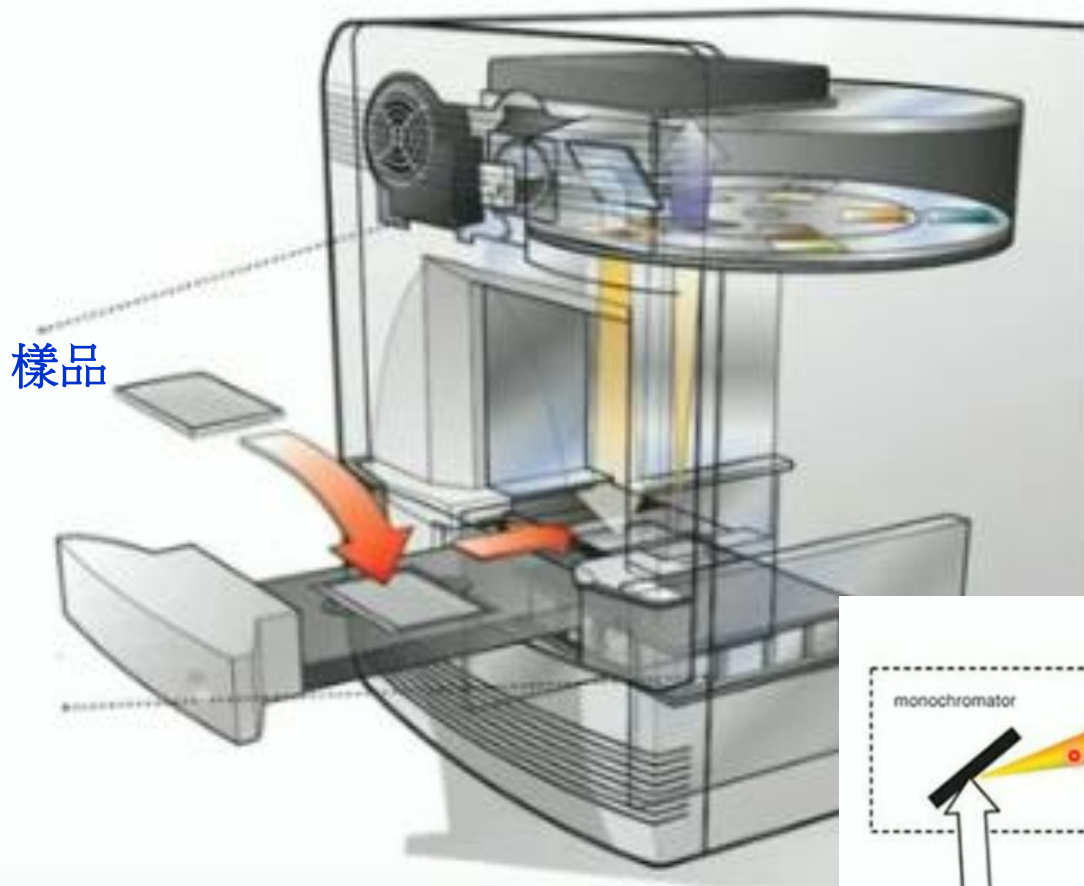
Quantstudio systems  
1,3,5,6,7



<https://www.thermofisher.com/tw/zt/home/life-science/pcr/real-time-pcr/real-time-pcr-instruments/quantstudio-systems/models/quantstudio-3-5.html>

<https://www.thermofisher.com/tw/zt/home/life-science/pcr/real-time-pcr/real-time-pcr-instruments/quantstudio-systems/accessories.html>

# 分光螢光計





# 實驗材料

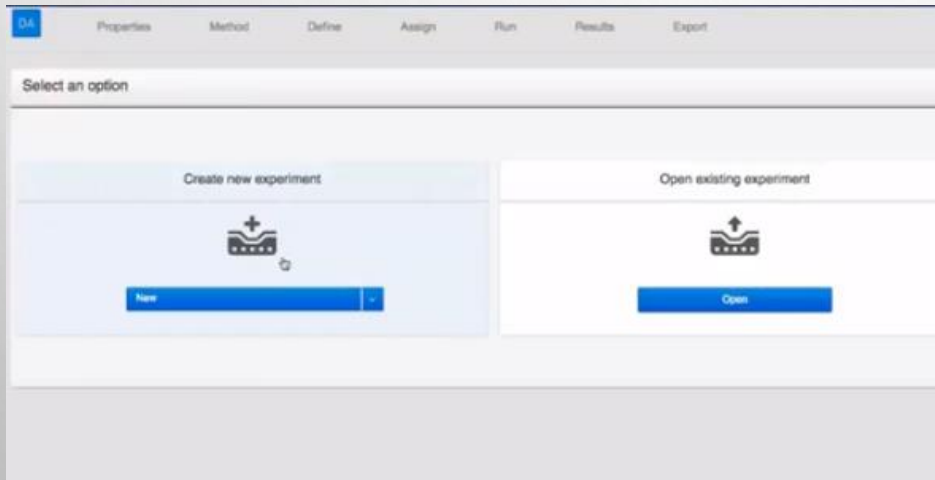


<https://www.youtube.com/watch?v=1uh2ZxZJtgs>



<https://youtu.be/K5flNp46wxo>

# 程式設置



Reagents: SYBR® Green Reagents Analyze Analysis Settings ?

View Plate Layout View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Wells View Legend

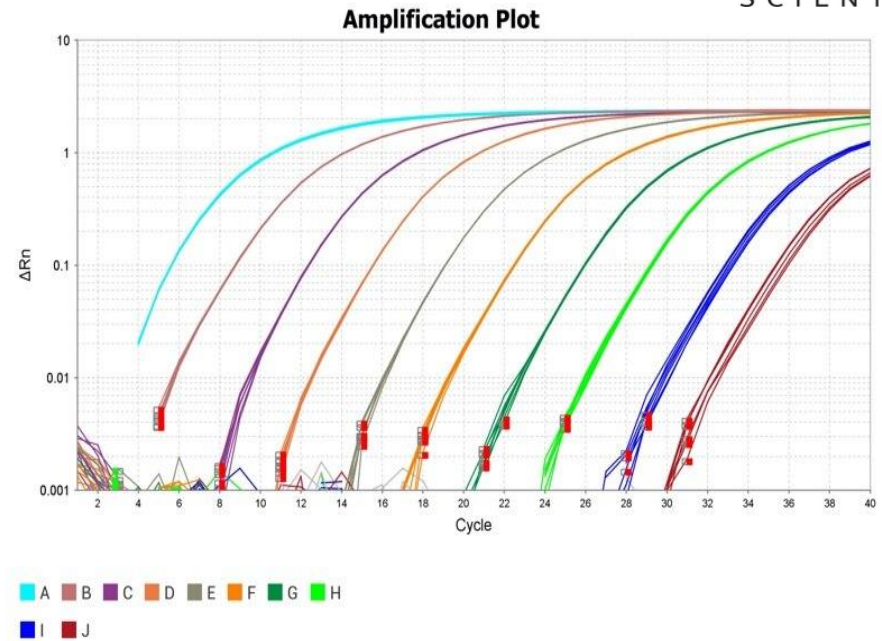
	1	2	3	4	5	6	7	8	9	10	11	12	
A	ARTIFICIA S CHIKV 7.51E8 Ct: 6.97	ARTIFICIA S CHIKV 7.51E7 Ct: 10.93	ARTIFICIA S CHIKV 7.51E6 Ct: 14.6	ARTIFICIA S CHIKV 7.51E5 Ct: 18.16	ARTIFICIA S CHIKV 7.51E4 Ct: 25.03	ARTIFICIA S CHIKV 7.51E3 Ct: 25.03	ARTIFICIA S CHIKV 7.51 Ct: 26.72	ARTIFICIA S CHIKV 7.51 Ct: 26.38					
B	ARTIFICIA S CHIKV 7.51E8 Ct: 10.97	ARTIFICIA S CHIKV 7.51E7 Ct: 14.69	ARTIFICIA S CHIKV 7.51E6 Ct: 17.91	ARTIFICIA S CHIKV 7.51E5 Ct: 21.82	ARTIFICIA S CHIKV 7.51E4 Ct: 24.99	ARTIFICIA S CHIKV 7.51E3 Ct: 26.6	ARTIFICIA S CHIKV 7.51 Ct: 26.55						
C	ARTIFICIA S CHIKV 7.51E9 Ct: 6.51	ARTIFICIA S CHIKV 7.51E7 Ct: 10.79	ARTIFICIA S CHIKV 7.51E6 Ct: 15.56	ARTIFICIA S CHIKV 7.51E5 Ct: 19.05	ARTIFICIA S CHIKV 7.51E4 Ct: 23.66	ARTIFICIA S CHIKV 7.51E3 Ct: 27.28	ARTIFICIA S CHIKV 7.51 Ct: 31.35	ARTIFICIA S CHIKV 7.51 Ct: 32.63					
D	ARTIFICIA S ZIKA 7.51E8 Ct: 8.31	ARTIFICIA S ZIKA 7.51E7 Ct: 11.83	ARTIFICIA S ZIKA 7.51E6 Ct: 15.56	ARTIFICIA S ZIKA 7.51E5 Ct: 19.05	ARTIFICIA S ZIKA 7.51E4 Ct: 23.66	ARTIFICIA S ZIKA 7.51E3 Ct: 27.28	ARTIFICIA S ZIKA 7.51 Ct: 31.35	ARTIFICIA S ZIKA 7.51 Ct: 32.63					
E	ARTIFICIA S ZIKA 7.51E9 Ct: 8.33	ARTIFICIA S ZIKA 7.51E7 Ct: 11.8	ARTIFICIA S ZIKA 7.51E6 Ct: 15.56	ARTIFICIA S ZIKA 7.51E5 Ct: 19.08	ARTIFICIA S ZIKA 7.51E4 Ct: 23.66	ARTIFICIA S ZIKA 7.51E3 Ct: 27.28	ARTIFICIA S ZIKA 7.51 Ct: 31.82	ARTIFICIA S ZIKA 7.51 Ct: 32.71					
F	ARTIFICIA S ZIKA 7.51E8 Ct: 8.37	ARTIFICIA S ZIKA 7.51E7 Ct: 11.98	ARTIFICIA S ZIKA 7.51E6 Ct: 15.91	ARTIFICIA S ZIKA 7.51E5 Ct: 19.05	ARTIFICIA S ZIKA 7.51E4 Ct: 23.66	ARTIFICIA S ZIKA 7.51E3 Ct: 27.95	ARTIFICIA S ZIKA 7.51 Ct: 31.82	ARTIFICIA S ZIKA 7.51 Ct: 32.71					
G													
H													

Wells: 0 Unknown 48 Standard 0 Negative Control 48 Empty



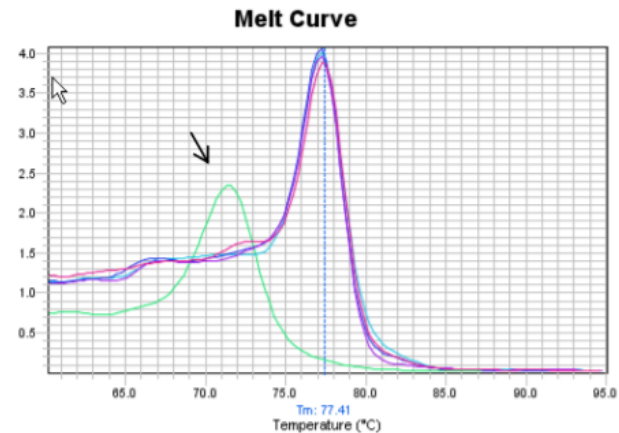
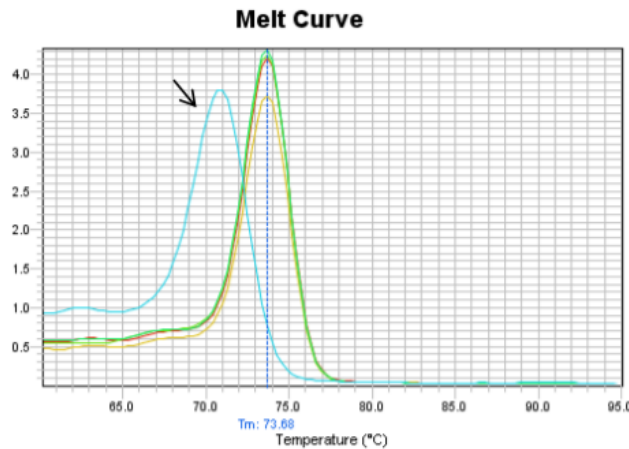


Amplification plots are displayed with the ability to drill down to a subset of sample wells.

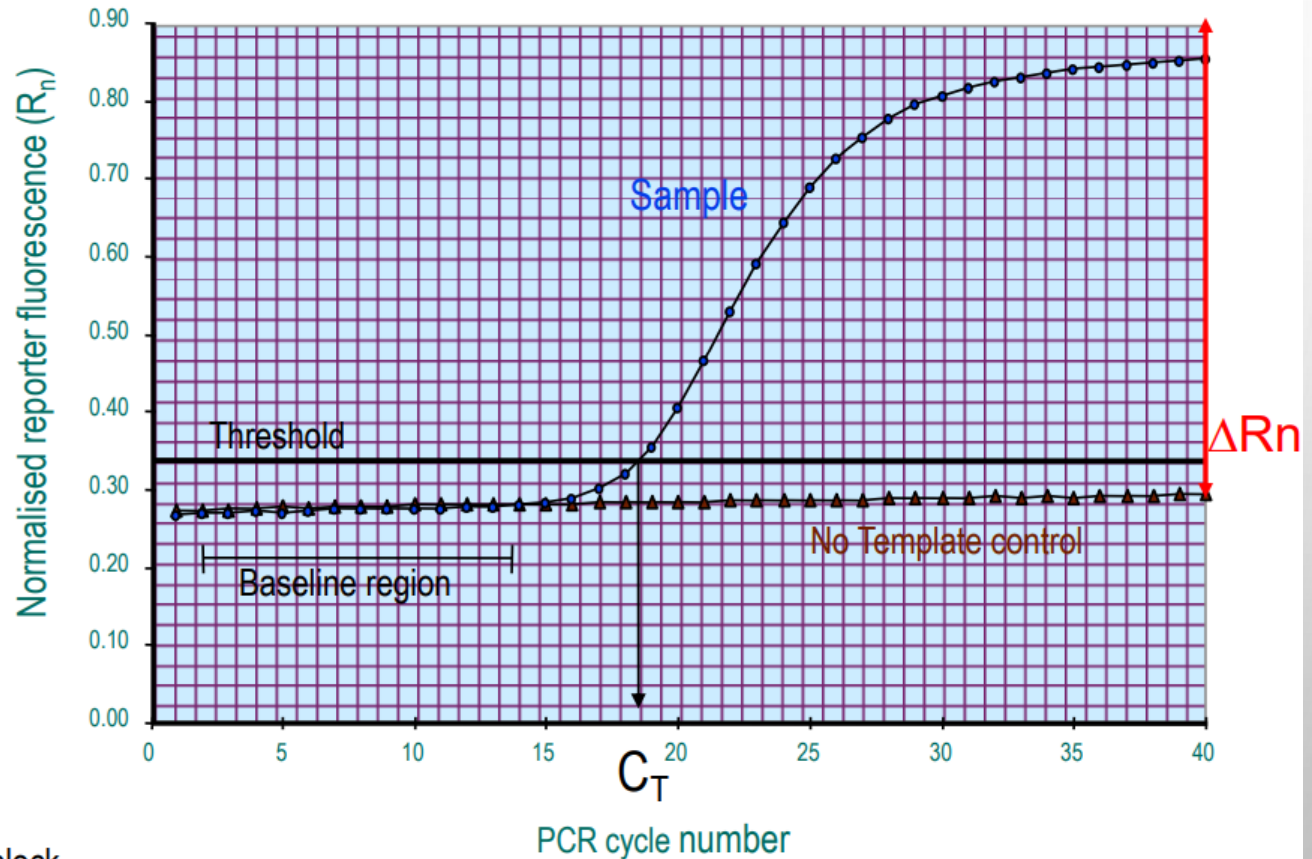
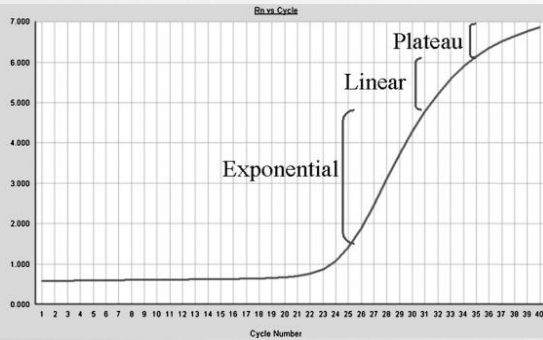


## 從Melt Curve確認是因為Primer Dimer或是有汙染

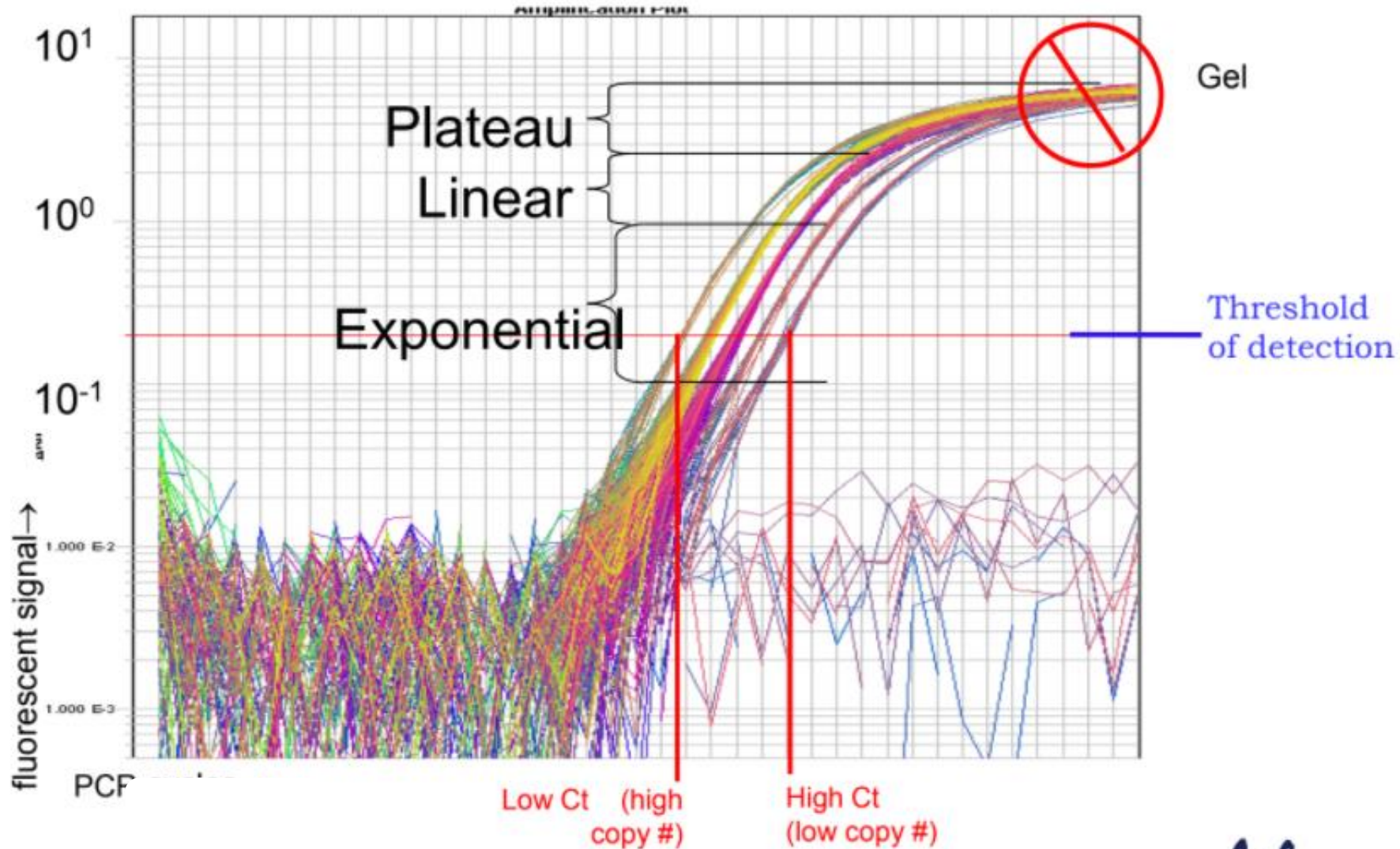
26



# Cycle Threshold (Ct)



$C_T$  = threshold cycle: the calculated fractional cycle number at which the PCR product crosses a threshold of detection

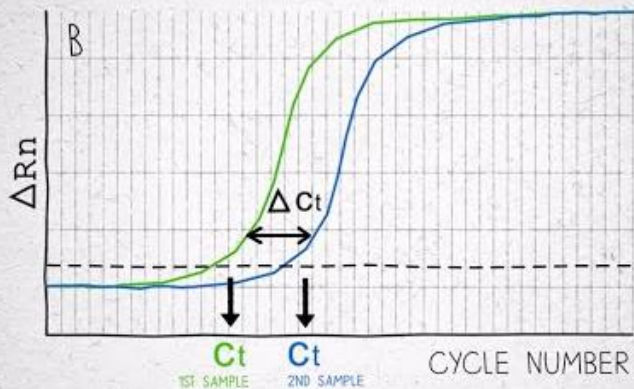


$Y = N_0 2^n$ ,  $C_T$  與起始濃度之對數值成反比





Samples	Raw Ct		Delta Ct	Delta Delta ct	2 <sup>delta d A</sup>
	GAPDH	p53			
Tumor cells 1	21.00	23.00	2.00	-3.93	=2 <sup>-(F3)</sup>
Tumor cells 2	20.50	22.00	1.50	-4.43	
Tumor cells 3	20.60	22.50	1.90	-4.03	
Normal cells 1	20.00	26.00	6.00	0.07	
Normal cells 2	20.50	26.20	5.70	-0.23	
Normal cells 3	20.30	26.40	6.10	0.17	
Avg delta Ct			5.93		



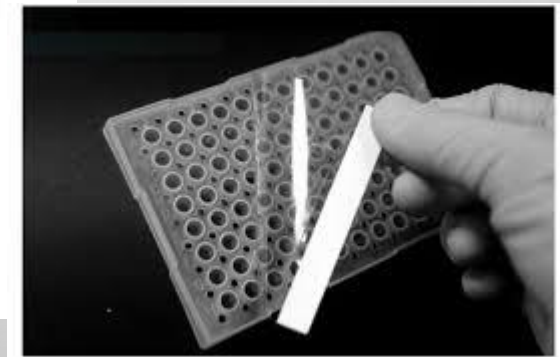
$$\Delta Ct = Ct_{\text{target}} - Ct_{18S rRNA}$$

$$\Delta\Delta Ct = \Delta Ct_{(\text{siRNA treated})} - \Delta Ct_{(\text{siRNA nontreated})}$$

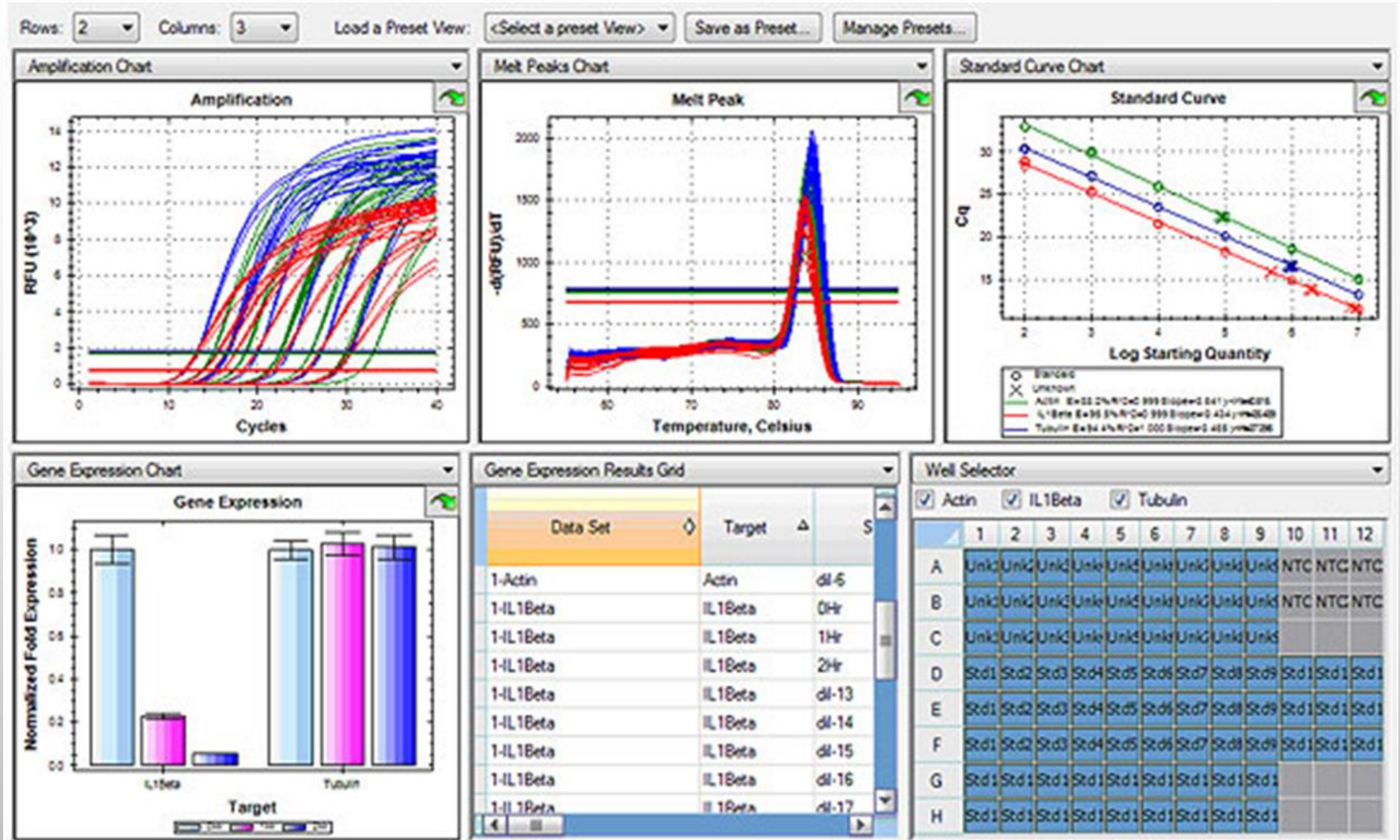
$$\text{Relative expression level} = 2^{-\Delta\Delta Ct}$$

$$\%KD = 100 \times (1 - 2^{-\Delta\Delta Ct})$$

## ( 2 ) Bio-Rad系統



# 程式設置



<https://www.youtube.com/watch?v=wUDysO8bFbA>



## (1) 核糖核酸品質

- 是成功製備cDNA並執行高效且可重複的Q-PCR的最關鍵步驟。
- RNA對RNA酶的降解非常敏感，在核酸分離步驟中必須小心處理。降解或污染的RNA會對Q-PCR實驗的效率和產量產生負面影響。
- 您的實驗室工作台、移液器和吸頭必須不含RNase，提取的RNA必須儲存在不含RNase的溶液中。
- 當您使用分光光度計評估RNA純度時，260和280nm ( $A_{260/280}$ ) 處的吸光度比應在1.8-2.0的範圍內。如果較低，則可能表明樣品被苯酚或蛋白質污染。



## (2) 預混液與表格使用

- 使用預混液可大幅減少實驗變異性，從而減少孔間和樣品間差異來提高再現性。
- 在執行Q-PCR實驗時最好事先製作一張準備要加入每項試劑的清單與樣品相對位置表格。

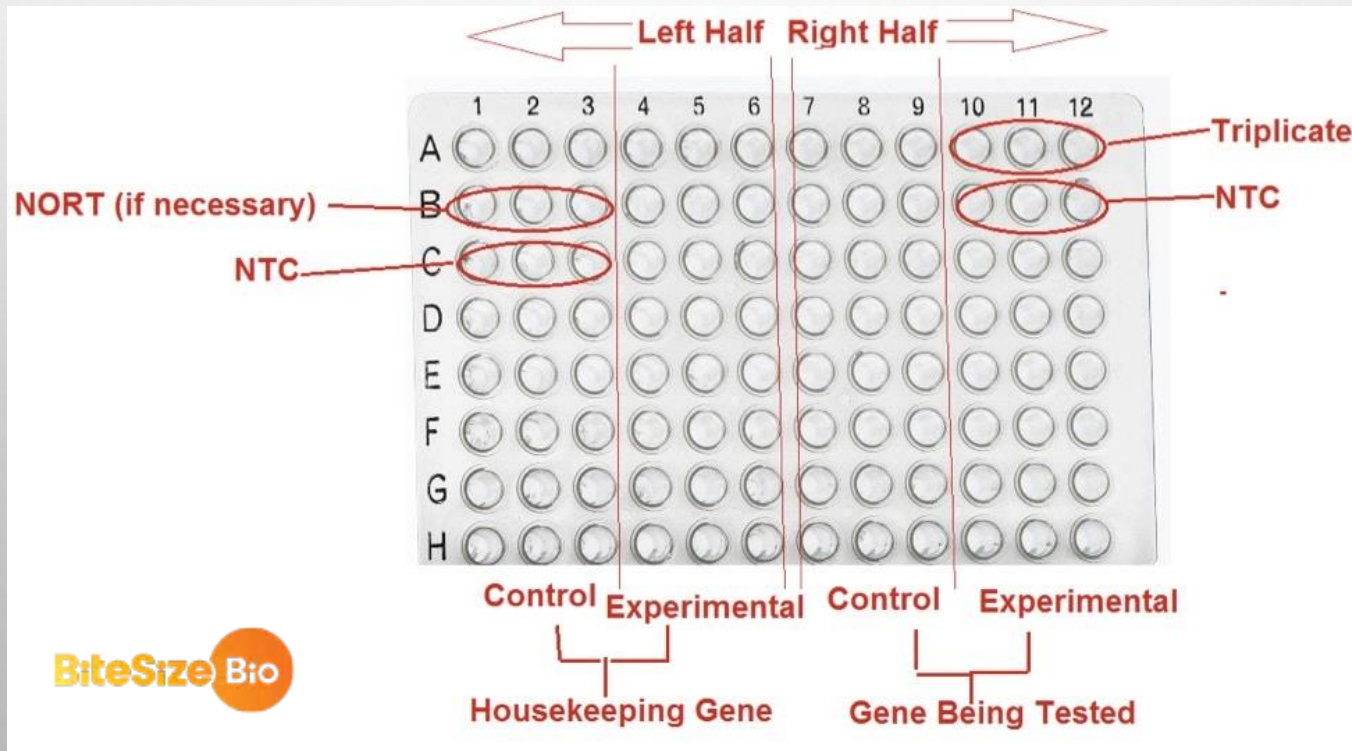


Component	Volume/ reaction
Master mix	20 $\mu$ l
Nuclease free water	12 $\mu$ l
5x Qiagen OneStep RT-PCR buffer	5 $\mu$ l
dNTP Mix (10 mM of each dNTP)	1 $\mu$ l
Primer Forward (100 pmol/ $\mu$ l)	0.5 $\mu$ l
Primer Reverse (100 pmol/ $\mu$ l)	0.5 $\mu$ l
Qiagen OneStep RT-PCR Enzyme Mix	1.0 $\mu$ l
Template RNA	5 $\mu$ l
Total	25 $\mu$ l



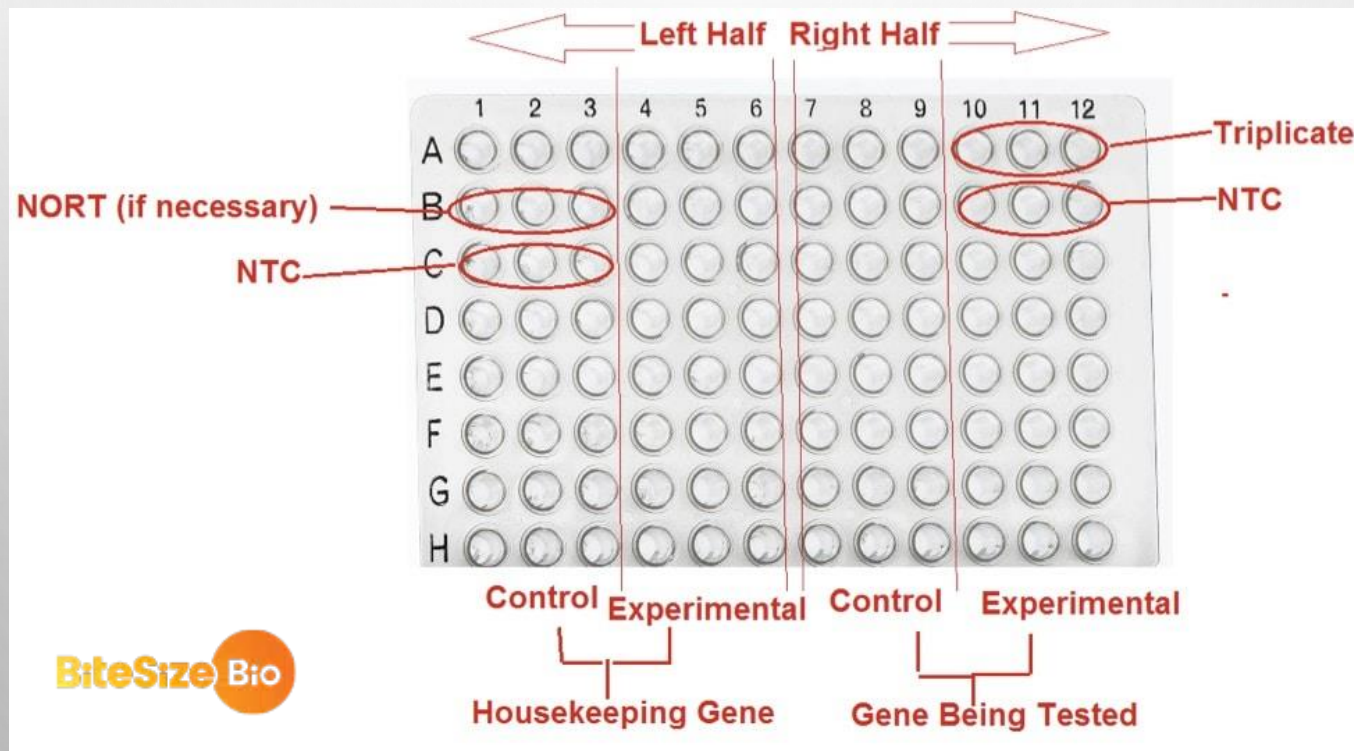
### (3) 控制組

- Q-PCR測定時，適當的**控制組**是很重要的。
- 應該包括一個**缺乏cDNA**的陰性對照組，可以檢測表面或試劑的交叉污染。
- 也需要一組**反轉錄的陰性對照組**，其中沒有逆轉錄酶，如果觀察到產品，表明您的樣品受到DNA污染。

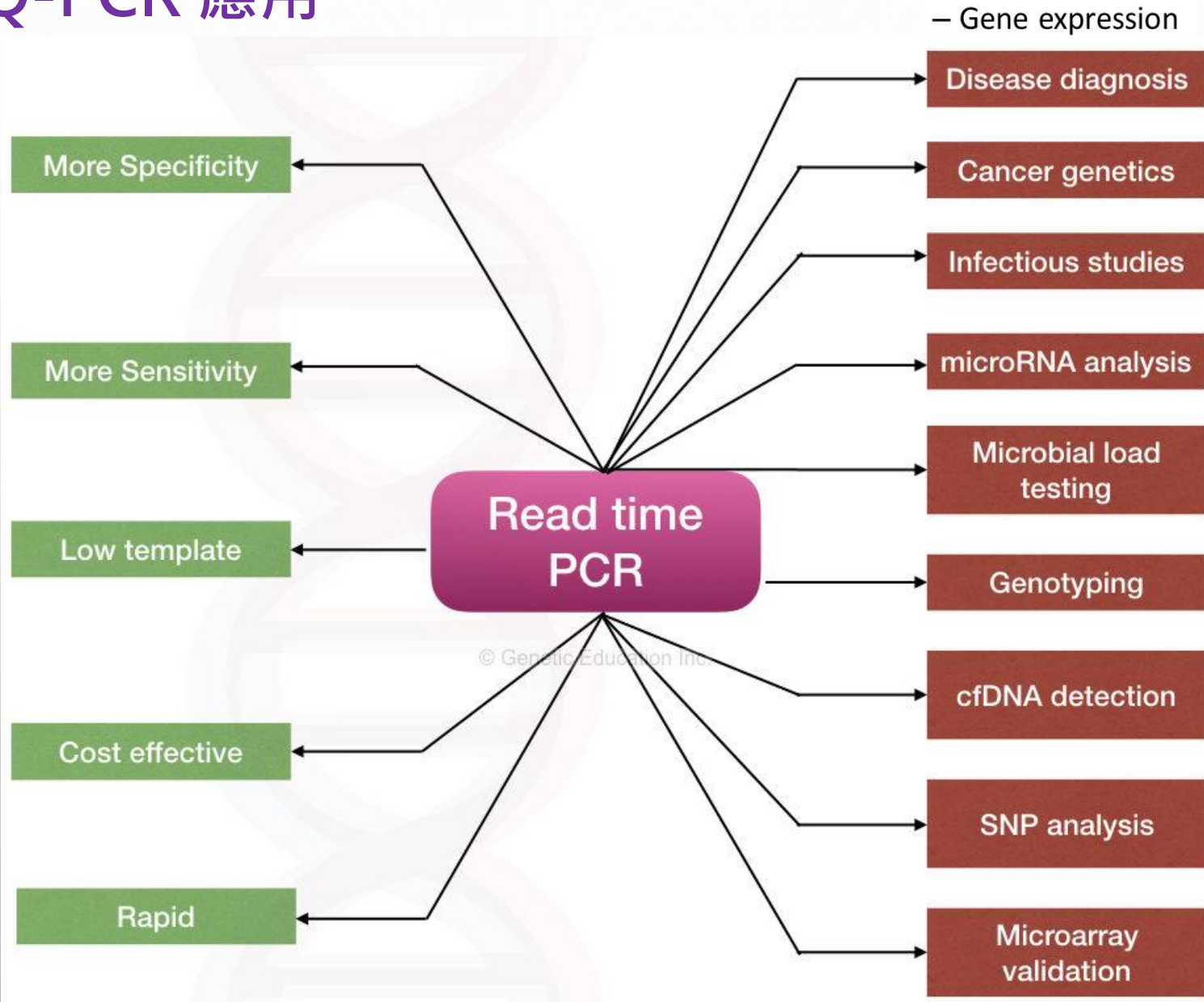


## (4) 參考基因

- 內源性對照基因的擴增可以解釋起始cDNA的數量或質量的差異，以及RNA製備方法或cDNA合成的差異。
- 可靠的參考基因是指其表達量不受實驗變數的影響，並且在樣品條件的相關生理狀態之間沒有差異的基因。

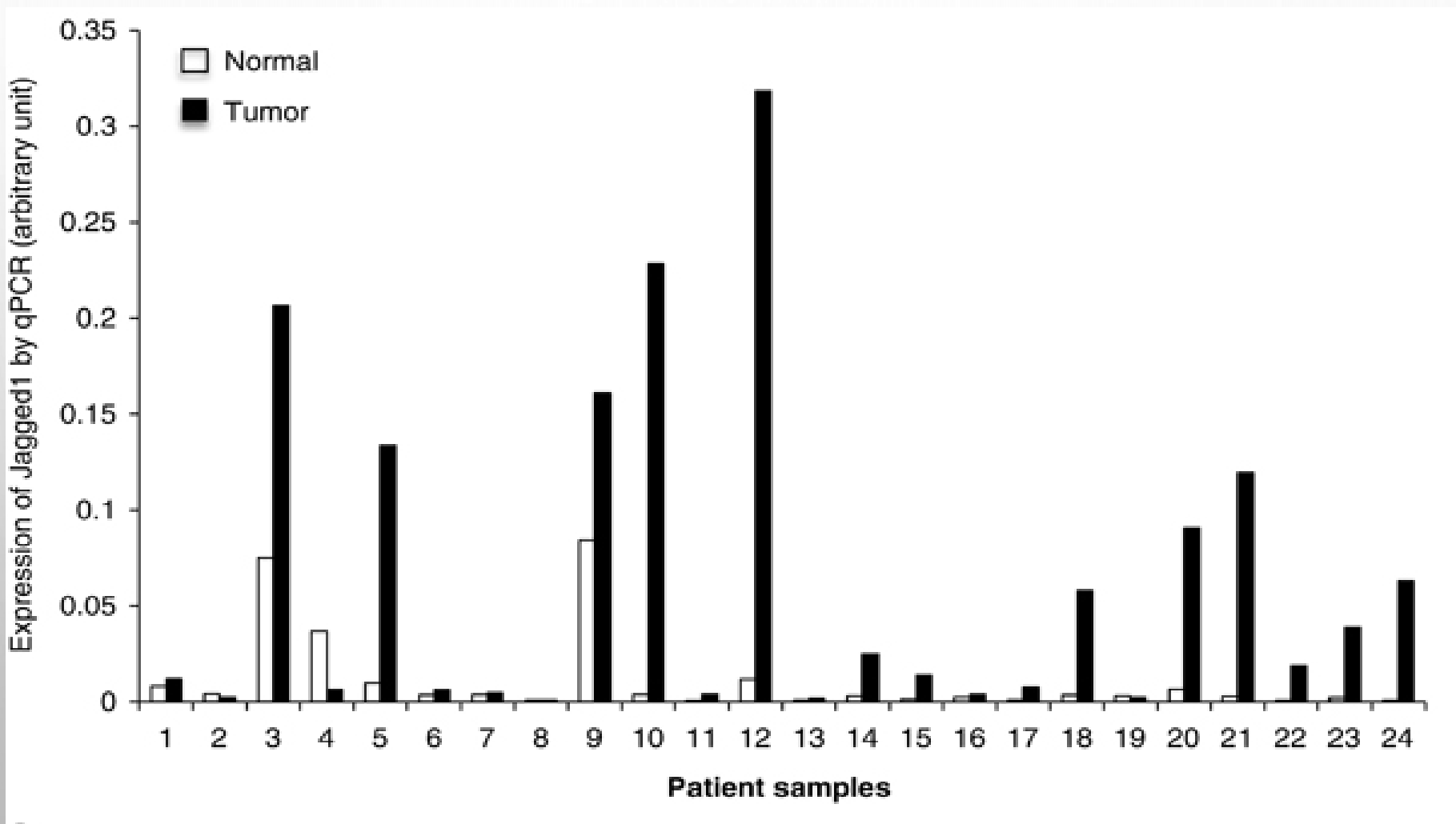


# Q-PCR 應用





## (1) 基因表達分析-例如病人正常細胞與癌細胞基因表現差異

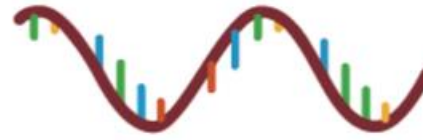


# ( 2 ) 微生物檢測量

a) sample collection



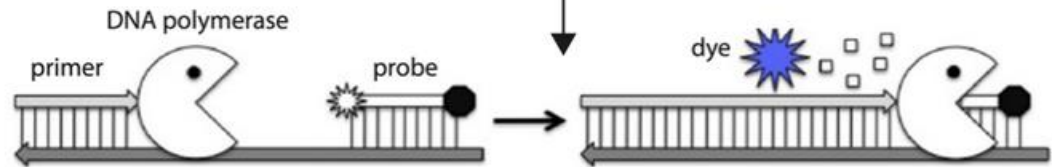
b) RNA extraction



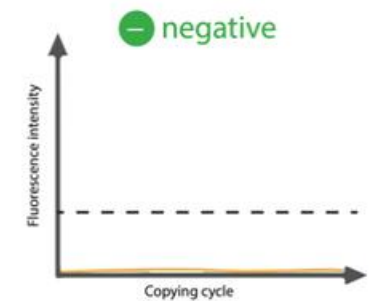
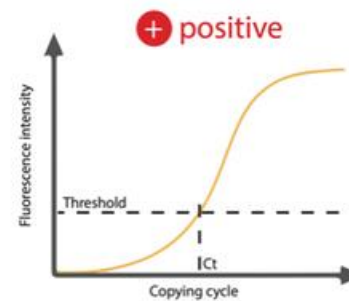
c) Reverse transcription



d) RT-PCR amplification



e) Results





COVID19

# 一次認識三種檢驗方式

健康醫療網



## 抗原檢測 (抗原快篩)

篩檢站  
所用

高風險地區，快速  
找出感染者的利器

- 檢測** 檢體中是否含有病毒的抗原
- 優點** 檢驗時間短  
快速得到結果
- 缺點** 準確率較**PCR**低，容易產生偽陽、偽陰性



## PCR檢測 (RT-PCR)

全球判斷染疫的  
標準檢測

- 檢測** 檢體中是否含有病毒的遺傳物質
- 優點** 準確度高，病毒量低也可檢驗出
- 缺點** 耗時、成本高，需要專業設備及人員執行



## 抗體檢測 (抗體快篩)

後續瞭解病毒的  
盛行率、研究用

- 檢測** 血清中是否含有病毒的抗體
- 優點** 可找出曾經感染過或打疫苗者是否有抗體
- 缺點** 感染後期才能驗出，也可能會有偽陰性產生

資料來源：疾管署

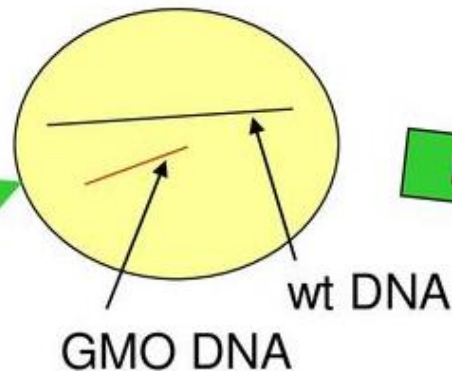
ICONS MADE BY FLATICON

## ( 3) 基因食品檢測

### Example: Determining percentage of GMO food content

Determination of percent GMO food content important for import / export regulations.

Labs use Real-Time PCR to measure amount of transgenic versus wild-type DNA.



International shipments depend on results!









## II. 報告基因分析 (Reporter gene assay)

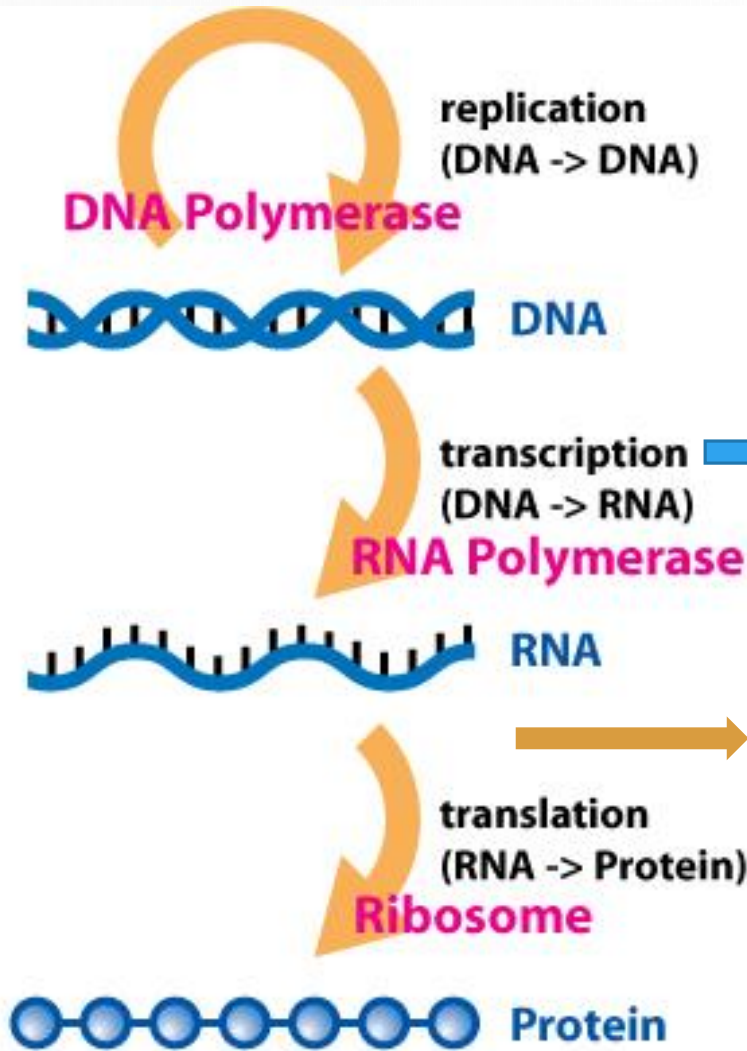
# 上課內容

- 基因表現調節
- 什麼是報告基因分析
- 常見的報告基因
- 報告基因分析的應用

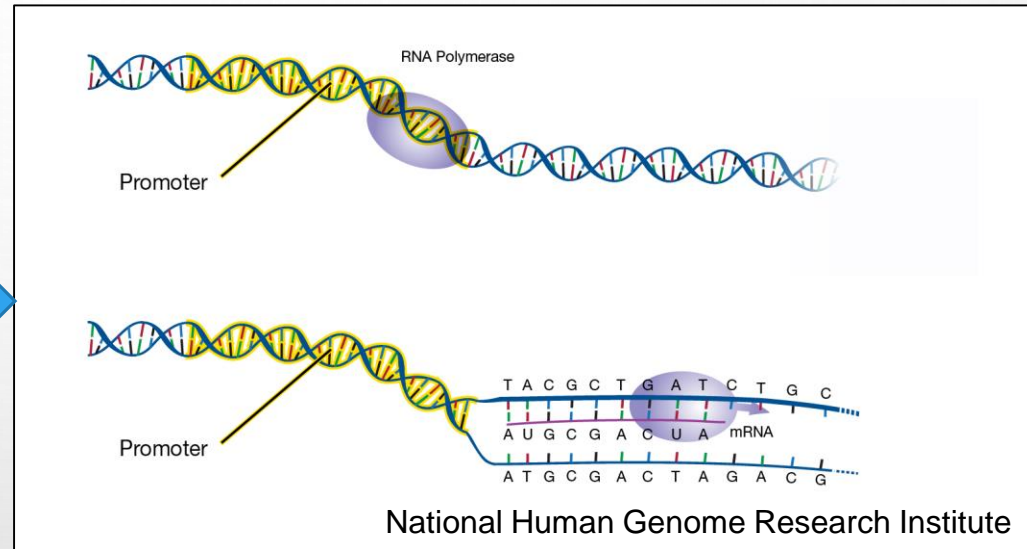


# 基因表現調節

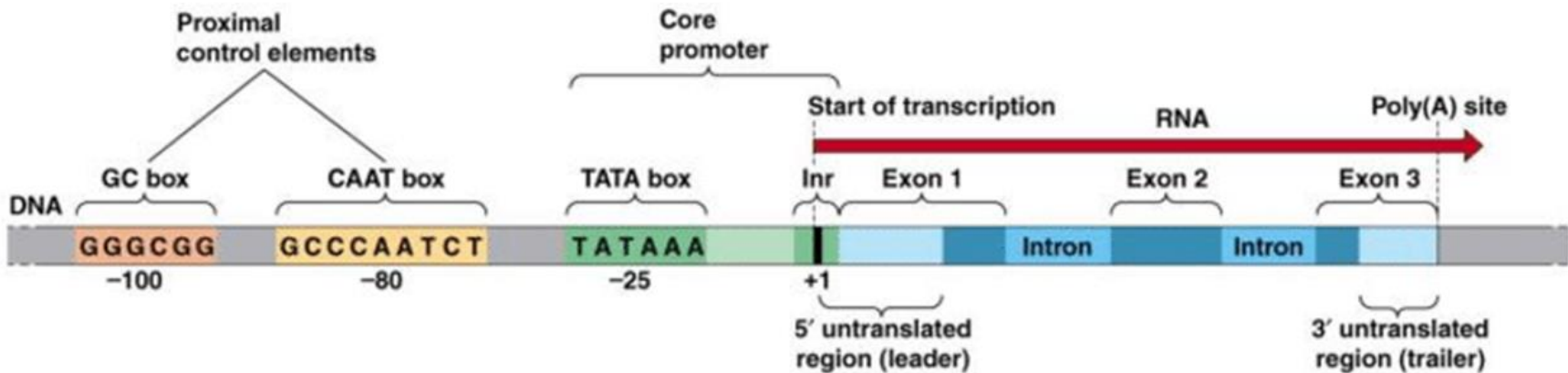
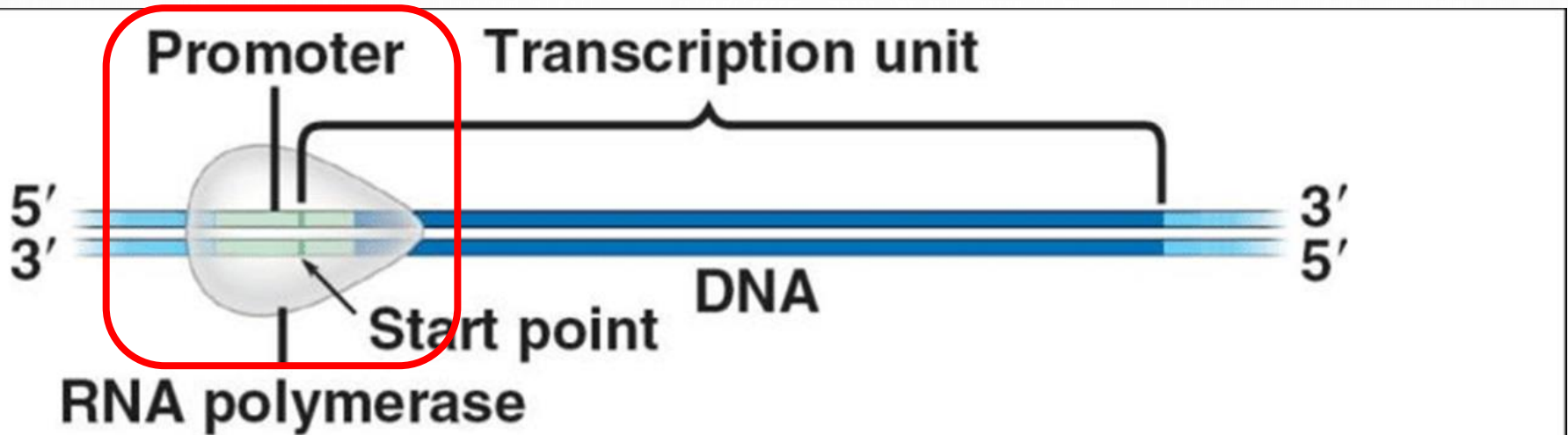
## 中心法則



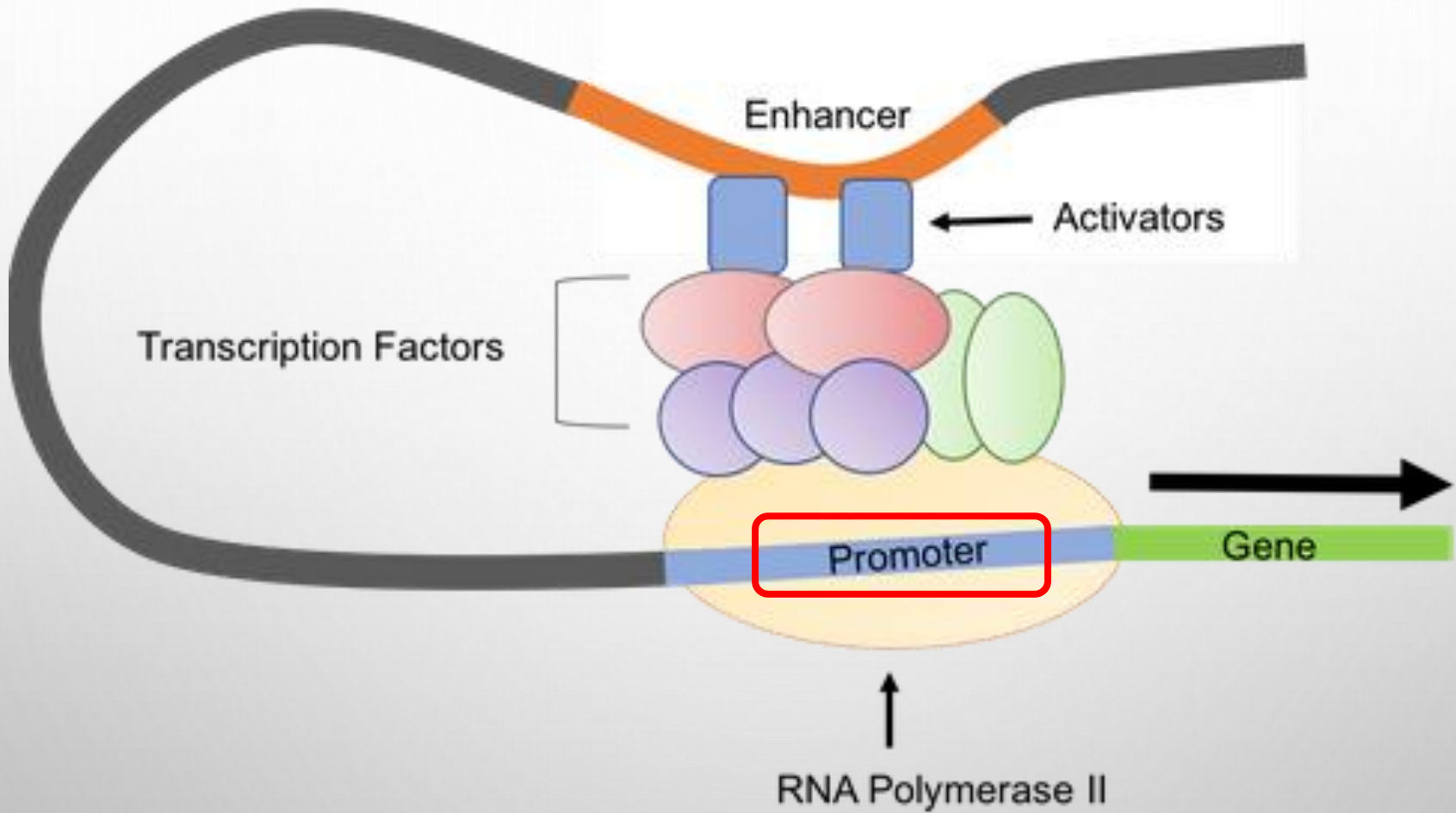
## DNA → RNA



# 啟動子 (Promoter)

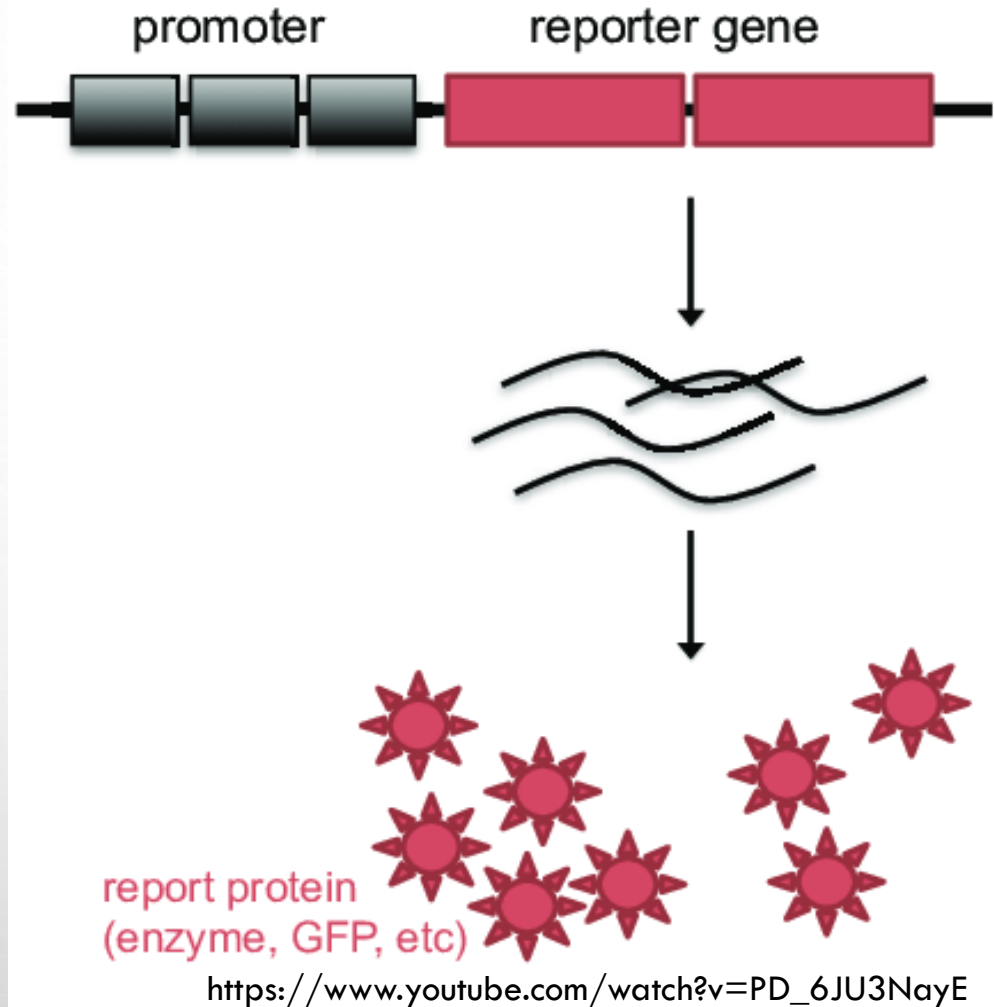


© 2012 Pearson Education, Inc.



# 什麼是報告基因分析

- 報告基因分析主要為**報告基因**的**啟動子**活性的測量。
- 報告基因是把**啟動子DNA**序列和**報告基因DNA**序列的融合。
- 報告基因可以為**酵素**或**螢光物**。





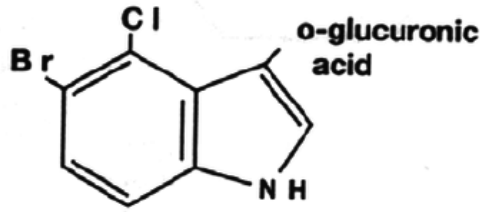
# 如何才是好的報告基因?

1. 易於檢測其功能。
2. 其表達產物能進行定量測定。
3. 不會影響實驗材料本身。
4. 表達產物在受體細胞中原本就不存在。

# 常見的報告基因

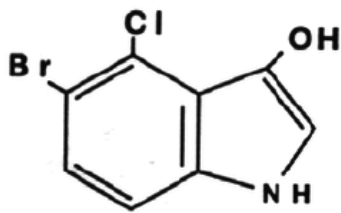
- $\beta$ -葡萄糖醛酸酶 (  $\beta$ -glucuronidase , GUS )
- $\beta$ -半乳糖苷酶 (  $\beta$ -galactosidase, LacZ )
- 鹼性磷酸酶 (alkaline phosphatase, ALP)
- 氯黴素乙醯轉移酶 ( Chloramphenicol acetyltransferase, CAT )
- 綠色螢光蛋白 ( Green fluorescent protein, GFP )
- 螢光素酶 (luciferase)

# (1) $\beta$ -葡萄糖醛酸酶 (GUS)



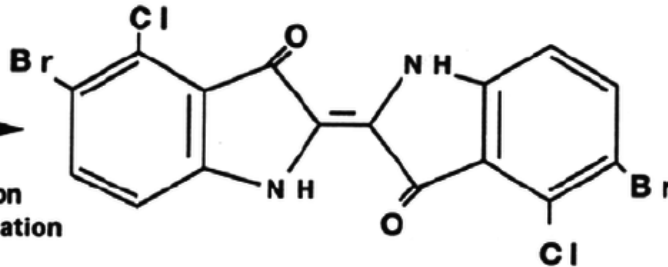
**X-Gluc** (5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide)

$\beta$ -glucuronidase



oxidation dimerization

5,5'-dibromo-4,4'-dichloro-indigo (insoluble, colored)



Susan Jean Karcher

Beta-glucuronidase

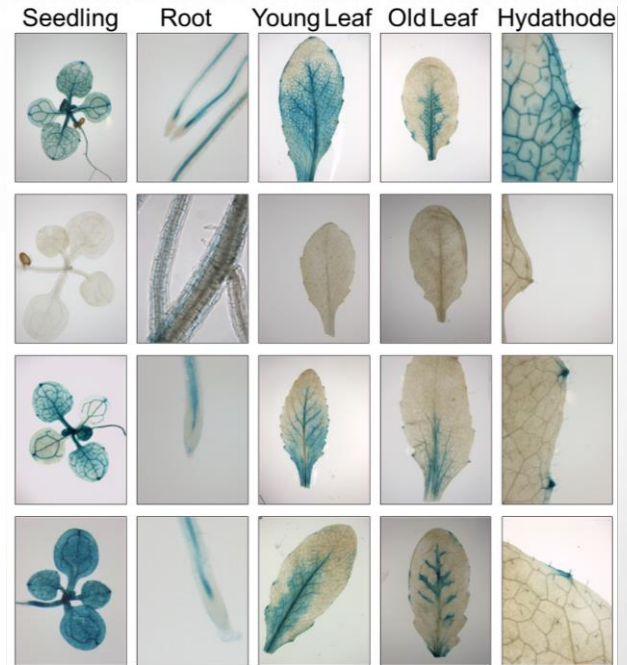
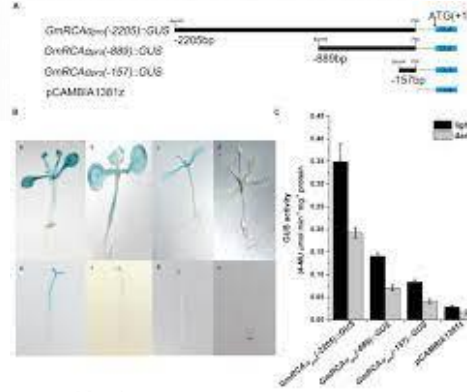


Hydrolysis of the X-Gluc substrate by the GUS enzyme

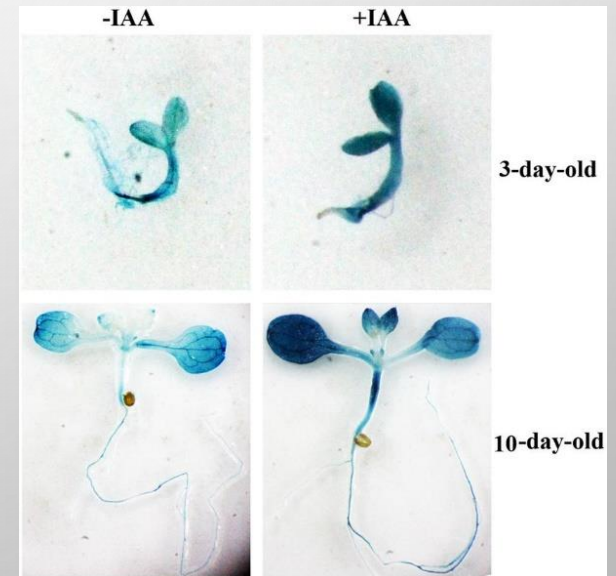


Dimerization of the Gluc product by reaction with O<sub>2</sub>

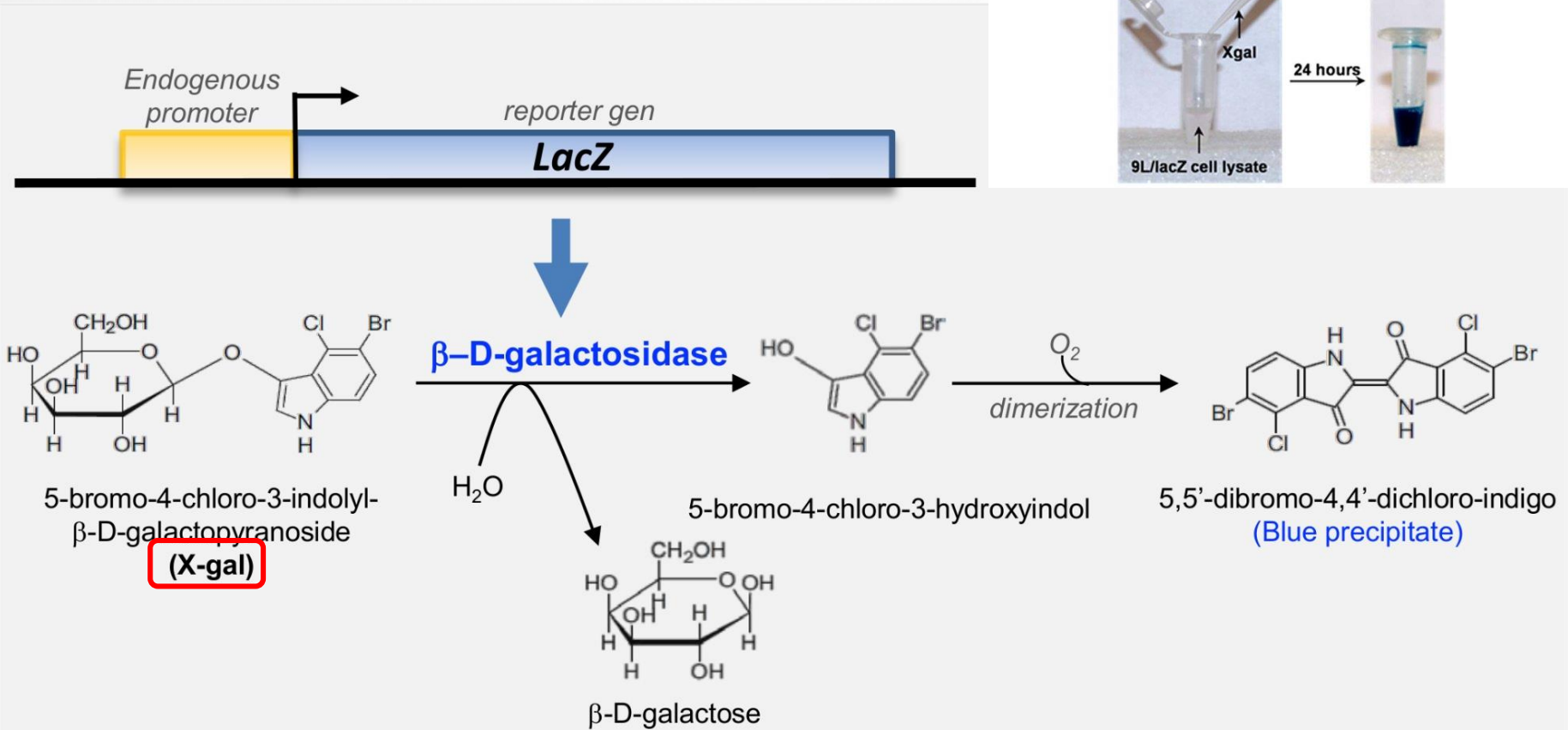
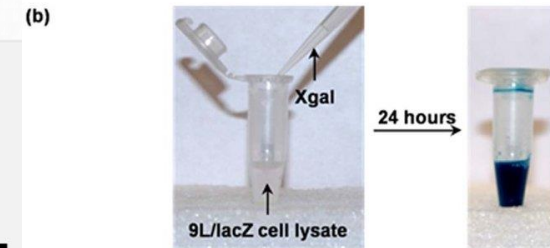
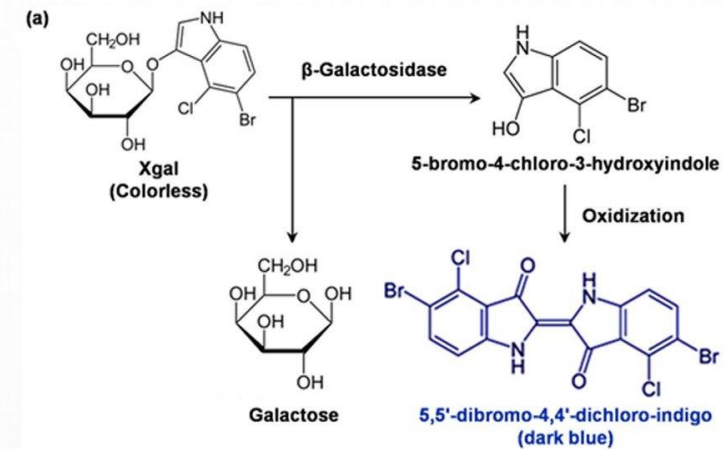
The GUS histochemical staining assay



doi: <https://doi.org/10.1371/journal.pone.0159875.g004>

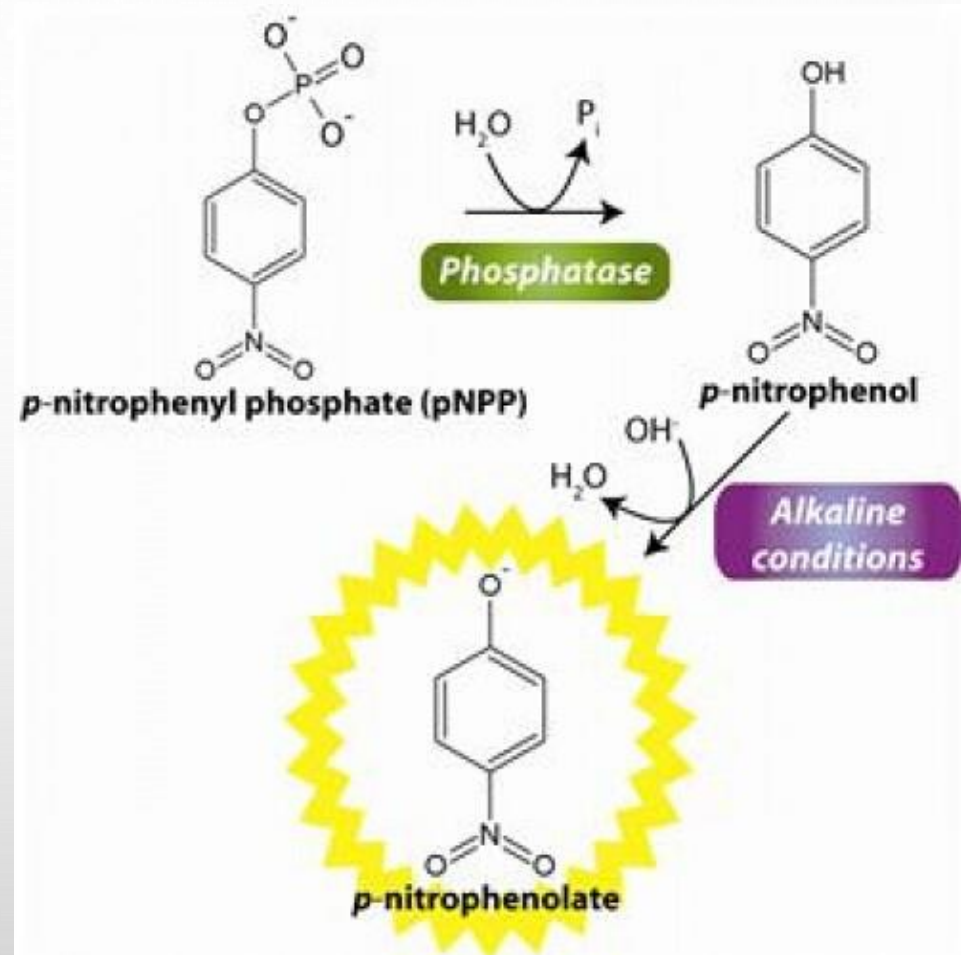
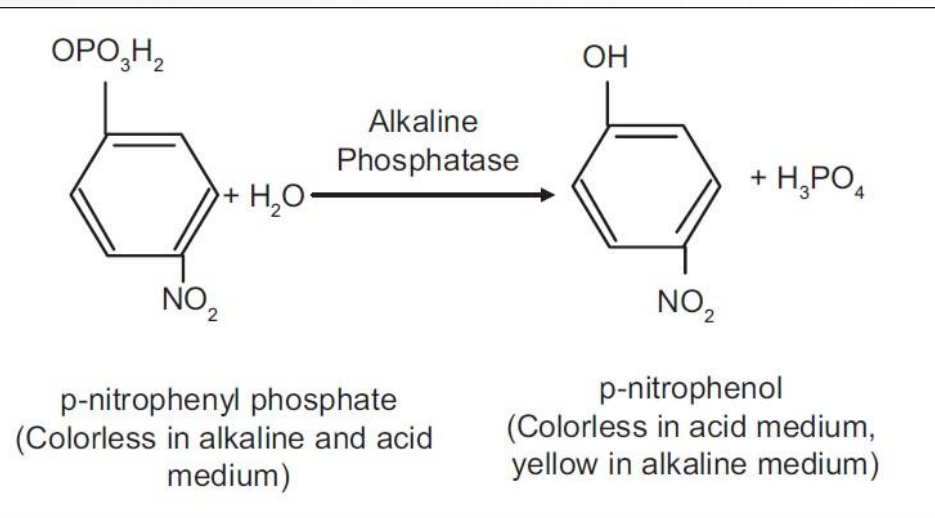


## (2) $\beta$ -半乳糖苷酶 (LacZ)

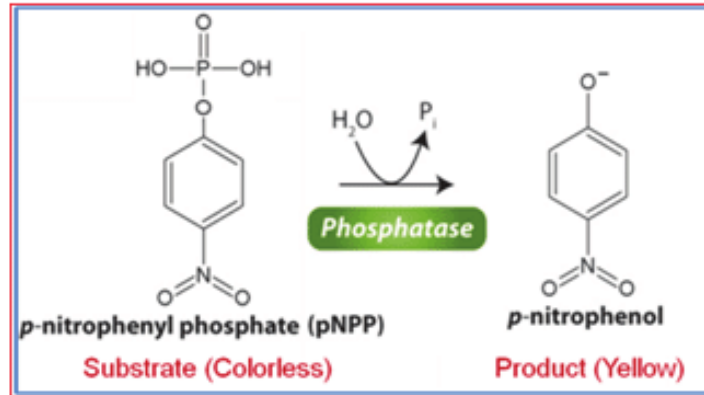




# ( 3 ) 鹼性磷酸酶 (ALP)

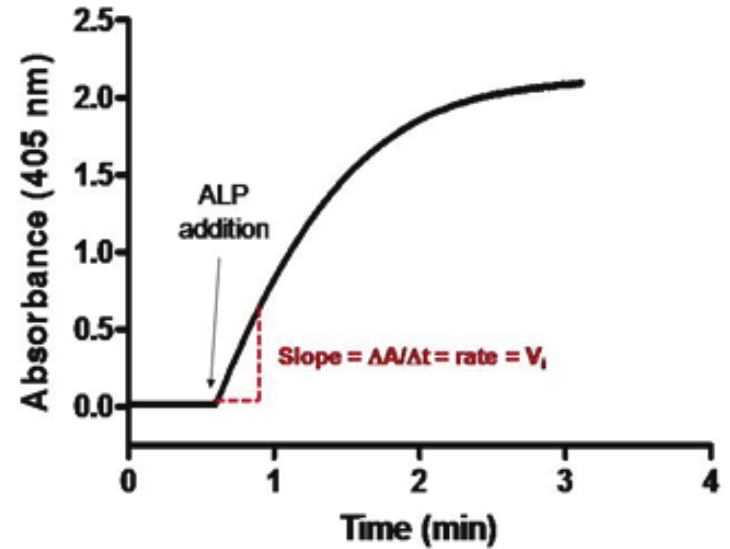
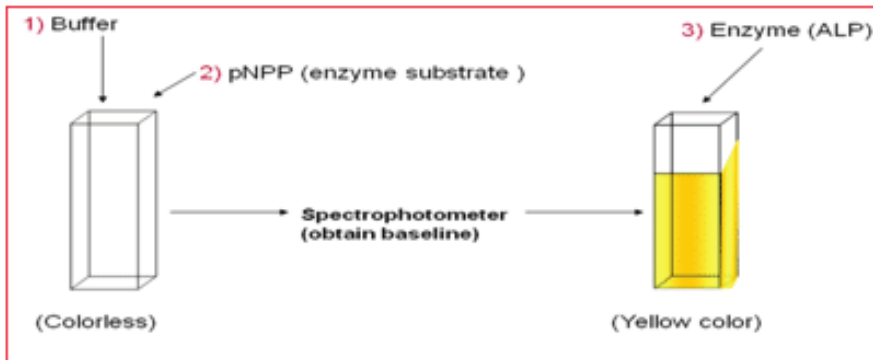


# A.



# B.

Alkaline Phosphatase (ALP)



# (4) 氯黴素乙醯轉移酶 (CAT)

CAT:  
Chloramphenicol (CAM)  
acetyl transferase

CAM: chloramphenicol (CAM)  
Protein synthesis inhibitor  
↓ acetylation by CAT  
Loss inhibitor activity

Other reporter enzymes:  
β-galactosidase  
luciferase

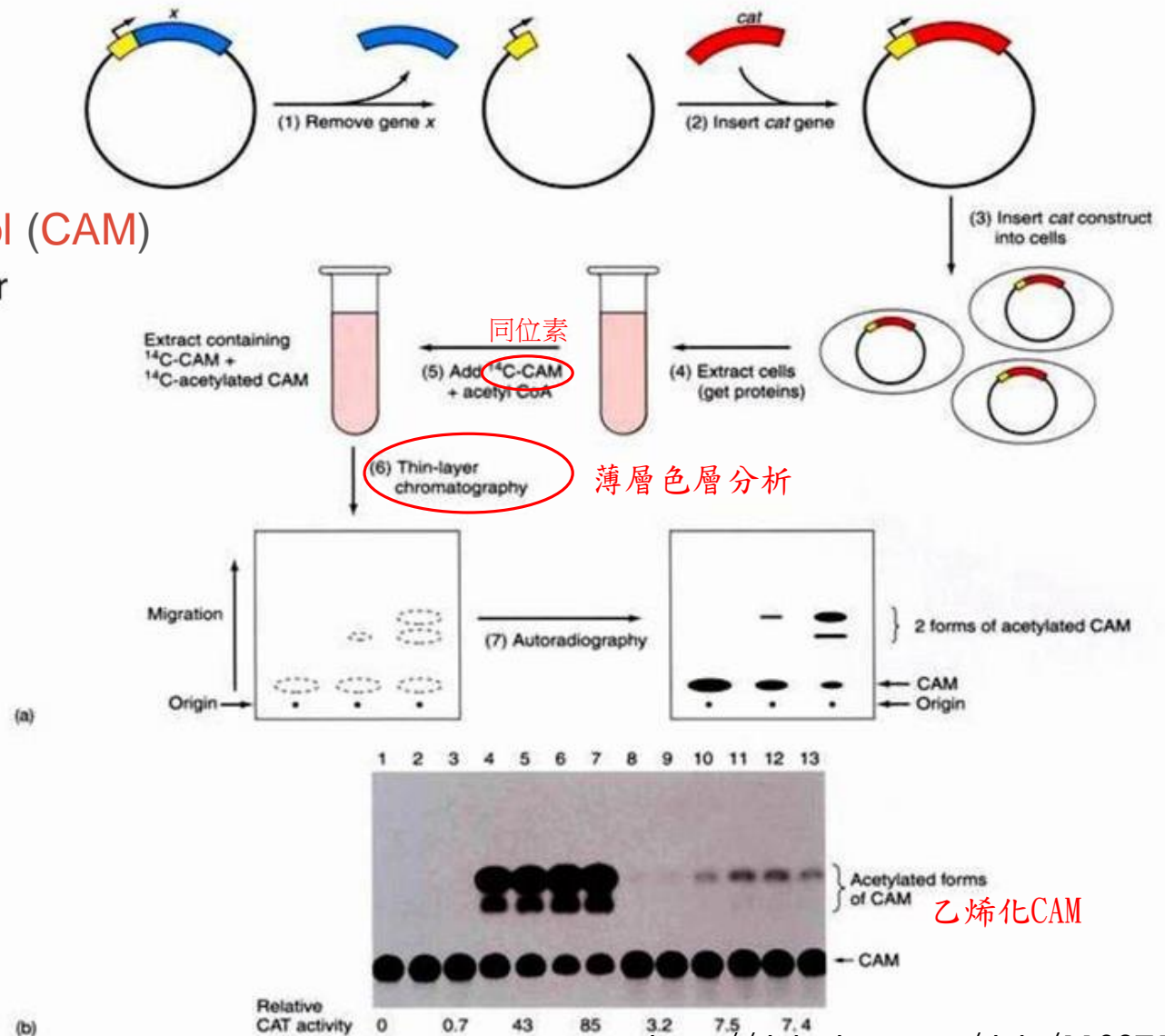
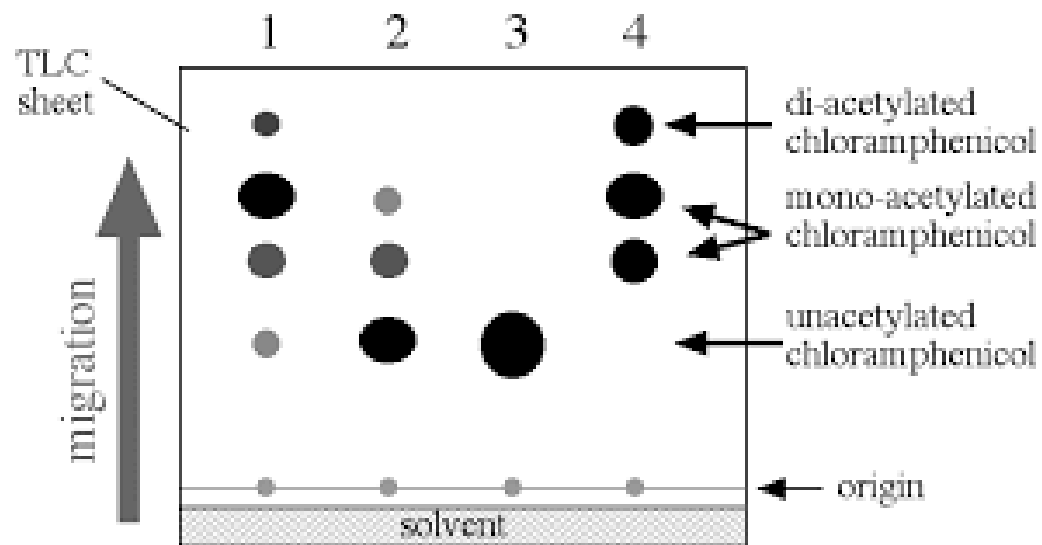
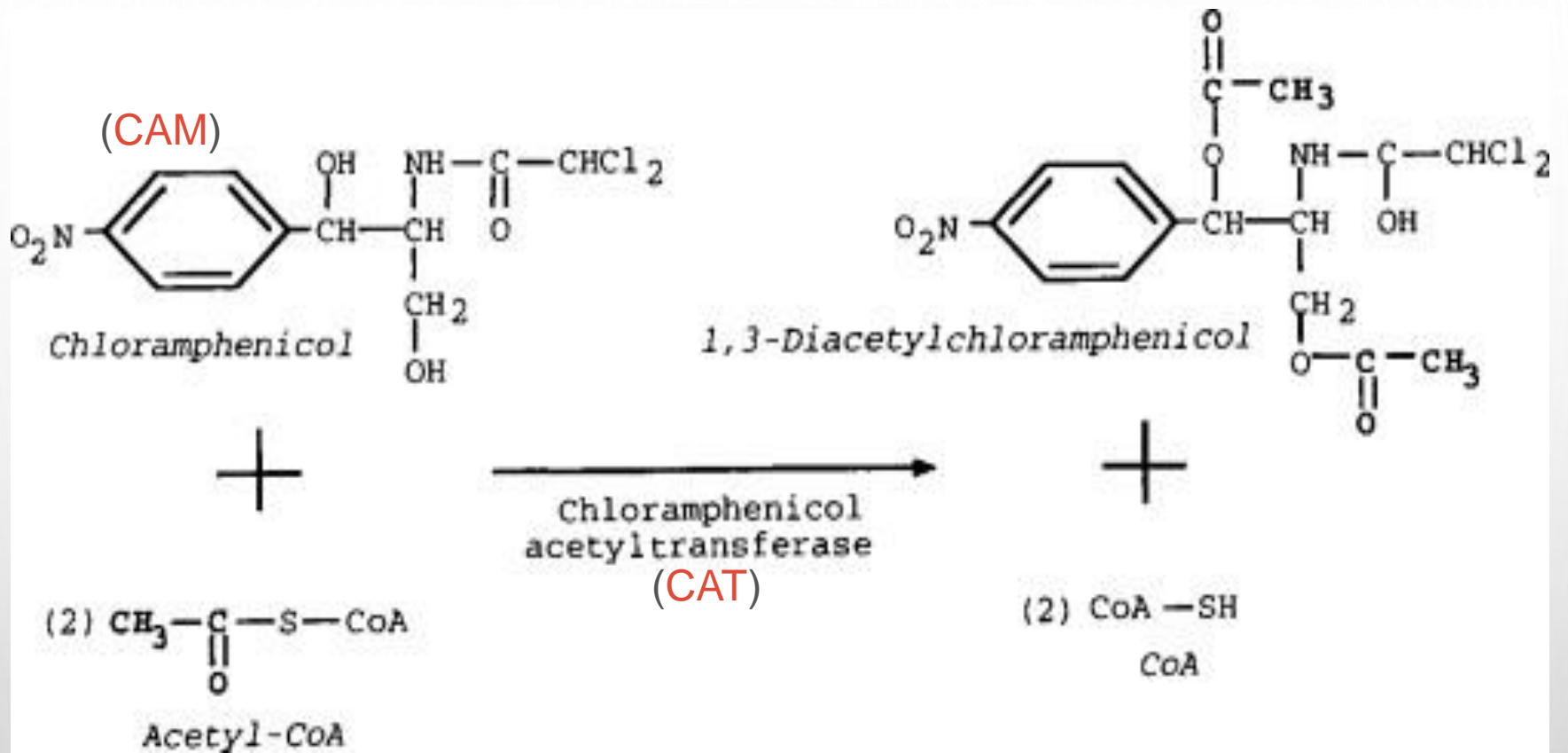


Figure 5.34 Using a reporter gene.

<https://slideplayer.com/slide/11837566/>







# 冷光與螢光反應

從微孔盤底部會不斷產生冷光自發光，這時後選擇反光效果較佳的白盤 可有效檢測樣品的冷光訊號值

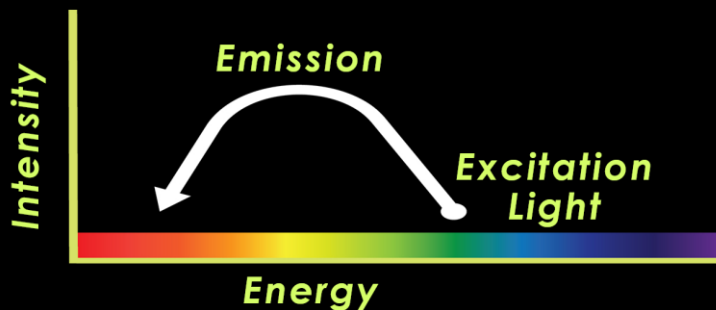
微孔盤底部會會有一道散射光，為了有效阻隔鄰近樣品間的干擾，螢光實驗建議選用遮蔽力較佳的黑盤來進行

<https://blog.xuite.net/clare690725/twblog/111356956>

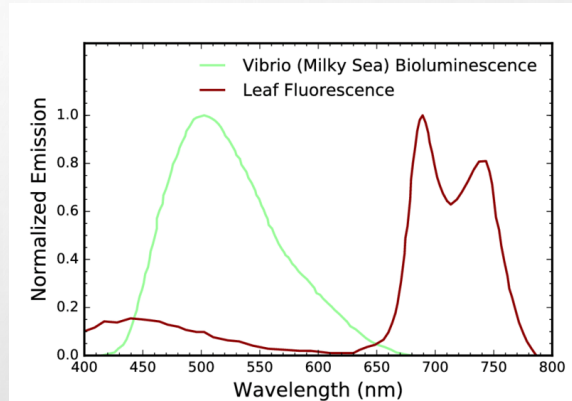
## LUCIFERIN - LUCIFERASE REACTION



## FLUORESCENCE REACTION



- Light is absorbed.
- Light emitted has less energy than light absorbed.



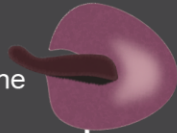
# DISCOVER BIOLUMINESCENT ORGANISMS



*Renilla reniformis*

Substrate: Coelenterazine

Wavelength: 480 nm



*Cypridina noctiluca*

Substrate: Vargulin

Wavelength: 465 nm



*Gussia princeps*

Substrate: Coelenterazine

Wavelength: 460 nm



*Photinus pyralis*

Substrate: D-Luciferin

Wavelength: 560 nm



*Pyrophorus*

*plagiophthalmus*

Substrate: D-Luciferin

Wavelength: 613 nm (red)

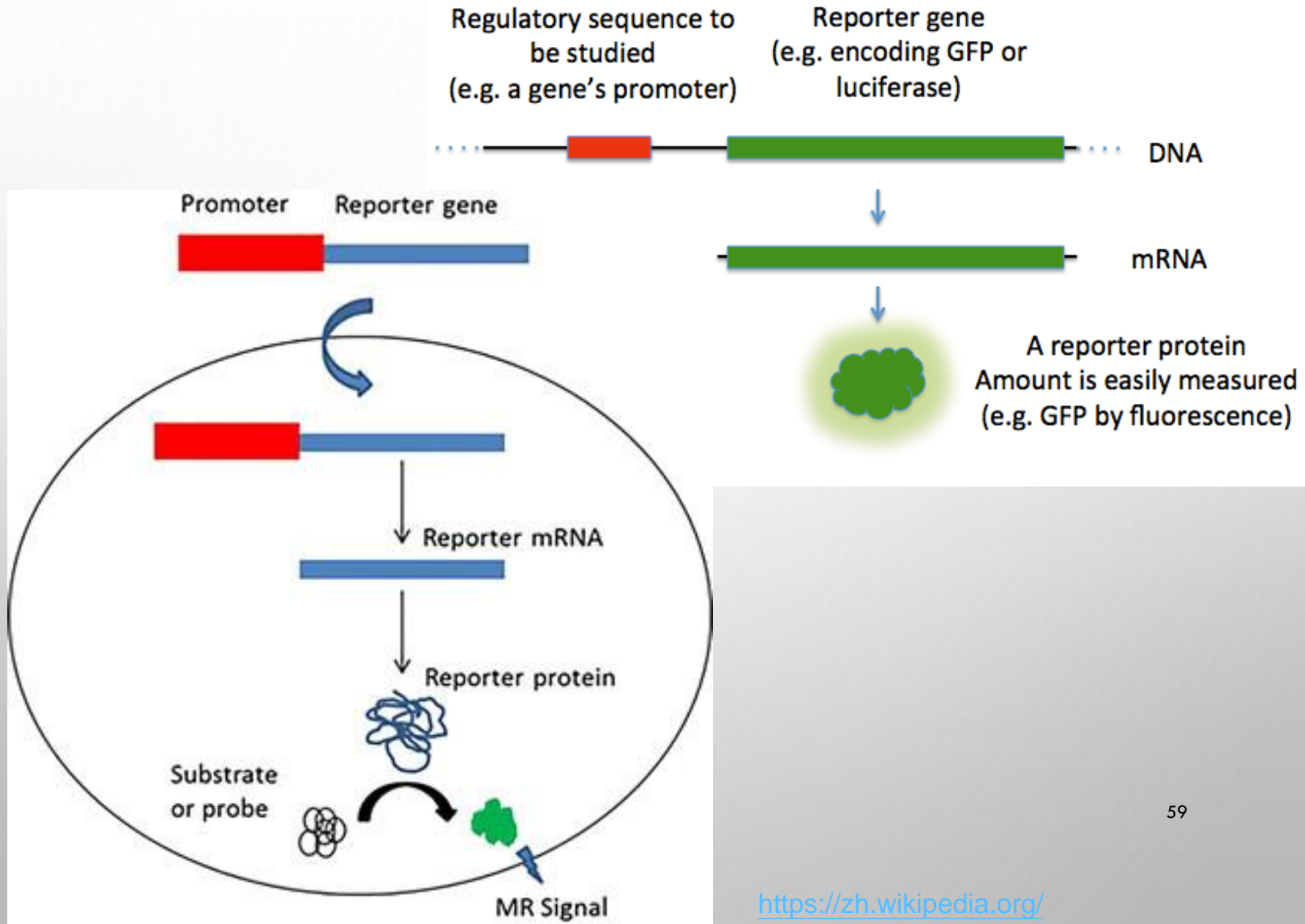


400

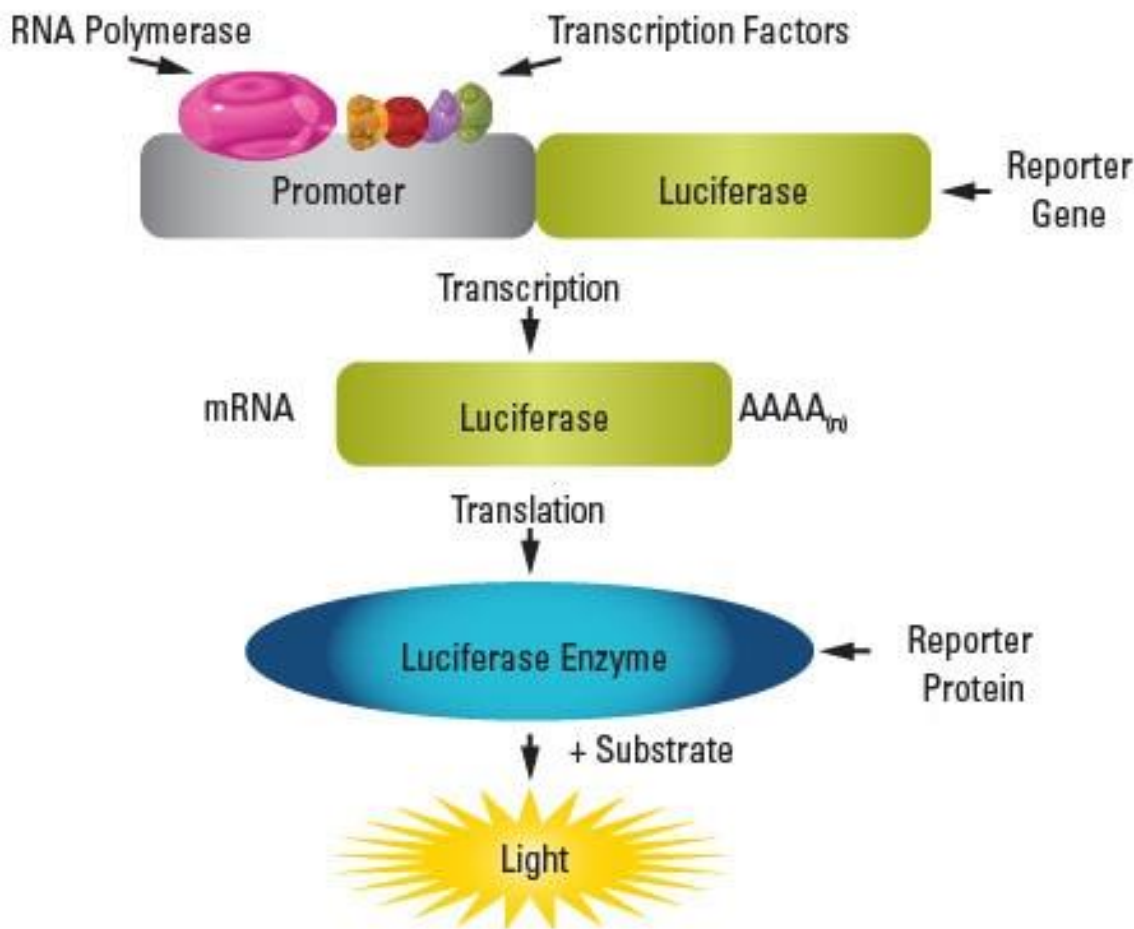
500

600

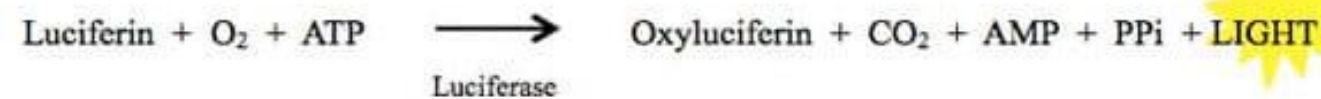
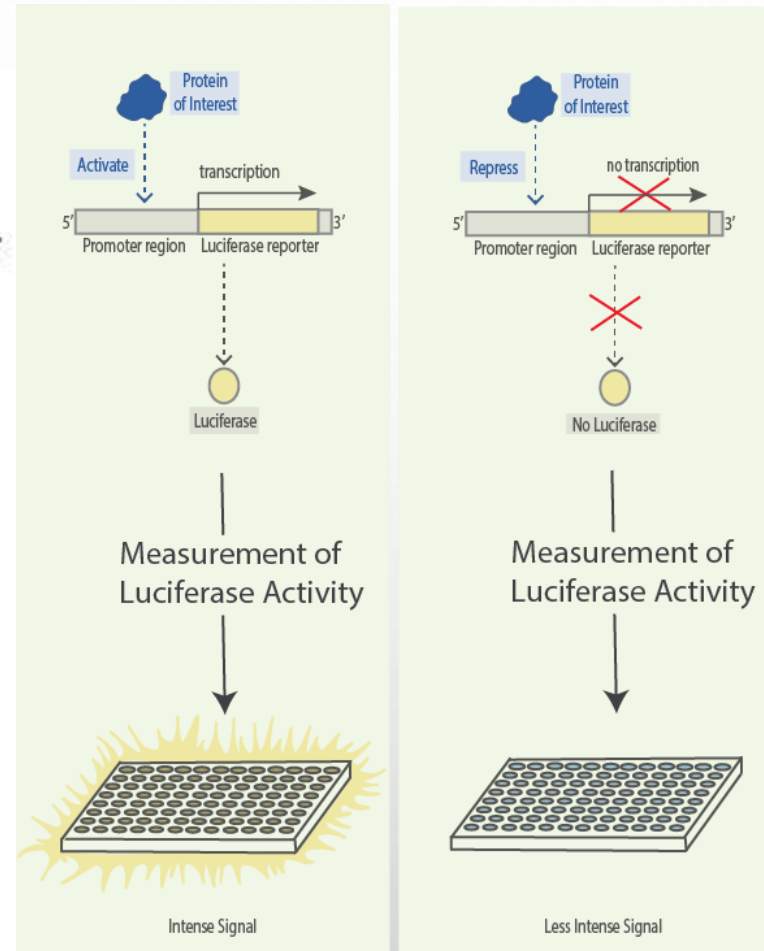
# ( 5 ) 綠色螢光蛋白 ( GFP )



# ( 6 ) 冷光素酶 (Luciferase)



Light Signal = Luciferase Expression = Promoter Activity







# The Nobel Prize in Chemistry 2008

"for the discovery and development of the green fluorescent protein, GFP"

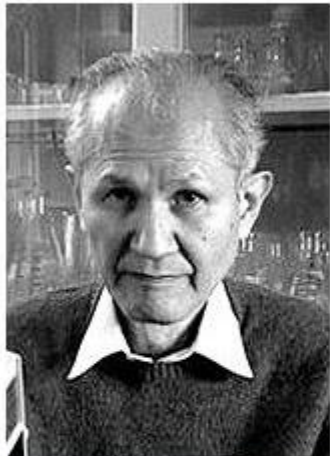


Photo: J. Henriksson/SCANPIX

**Osamu Shimomura**

🕒 1/3 of the prize

USA

Marine Biological Laboratory (MBL)  
Woods Hole, MA, USA;  
Boston University Medical School  
Massachusetts, MA, USA



Photo: J. Henriksson/SCANPIX

**Martin Chalfie**

🕒 1/3 of the prize

USA

Columbia University  
New York, NY, USA



Photo: UCSD

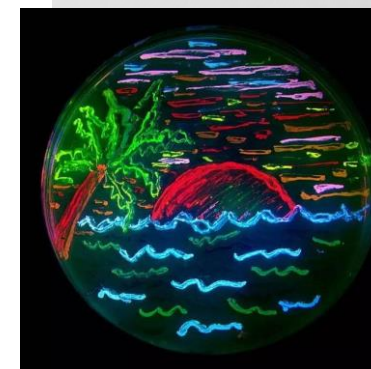
**Roger Y. Tsien**

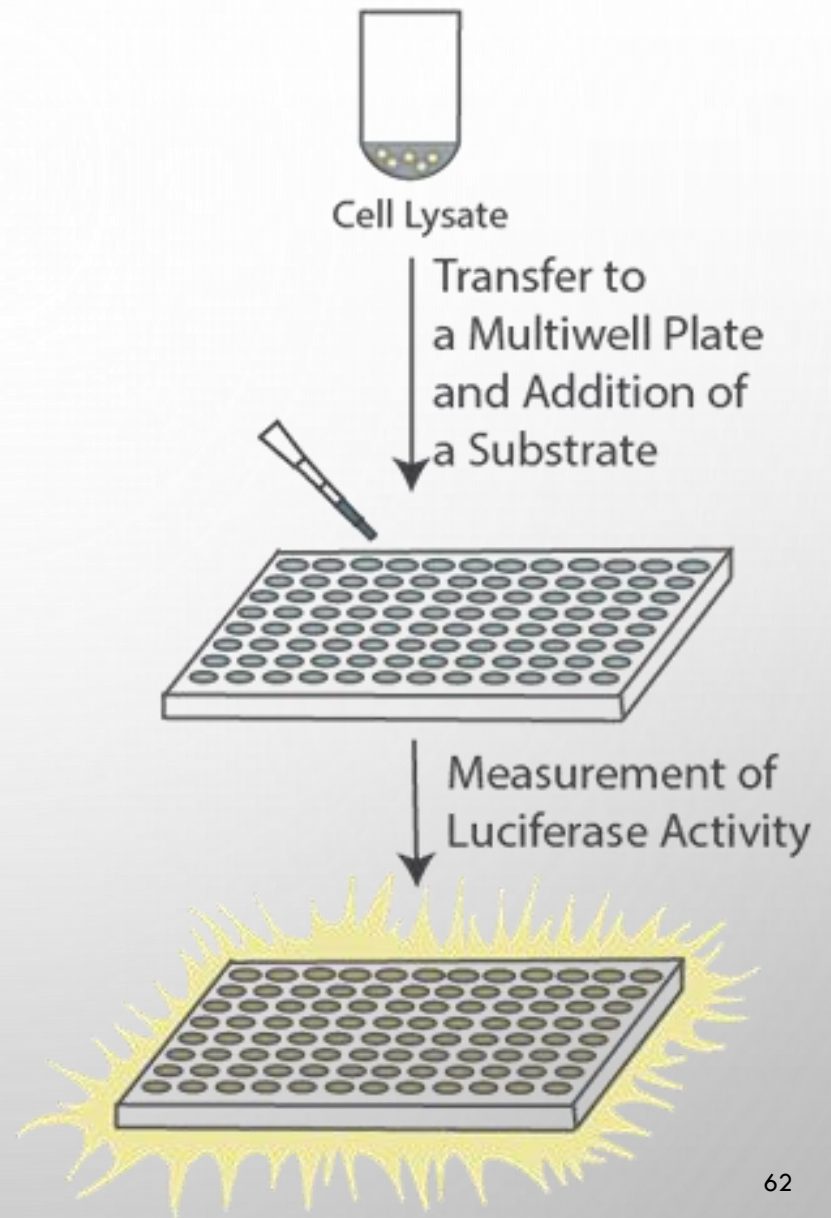
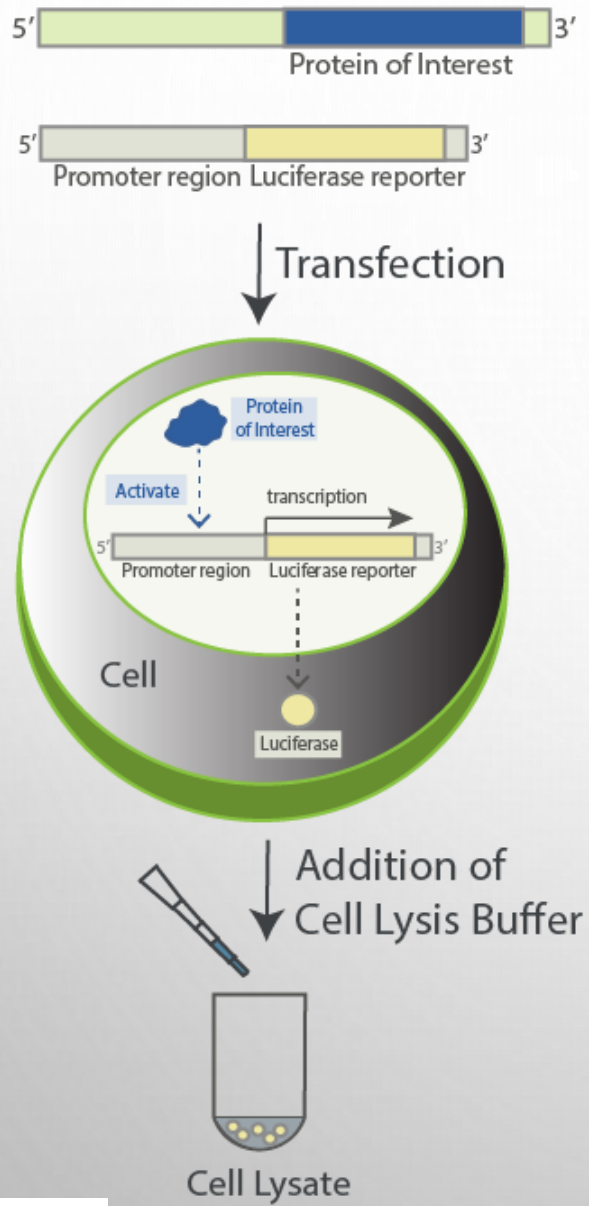
🕒 1/3 of the prize

USA

University of California  
San Diego, CA, USA;  
Howard Hughes Medical Institute

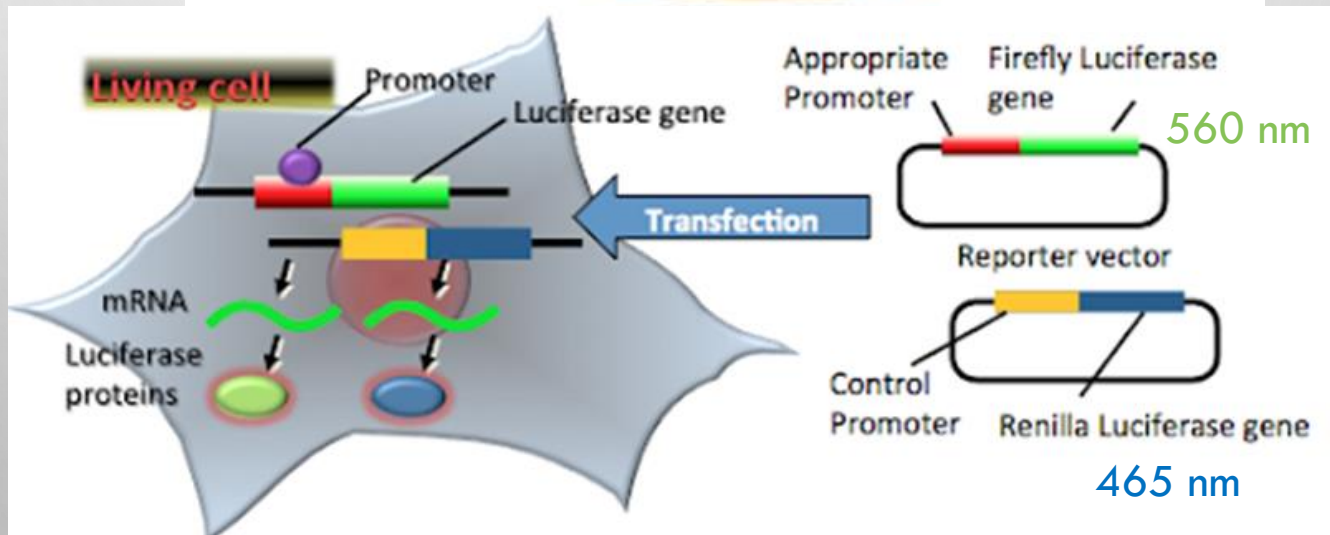
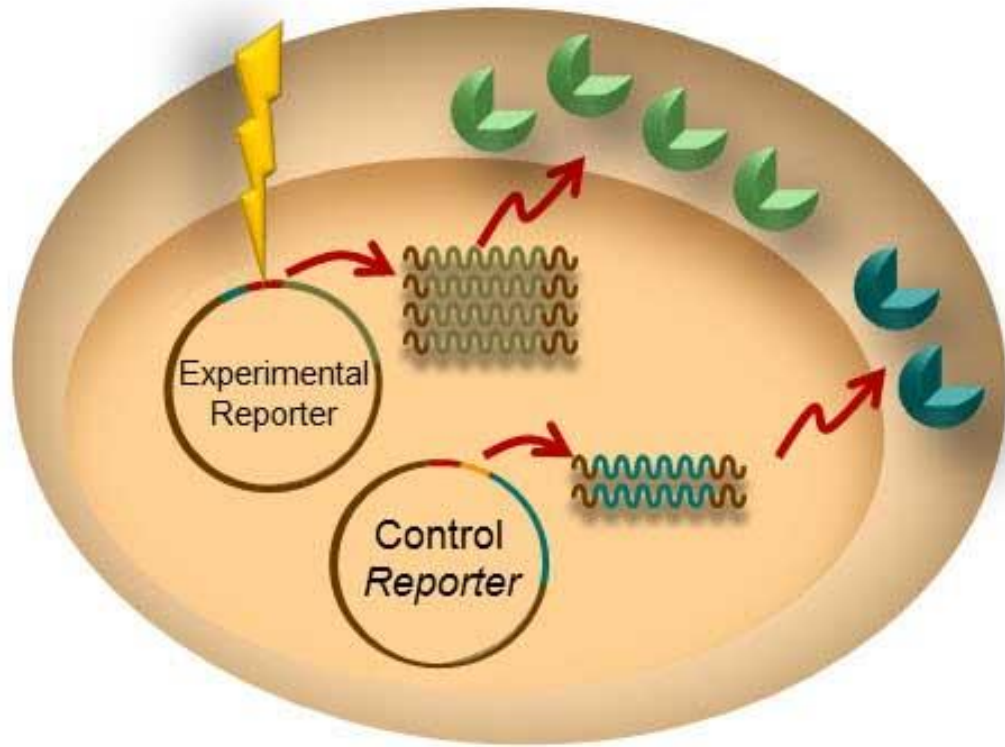
2008年10月8日，瑞典皇家科學院在瑞典首都斯德哥爾摩宣布，日本科學家下村修、美國科學家馬丁·查爾菲和美籍華裔科學家錢永健獲得2008年諾貝爾化學獎。今年得獎的三人代表在「綠色螢光蛋白(GFP)」研究上的三個不同時期。下村脩是這種蛋白的發現者，查爾菲辨識出基因，錢永健則開發出能釋放更強光線、色彩更多樣化的蛋白變形，讓研究者能以不同顏色標示不同蛋白，並看到彼此交互作用。





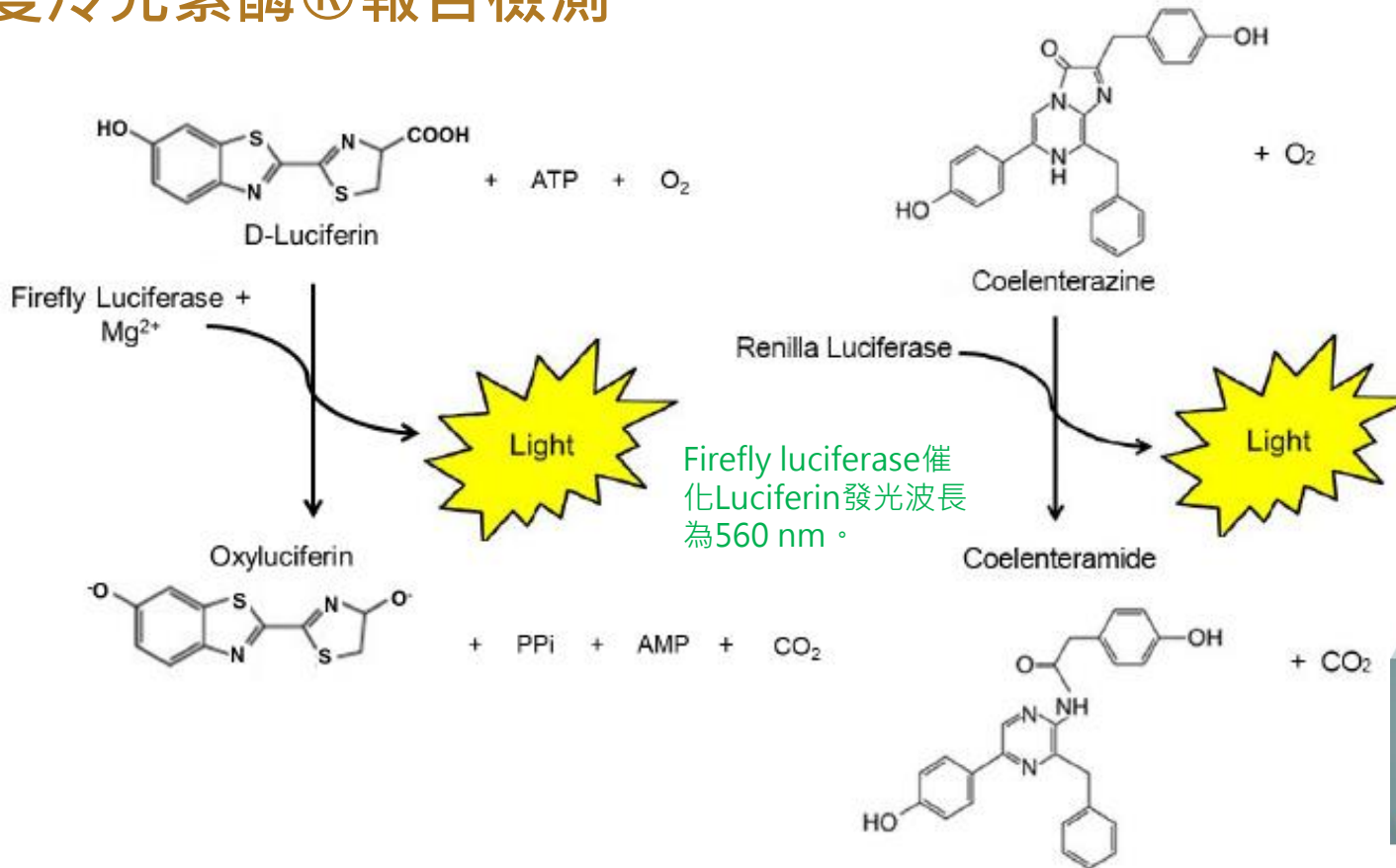
# 雙報告書檢測

TREATMENT



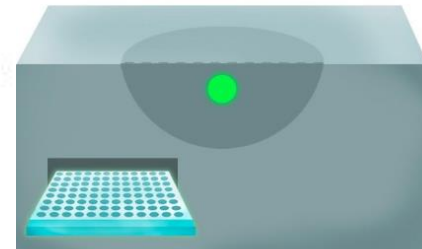


# 雙冷光素酶® 報告檢測

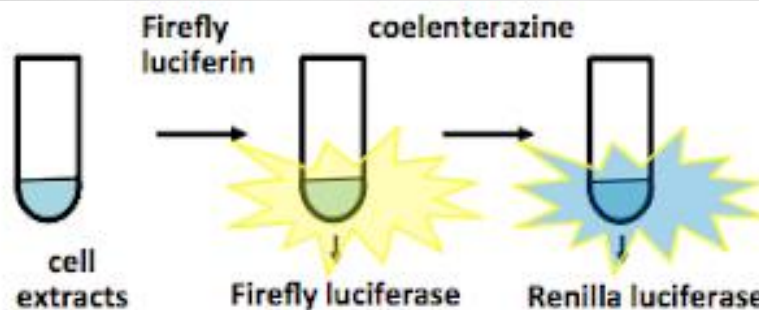


Renilla luciferase作用為校正轉染效率的內參，以消除孔間細胞數量和轉染效率的差異。

Renilla luciferase催化Coelenterazine發光波長為465 nm



Read results on any luminometer



$$\text{Promoter activity} = \frac{\text{Firefly luciferase activity}}{\text{Renilla luciferase activity}}$$

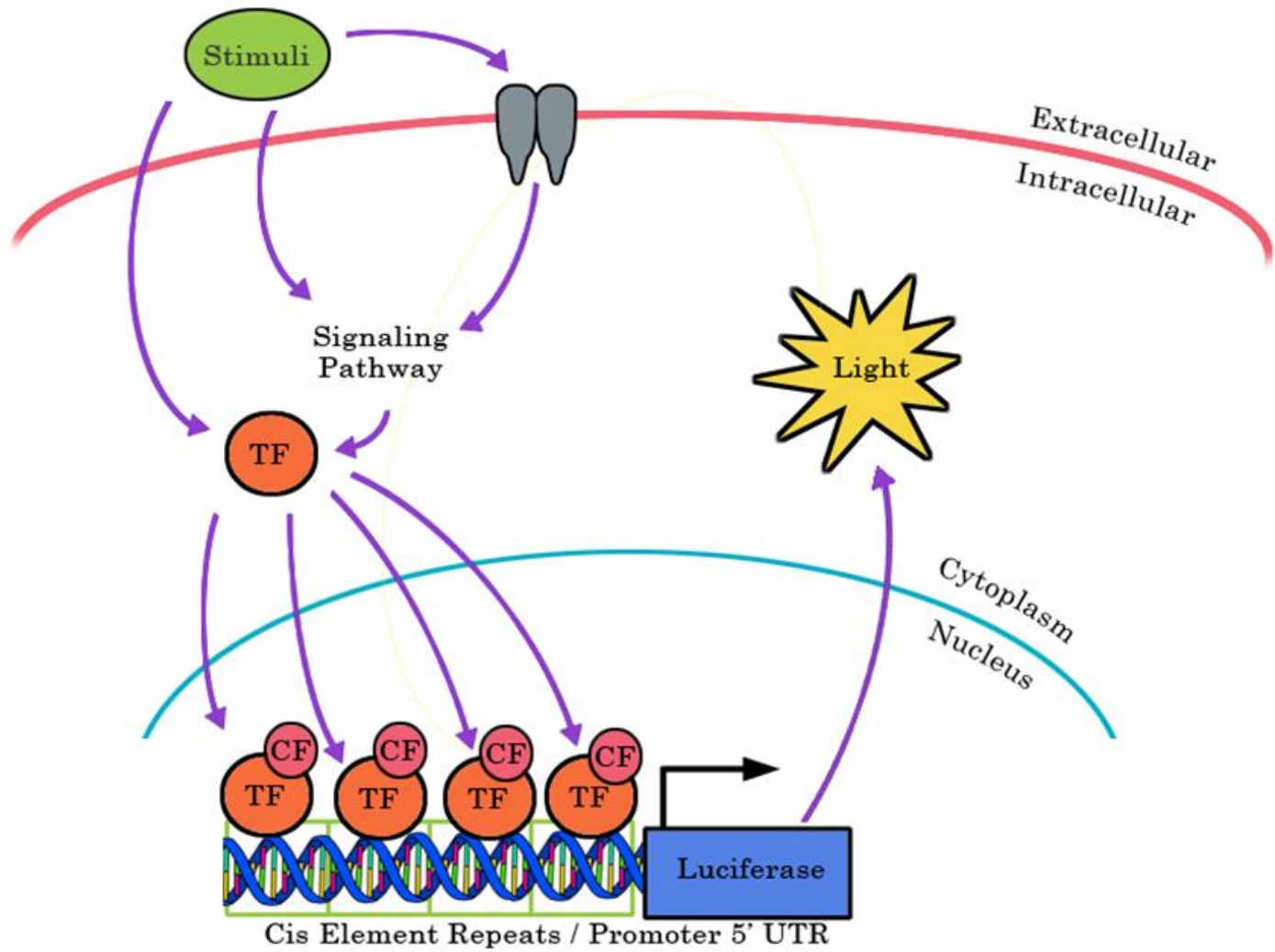




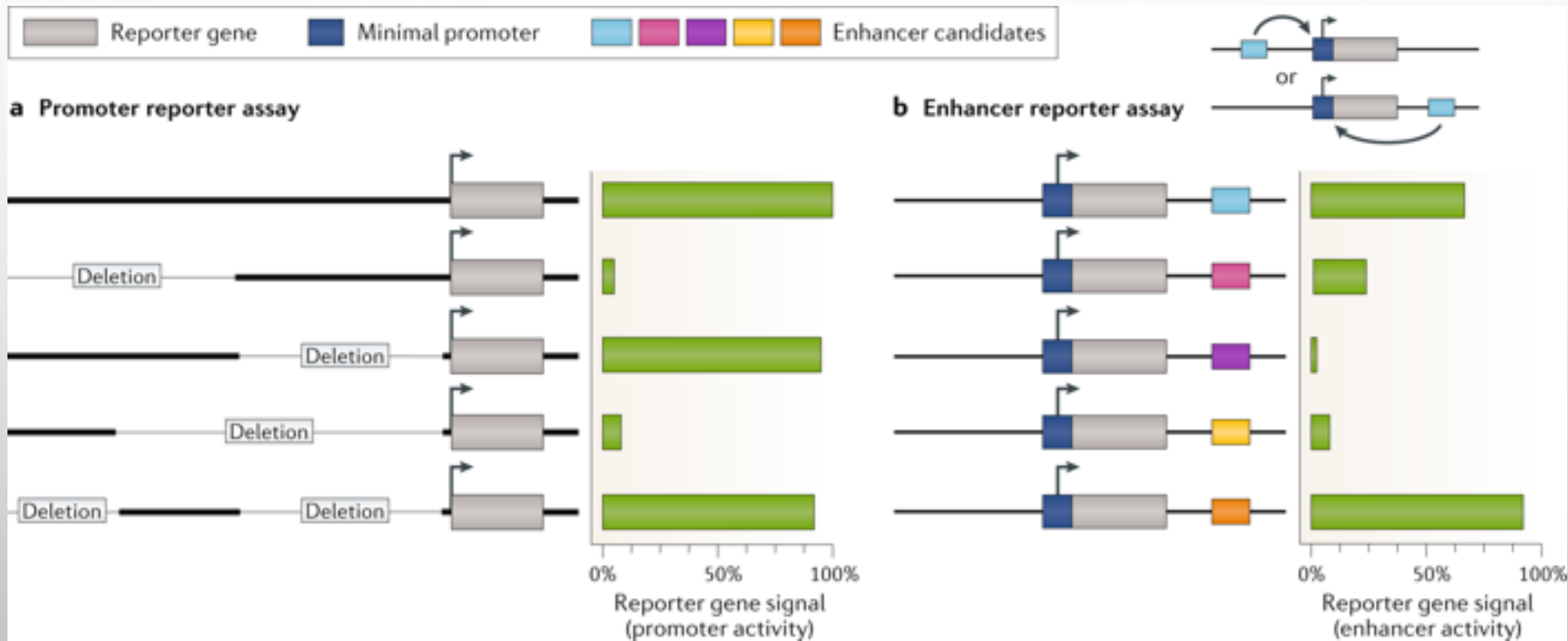
# 報告基因分析的應用

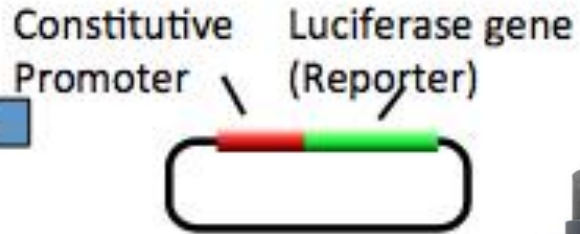
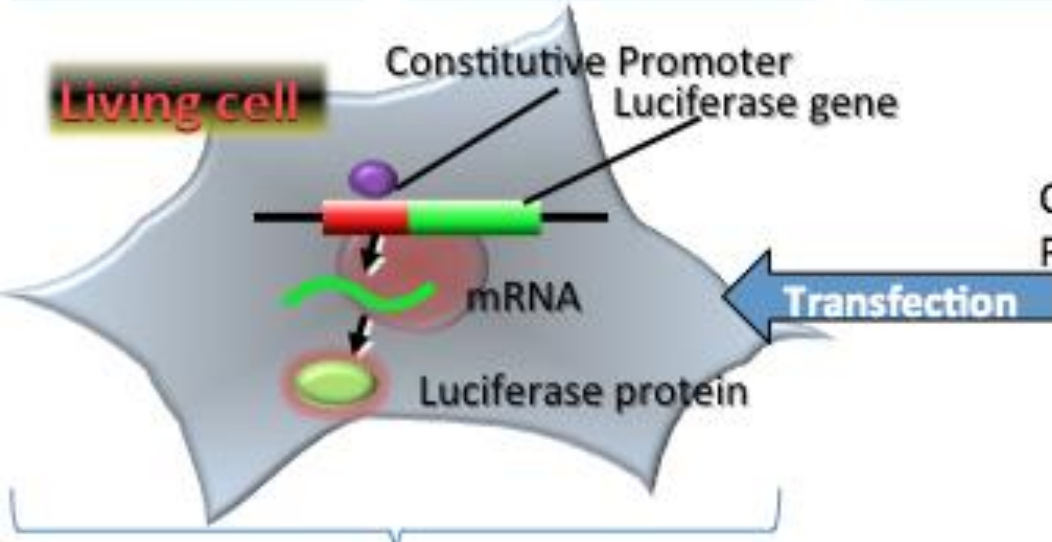
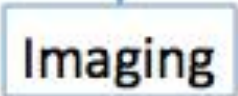
- 分析基因調控
- 分析miRNA靶標
- 分析核受體信號傳導
- 研究蛋白質相互作用
- 高通量篩選

# ( 1 ) 分析基因調控

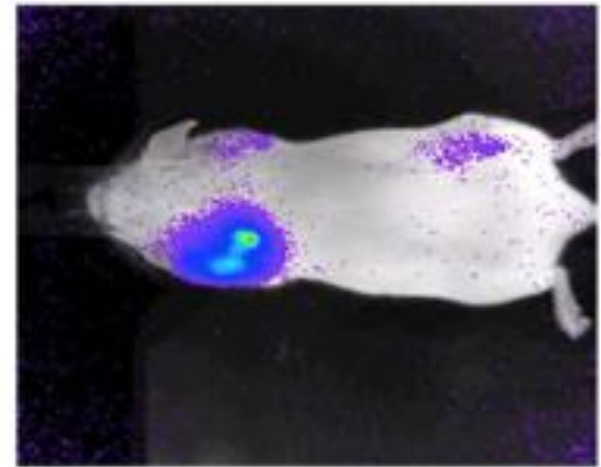
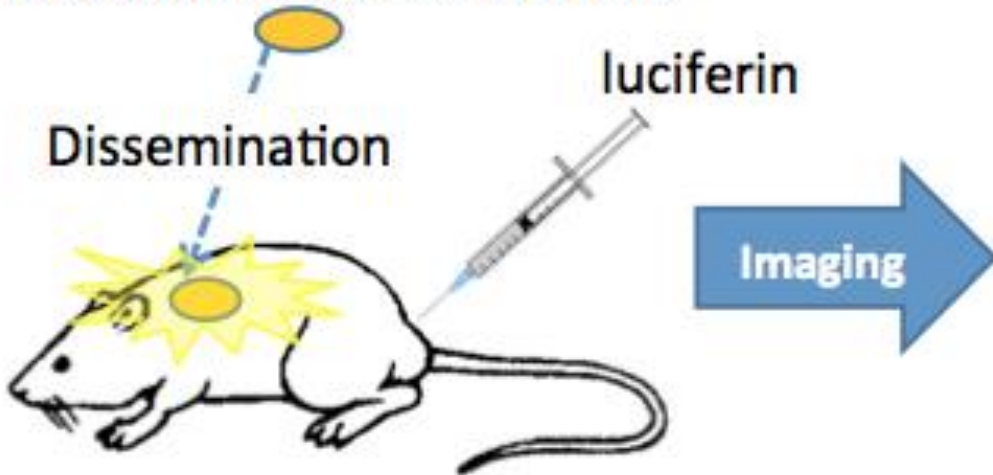


TF = Transcription Factor  
CF = Co-factor





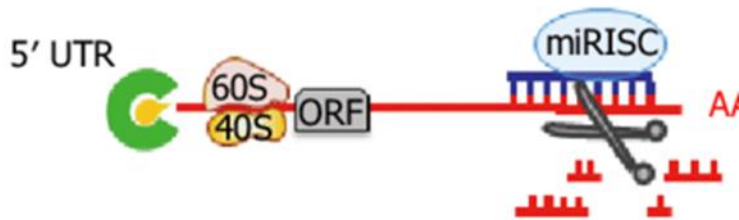
Establish for stable cell line



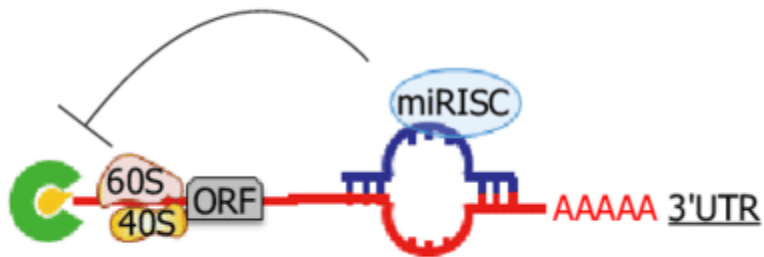
## (2) 分析MiRNA靶標

MicroRNA ( miRNA ) 是短RNA，與轉錄本3' UTR中的靶標相互作用，導致mRNA降解或抑制翻譯。

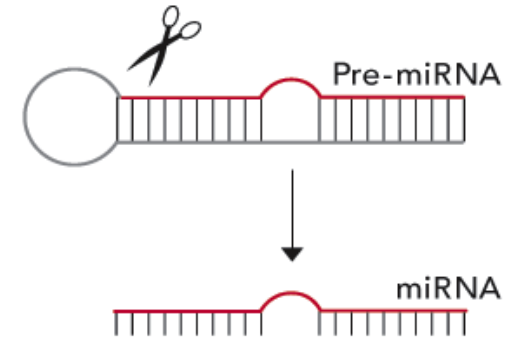
mRNA degradation



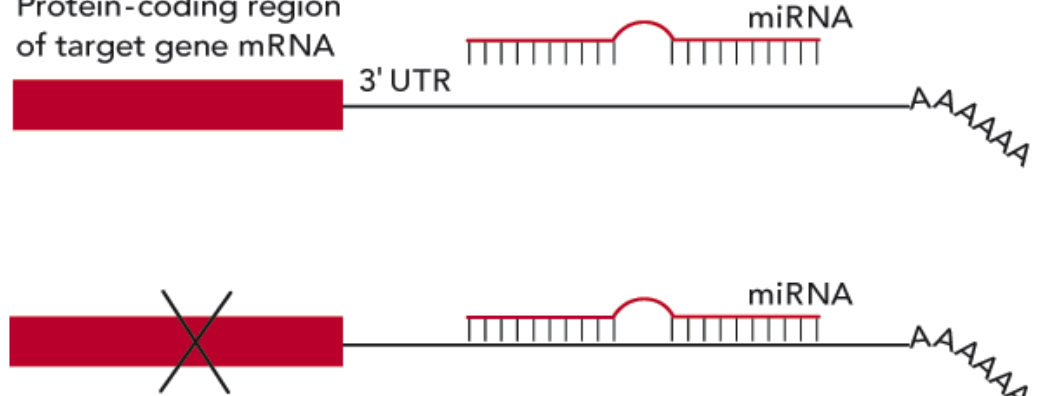
Translation inhibition



DOI:10.4331/wjbc.v8.i1.45



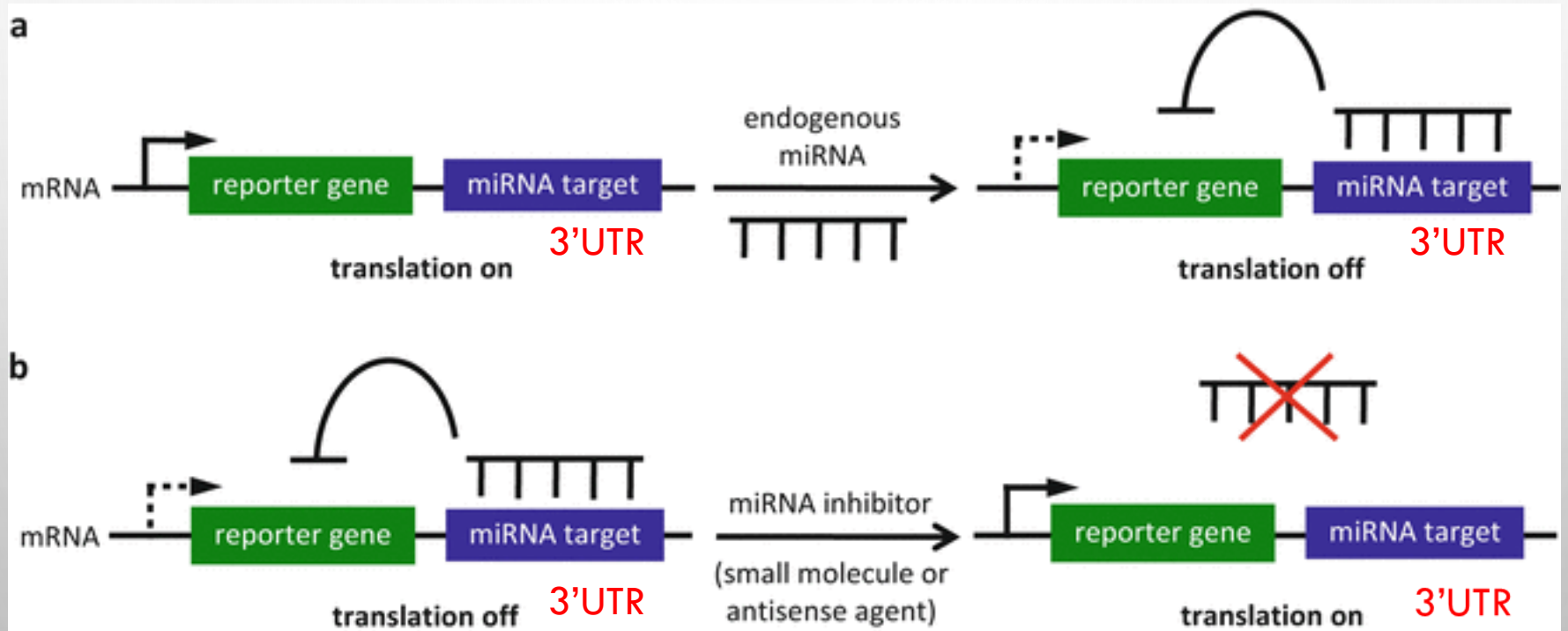
Protein-coding region of target gene mRNA

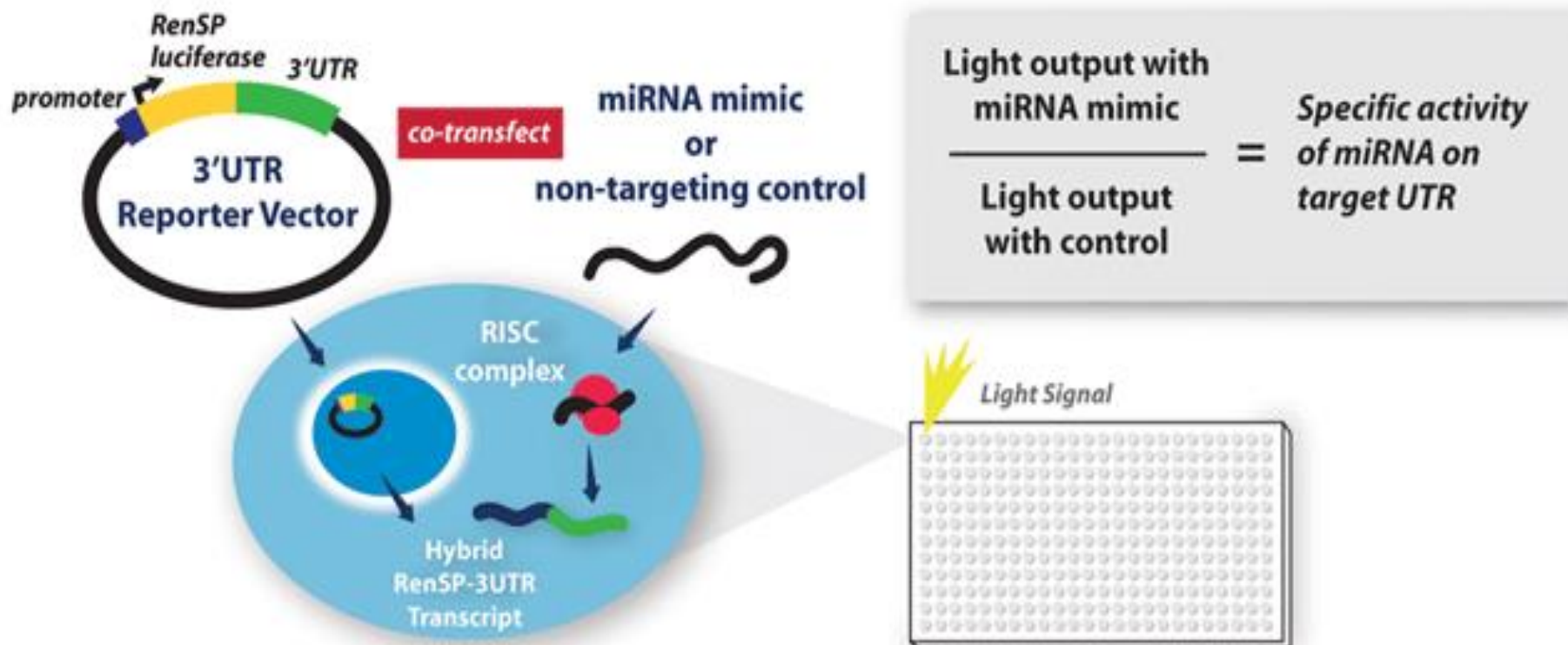


DOI:10.13140/RG.2.1.1422.1926



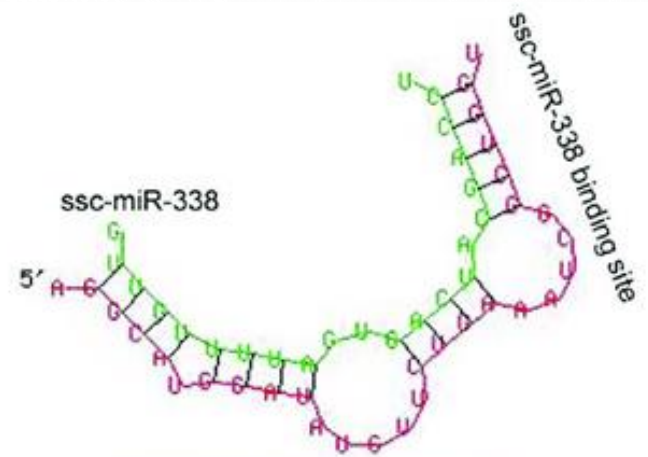
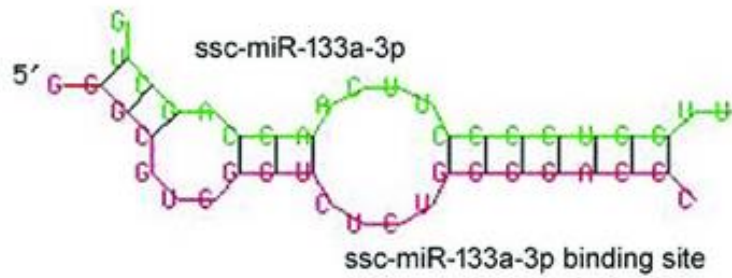
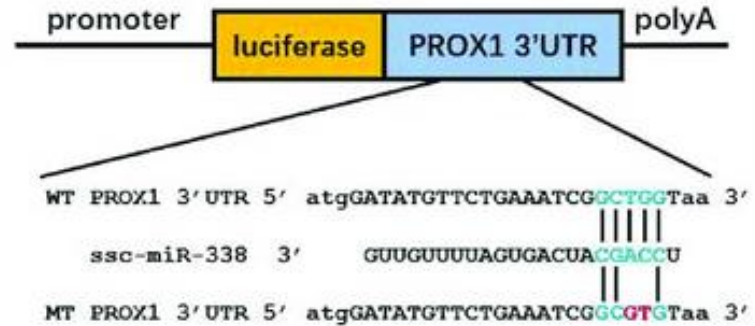
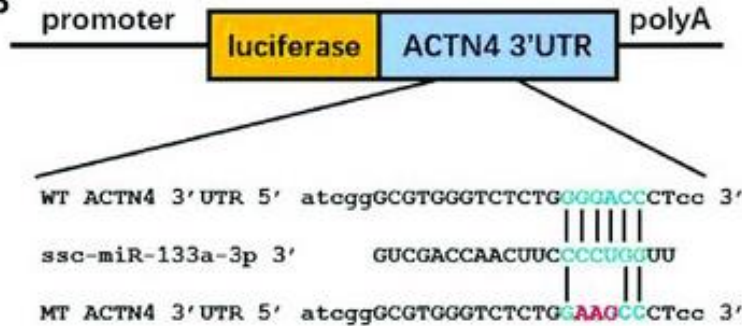
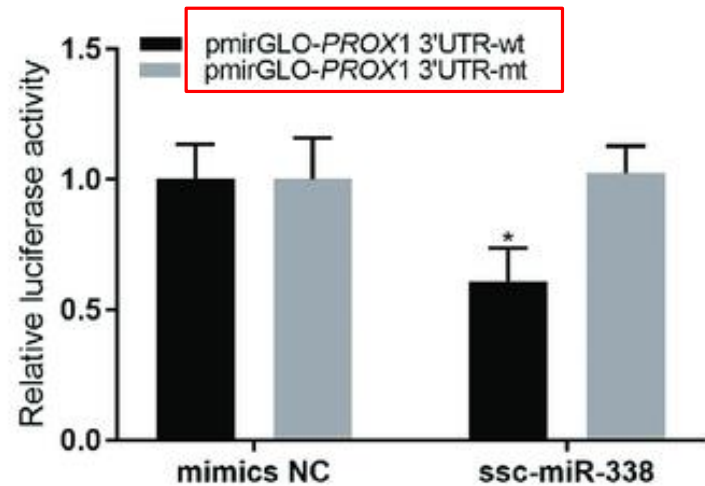
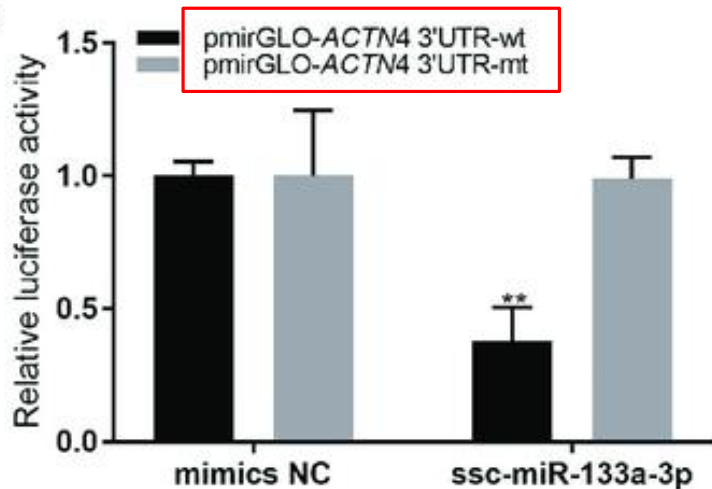
觀察MiRNA介導的效應需要一個在較弱的啟動子的控制下的報告者，以便可以觀察到基因表達的細微變化。



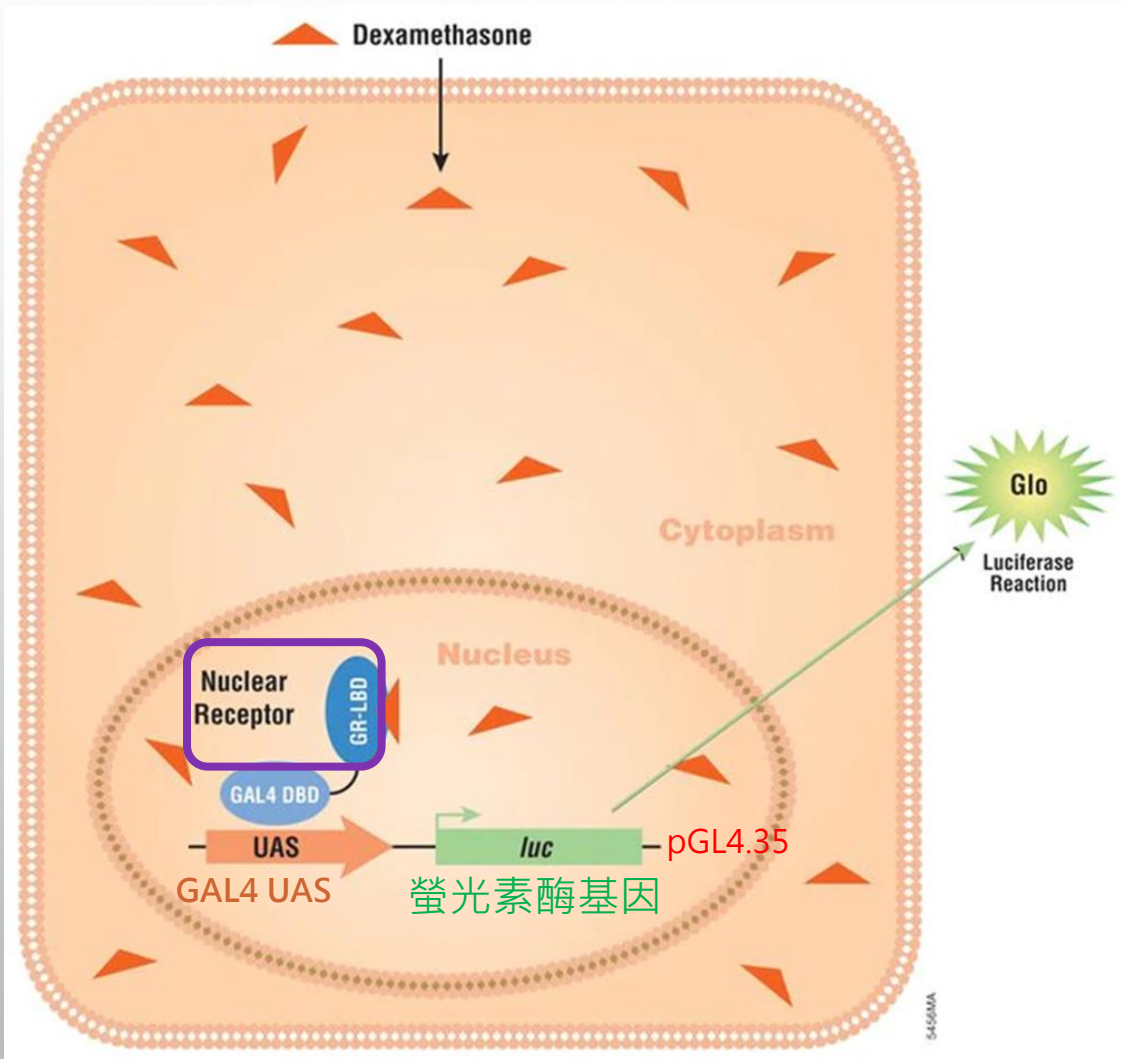
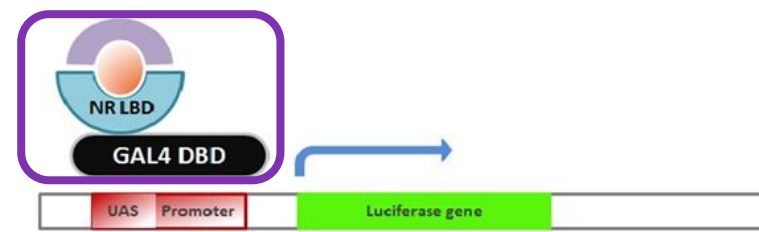
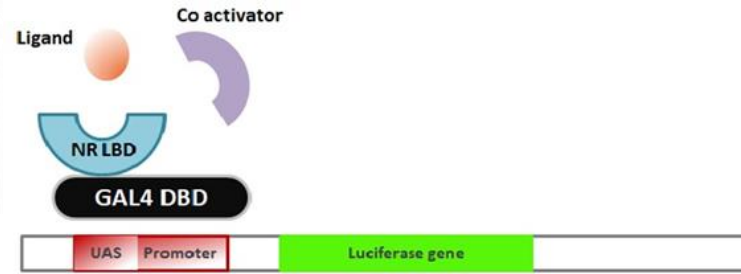


$$\frac{\text{Light output with miRNA mimic}}{\text{Light output with control}} = \text{Specific activity of miRNA on target UTR}$$

1. Transfect the GoClone 3'UTR construct into your cell line
2. A constitutive promoter drives production of a hybrid RenSP-human 3'UTR transcript
3. Total luciferase output depends on the effect of the human 3'UTR on the hybrid transcripts stability and/or translation efficiency
4. Include a co-transfected synthetic miRNA or miRNA inhibitor to measure its impact on transcript stability and/or translation efficiency
5. Use optimized LightSwitch Luciferase Reagents to obtain maximum sensitivity and dynamic range

**A****B****C**

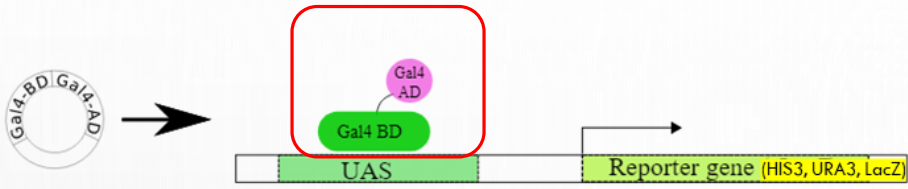
### ( 3 ) 分析核受體信號傳導



- 關鍵是一個優化的PGL4載體，其中包含9個重複的**GAL4 UAS**，一個最小的啟動子和**LUC2P**螢光素酶基因（**PGL4.35**）。
- 製備兩個含有**雌激素受體配體結合域**（PBIND-ER $\alpha$ ）或**糖皮質激素受體配體結合域**（PBIND-GR）的**PFN26A**載體。
- 用載體、培養和檢測轉染細胞。



# (4) 研究蛋白質相互作用



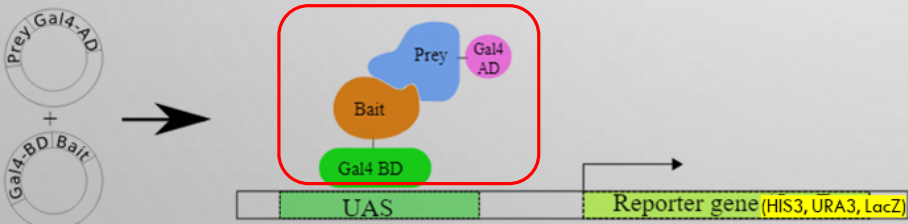
A. Regular transcription of the reporter gene



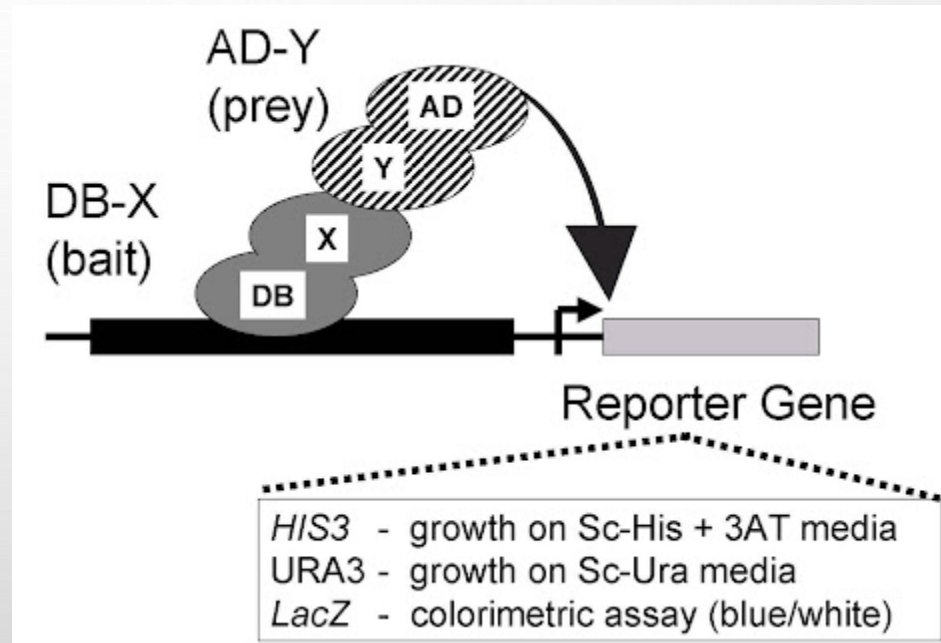
B. One fusion protein only (Gal4-BD + Bait) - no transcription



C. One fusion protein only (Gal4-AD + Prey) - no transcription

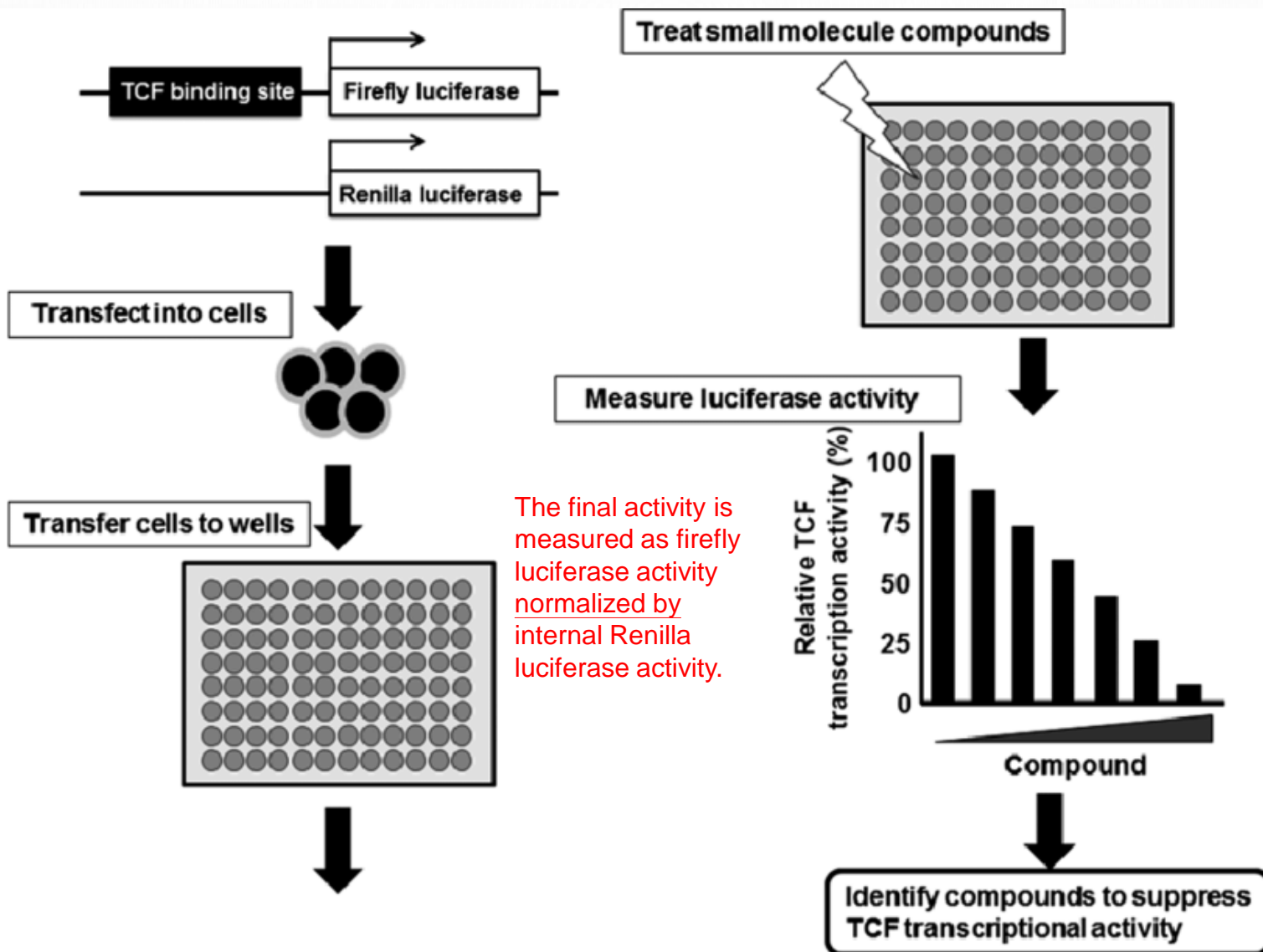


D. Two fusion proteins with interacting Bait and Prey





## ( 5 ) 高通量篩選







# III. 核糖核酸干擾 (RNAi)

# 上課內容

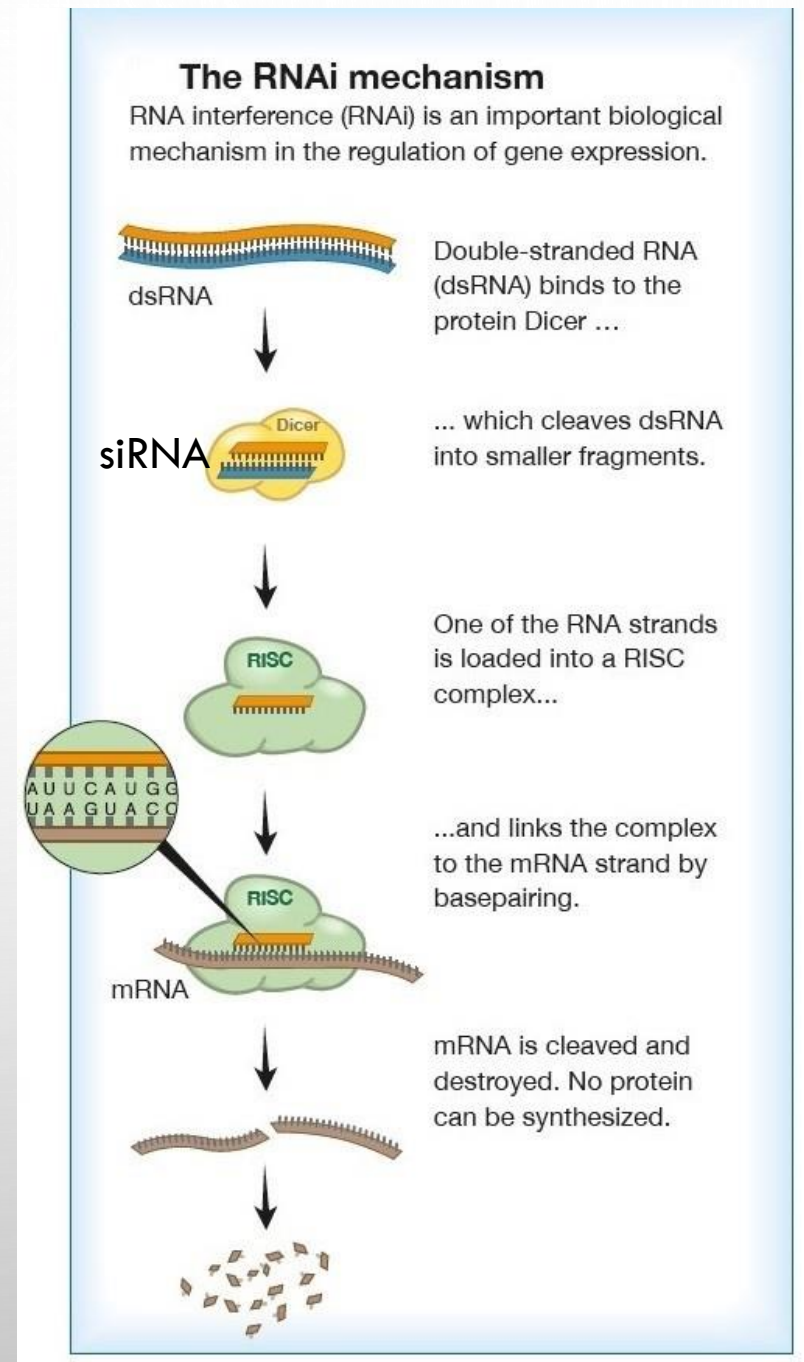
- RNAi原理
- 誰發現了RNAi
- RNAi技術
- RNAi的應用



# RNAi原理

- 雙鏈RNA ( dsRNA ) 由RNase III家族成員Dicer處理，產生21-23nt小干擾RNA。
- siRNA由稱為RNA誘導的沉默複合物 ( RISC )的多組分核酸酶操縱。

siRNA: small interfering RNA



# 誰發現了RNAi

2006 Nobel Prize in Medicine or Physiology

## RNA Interference

NATURE | VOL 391 | 19 FEBRUARY 1998

克雷格·梅洛

安德魯·菲爾

**Potent and specific  
genetic interference by  
double-stranded RNA in  
*Caenorhabditis elegans***

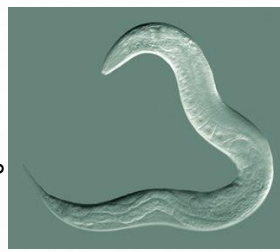
Andrew Fire<sup>\*</sup>, SiQun Xu<sup>+</sup>, Mary K. Montgomery<sup>+</sup>,  
Steven A. Kostas<sup>++</sup>, Samuel E. Driver<sup>‡</sup> & Craig C. Mello<sup>‡</sup>

A naturally occurring mechanism

Destruction of mRNA results in the post transcriptional inhibition of gene expression and the prevention of protein synthesis.



# 動物模型-線蟲



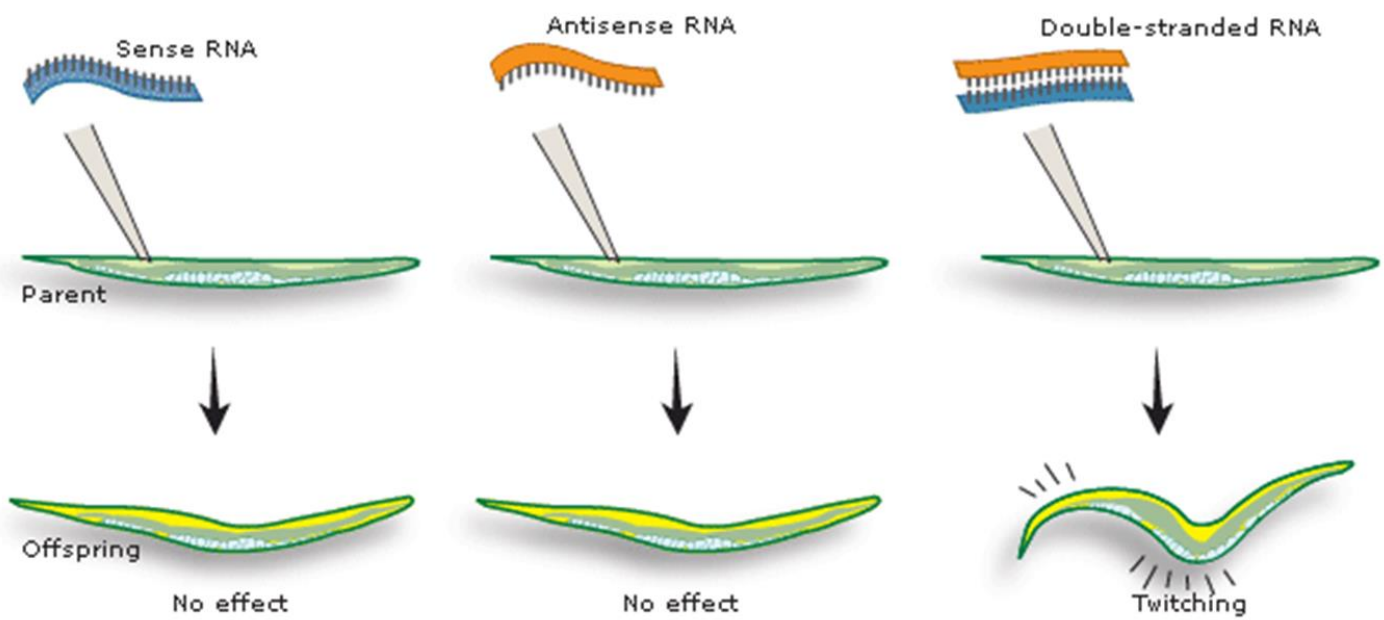
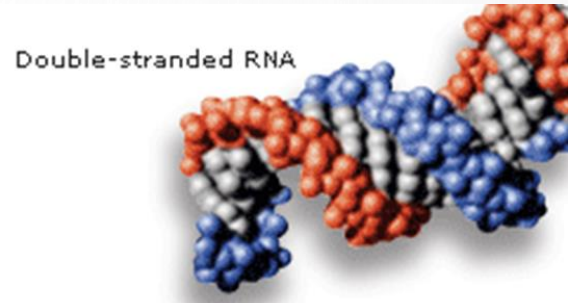
- RNAi最初被發現於秀麗隱桿線蟲 (*Caenorhabditis elegans*, *C. elegans*)。
- 觀察到雙鏈RNA ( dsRNA ) 在沉默標靶基因表達方面比單獨使用反股RNA (anti-sense RNA)有效10倍。

## Key experiments

### Gene silencing

Fire and Mello injected RNA corresponding to a gene important for muscle function in the worm *C. elegans*.

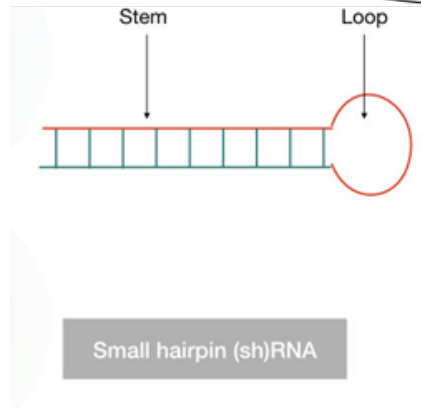
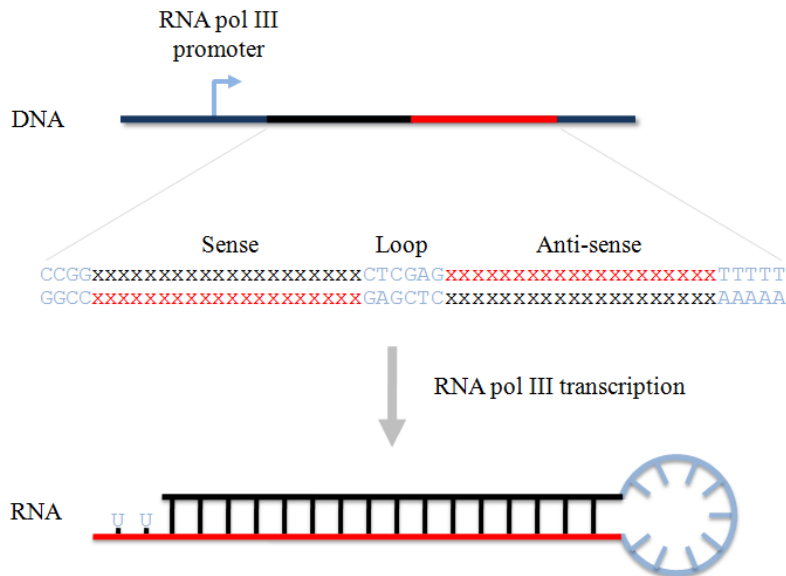
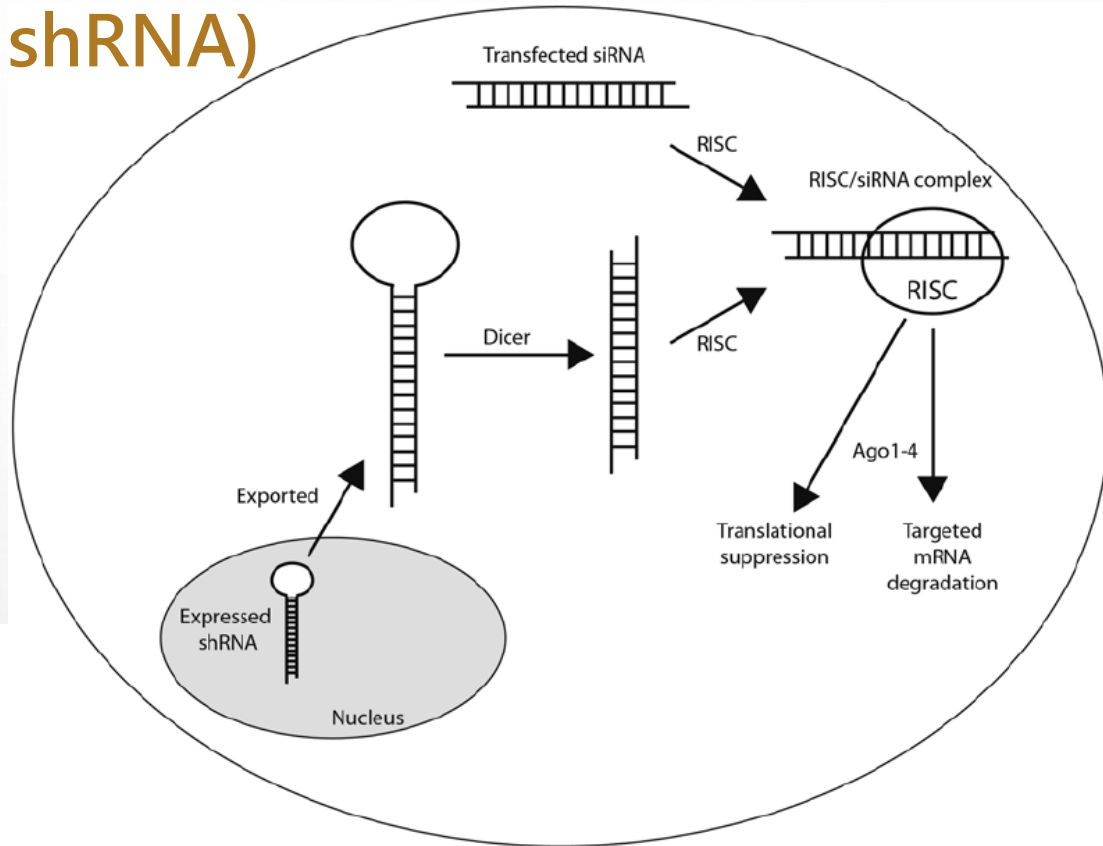
Single-stranded RNA (sense or antisense) had no effect. But double-stranded RNA caused the worm to twitch in a similar way to worms that lack a functional gene for the muscle protein.



# 小髮夾RNA (short hairpin RNA, shRNA)

19–29 個核苷酸

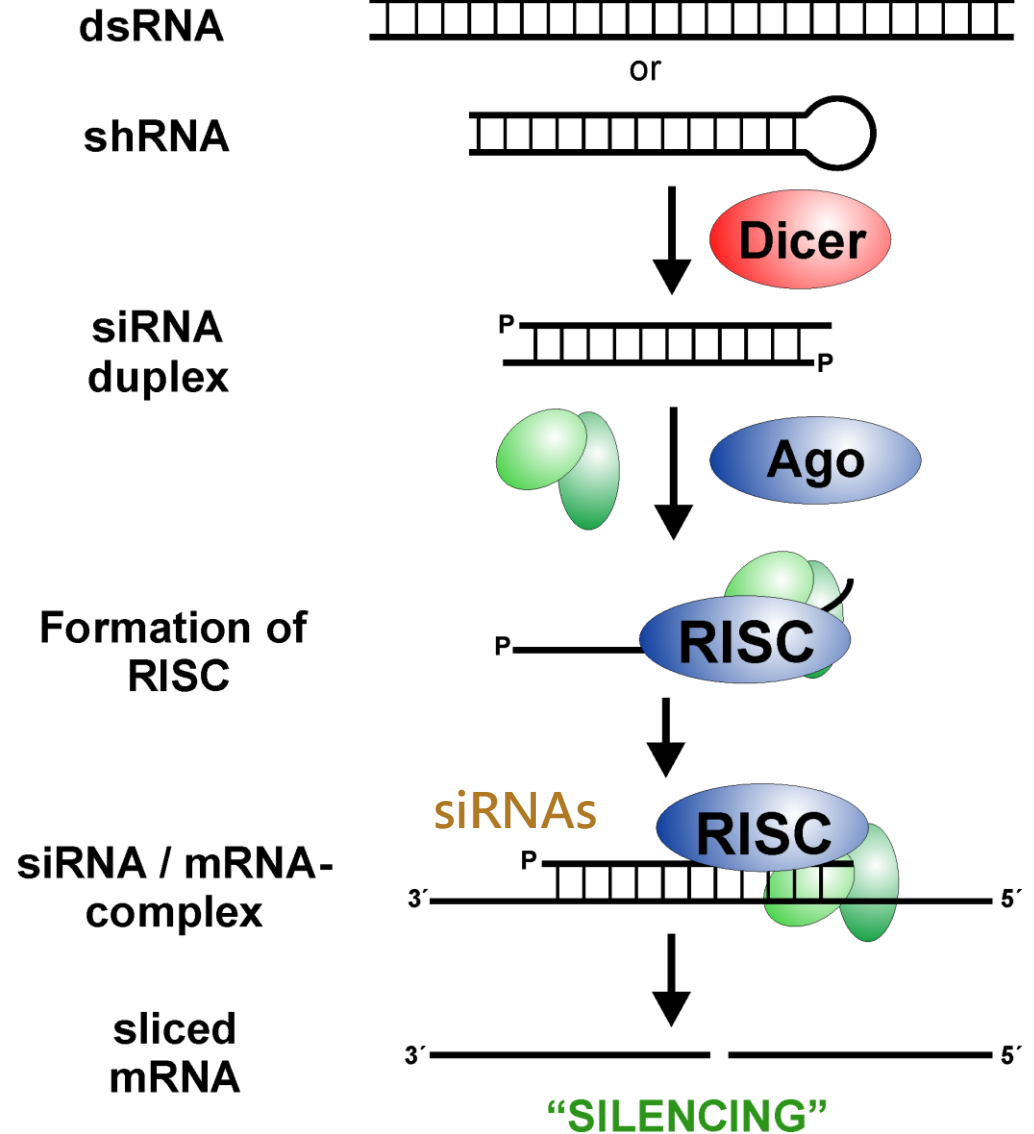
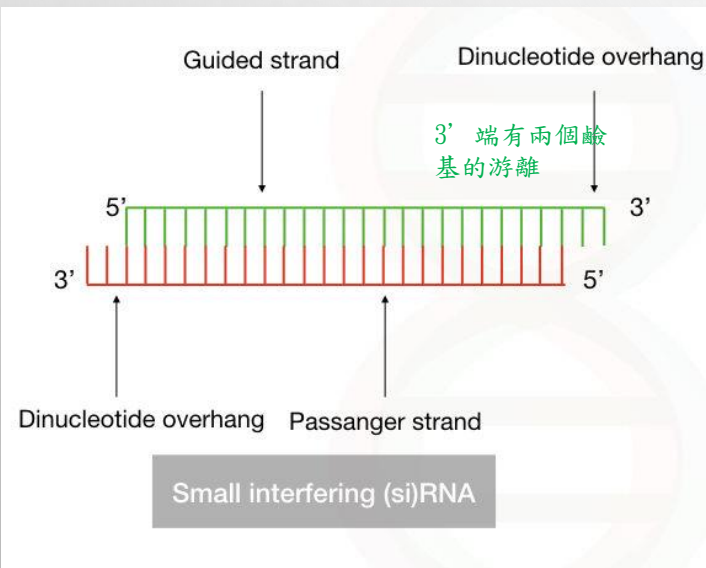
短髮夾 RNA (shRNA) 包含一個環結構，該環結構被加工成 siRNA，並且還導致 mRNA 以依賴於靶 mRNA 的互補結合的序列特異性方式降解。





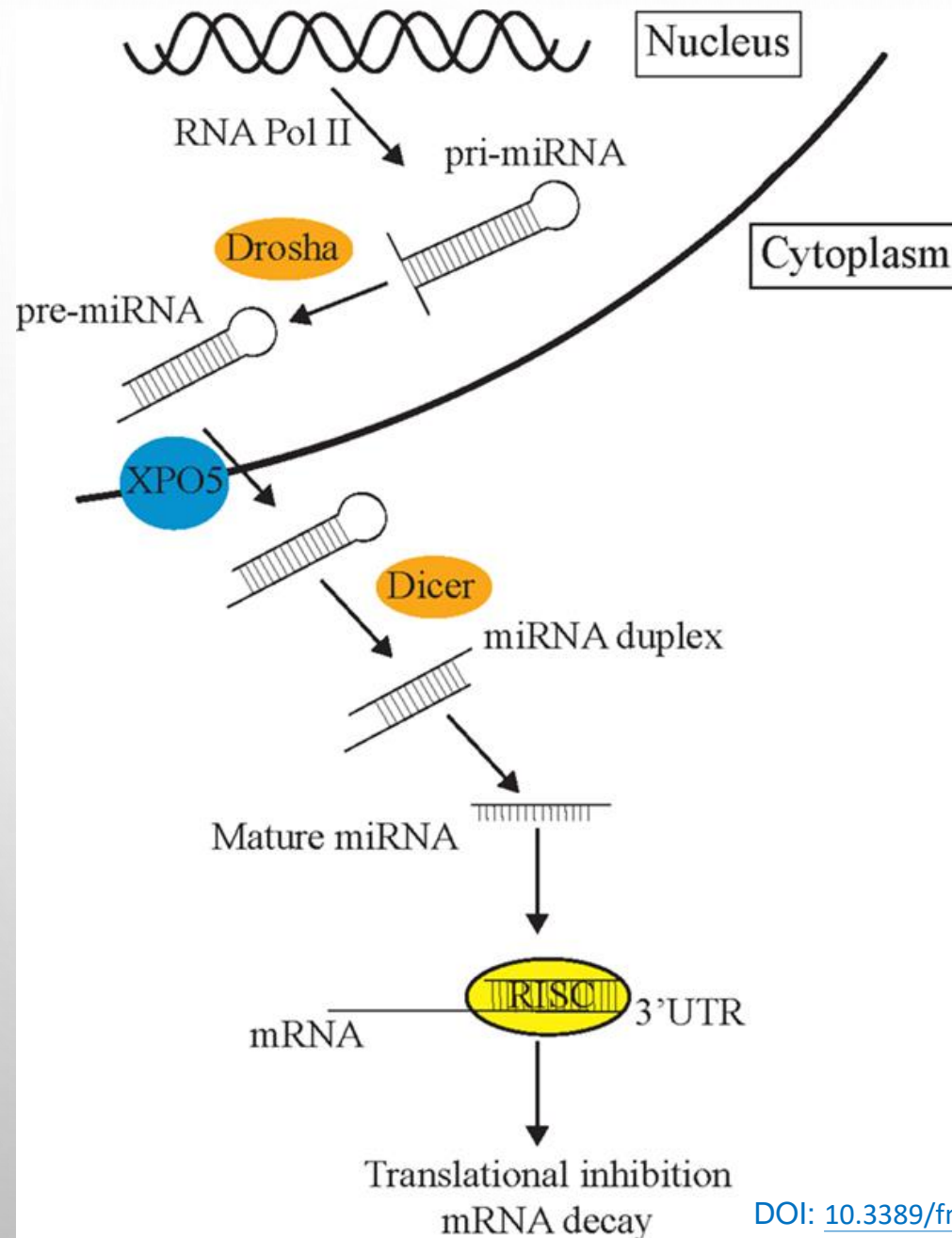
# 小干擾 RNA (small interfering RNAs, siRNAs)

小干擾 RNA (siRNA)。具有 20-25 個核苷酸，3' 末端突出的 siRNA 可激活 RNAi，導致 mRNA 以依賴於靶 mRNA 的互補結合的序列特異性方式降解。



# 小分子核糖核酸 ( microRNA, miRNA)

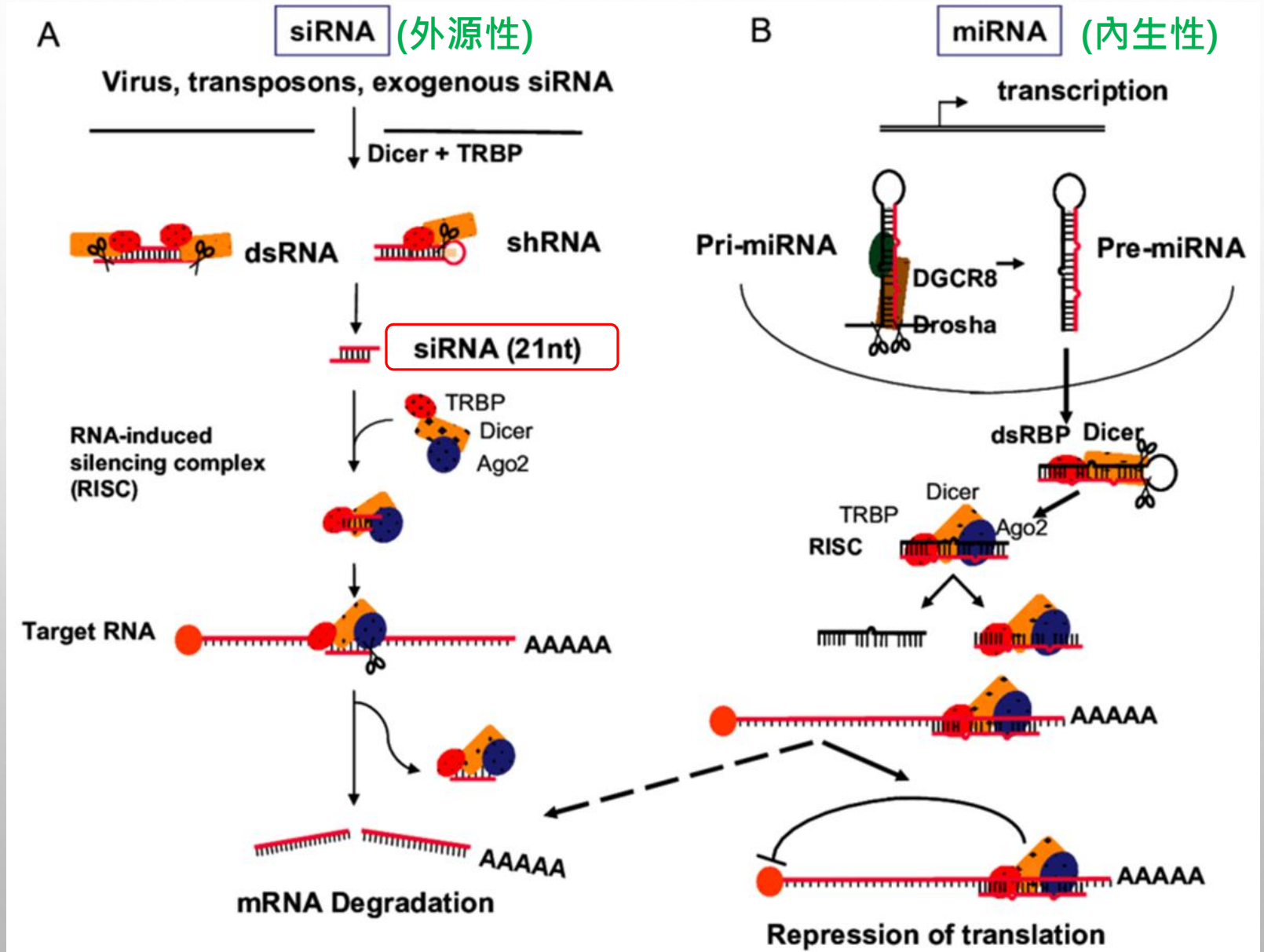
小分子核糖核酸 ( miRNA )，是真核生物中廣泛存在的一種長約19-25個核苷酸的RNA分子，可調節其他基因的表現



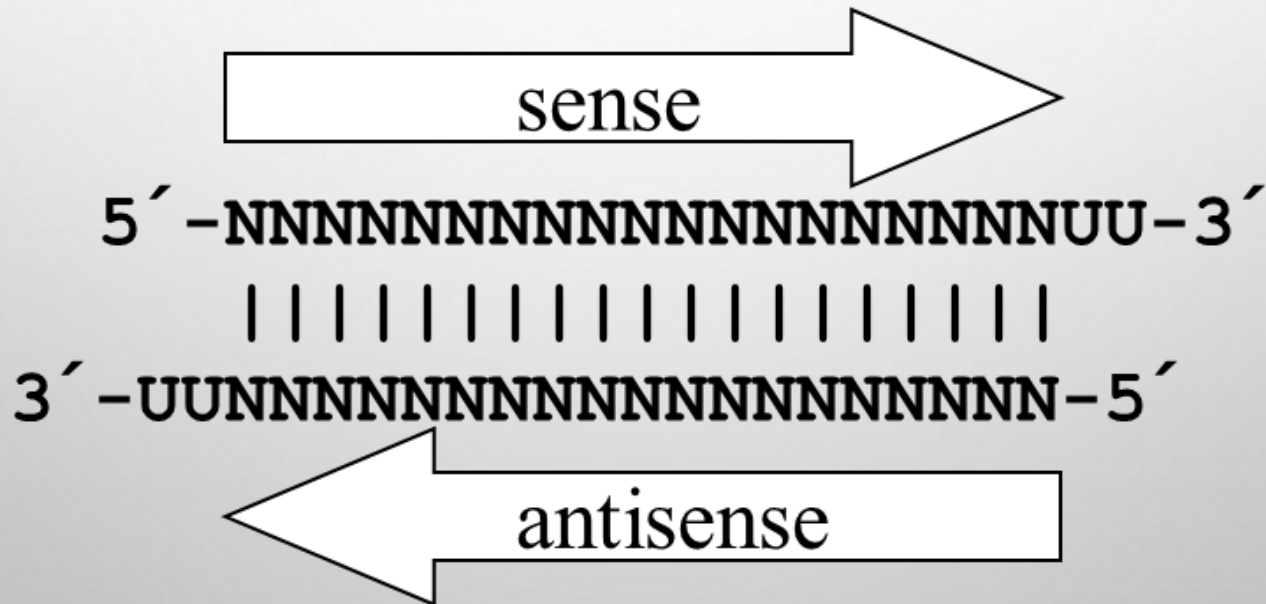
miRNA主要作用於靶標基因3'-UTR區，而siRNA可作用於mRNA的任何部位。

# RNAi相關技術

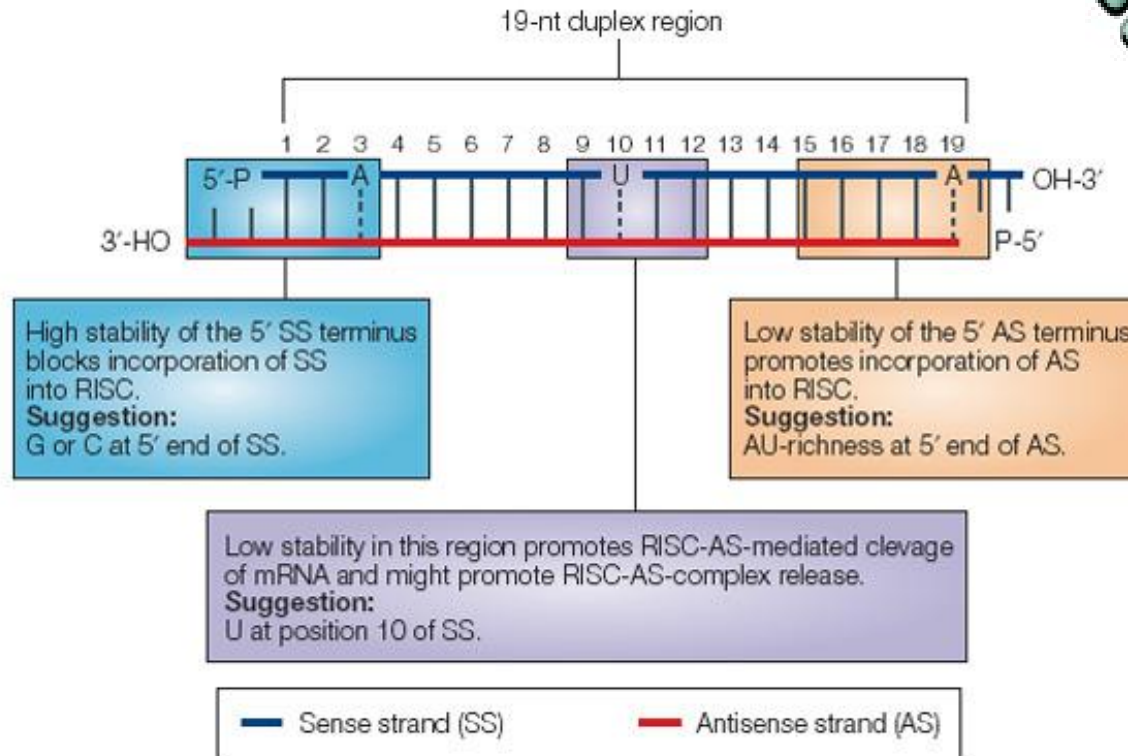
## 1. siRNA化學合成



- 靶向任何序列的合成siRNA可以通過化學合成製備
- 在哺乳動物細胞中，siRNA在敲低靶基因表達方面的有效性範圍 ( 50-95% )
- siRNA的有效性取決於標靶序列







1. 低至中等GC含量 ( 30-50% ) 。
2. 無內部重複或回文 。
3. 在感應鏈的位置 3 處存在 A 。
4. 在感應鏈的位置 19 處存在 A 。
5. 在感測鏈的位置 19 處沒有 G 或 C 。
6. 在感應線的位置 10 處存在 U 。
7. 感應鏈位置 13 處沒有 G 。
8. 在感應鏈的 15-19 位置至少有 3 個 A/U 。

**Table 2.** Names and addresses of siRNA design computational soft wares

Soft wares	Address
siDirect	<a href="http://genomics.jp/sidirect/index.php?type=fc">http://genomics.jp/sidirect/index.php?type=fc</a>
siDESIGN Center	<a href="http://dharmacon.gelifesciences.com/design-center">dharmacon.gelifesciences.com/design-center</a>
siRNA Design Software	<a href="http://www.genscript.com/ssl-bin/app/rnai">http://www.genscript.com/ssl-bin/app/rnai</a>
Block-iT RNAi Designer	<a href="https://rnaidesigner.invitrogen.com">https://rnaidesigner.invitrogen.com</a>
siRNA Target Finder	<a href="http://www.ambion.com/techlib/misc/siRNA_finder.html">http://www.ambion.com/techlib/misc/siRNA_finder.html</a>
RNAi explorer	<a href="http://www.genelink.com/sirna/siRNAorder.asp">www.genelink.com/sirna/siRNAorder.asp</a>

# 靶向miRNA活性的不同方法

(內生性)

(外源性)

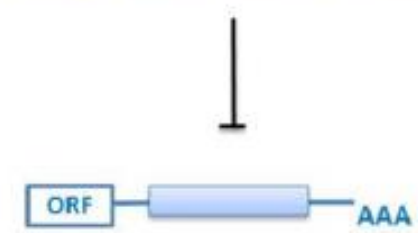
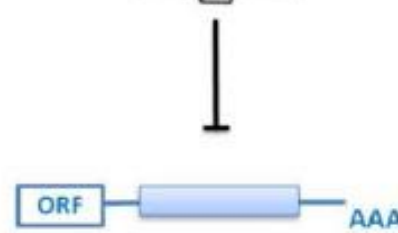
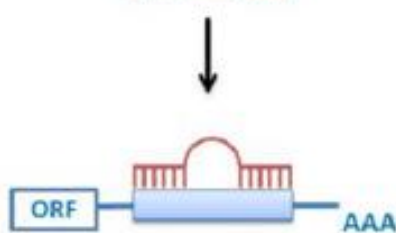
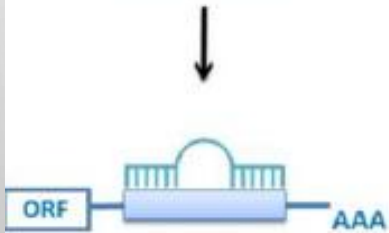
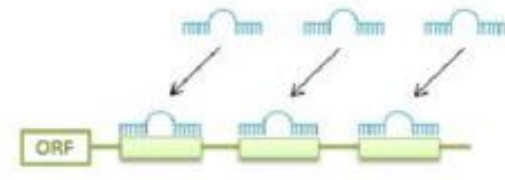
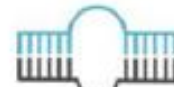
(外源性)

1. endogenous miRNA

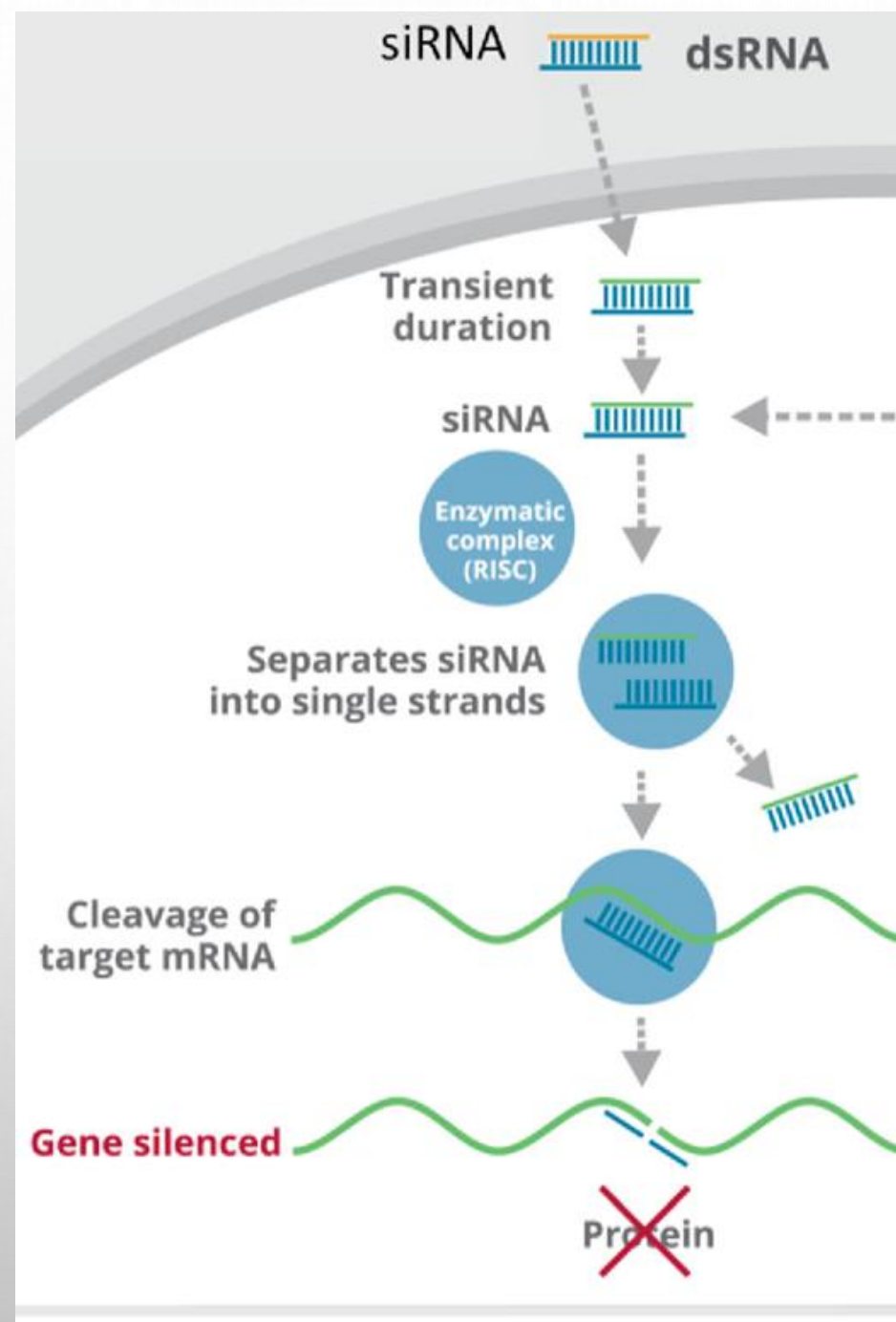
2. miRNA mimic

3. antagomiR

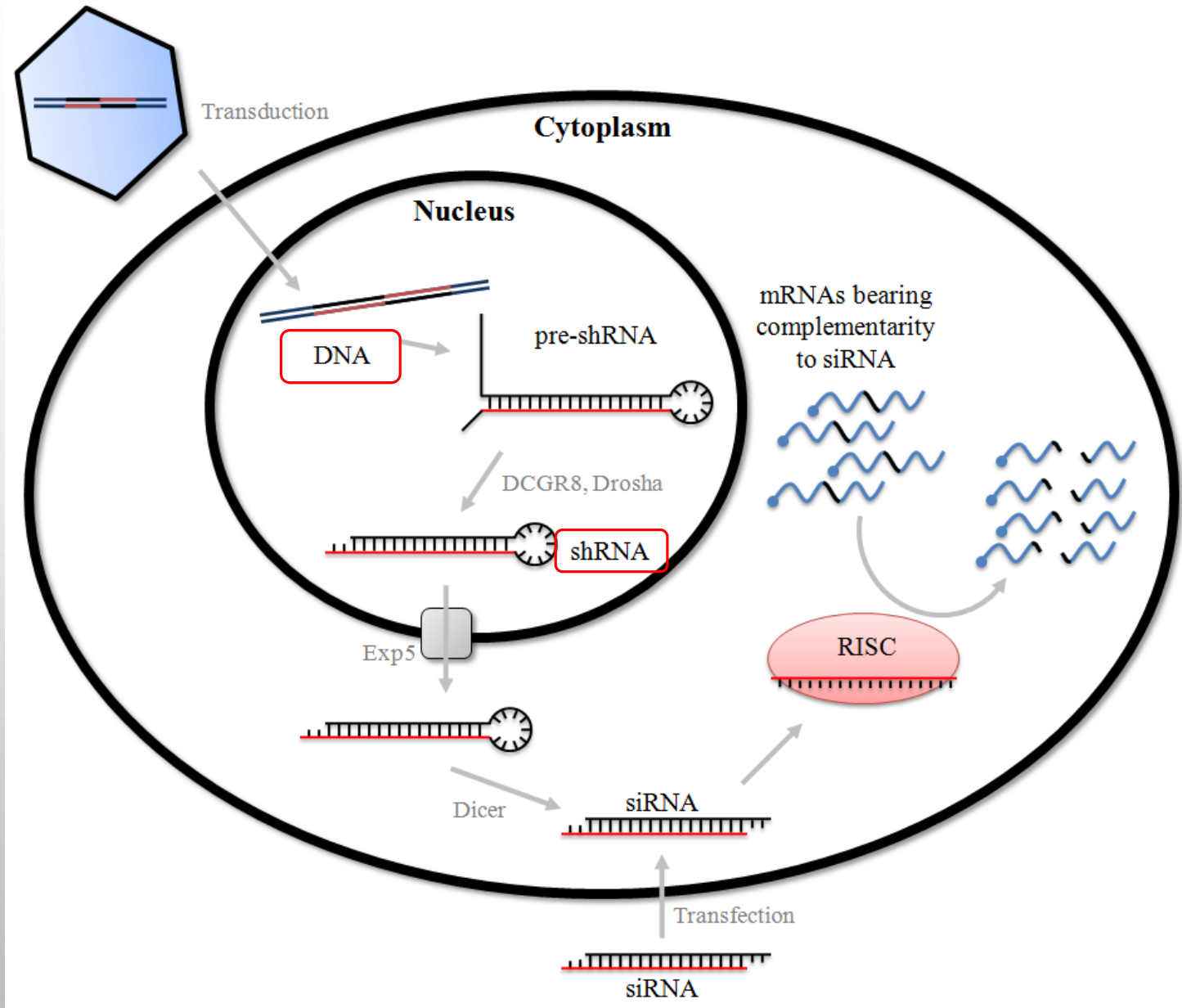
4. miRNA sponge



## 2. siRNA轉染



### 3. shRNA轉染







## 線上回報 knockdown feedback information



免費獲贈 shRNA glycerol stock



會員登入

Account

Password



帳號申請

Login

C6-1 shRNA質體菌株分讓

→ 訂購 control shRNA質體菌株

**Bacteria**



C6-2 盤式VSV-G pseudotyped lentivirus

C6-3 混合型VSV-G pseudotyped lentivirus

C6-4 單株VSV-G pseudotyped lentivirus

C6-9 客製化VSV-G pseudotyped lentivirus

C6-10 Genome-wide pooled lentivirus

C6-13 shRNA細菌株到病毒或DNA製備服務

**Virus**

◆ 服務項目清單及收費準則

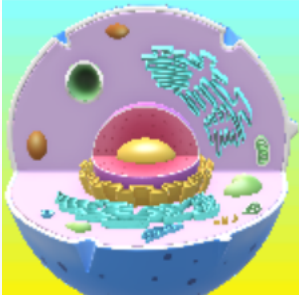
核心公告 new

C6-22 AAV 客製化服務說明 +new+

2019/11/26

108年12月停止取件服務

2019/11/1



# 新服務其他客製化服務

服務項目訂購(核心服務項目&收費準則查詢)

會員登入: 帳號  密碼   申請會員

- [C6-1](#)
- [C6-2](#)
- [C6-3](#)
- [C6-4](#)
- [C6-5](#)
- [C6-6](#)
- [C6-7](#)
- [C6-8](#)
- [C6-9](#)
- [C6-10](#)
- [C6-11](#)
- [C6-12](#)
- [C6-13](#)
- [C6-14](#)
- [C6-15](#)
- [C6-16](#)
- [C6-17](#)
- [C6-18](#)
- [C6-19](#)
- [C6-20](#)
- [C6-21](#)

shRNA 質體細菌株分讓 (點選可展開) [ [產品簡介](#) ] [ [使用須知](#) ] [ [技術支援](#) ]

## shRNA 關鍵字查詢

可利用 ClonId / Symbol / targetSeq 作查詢

Search [Entrez Gene](#) to find official symbols.

TRC1 vector



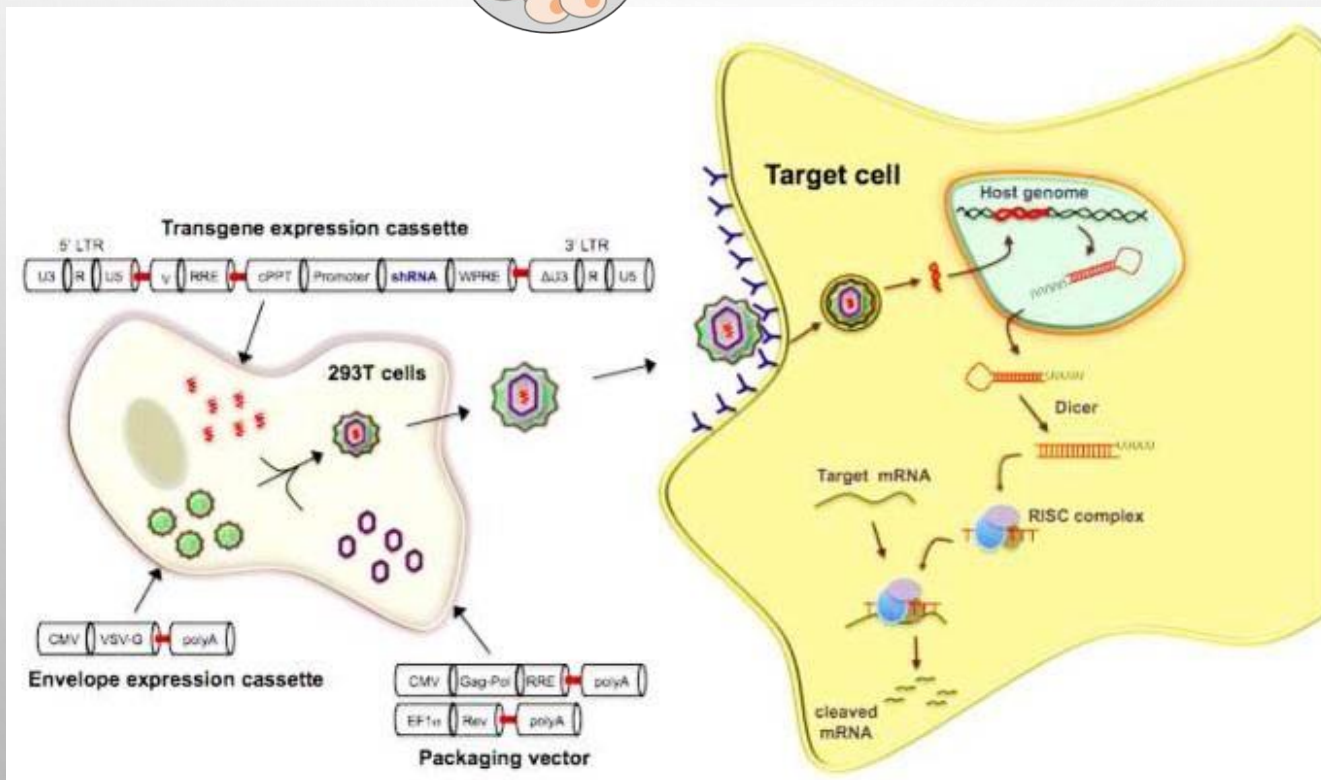
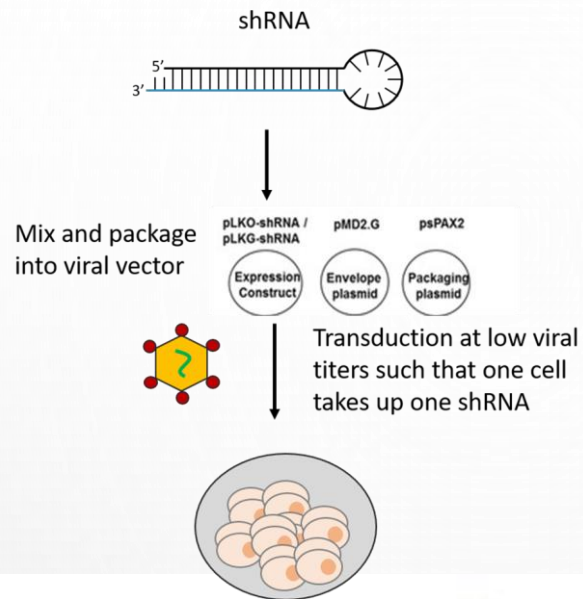
TRC2 vector



訂單取貨方式	收單(繳費完成)時間	出貨時間說明
核心取貨	※ 每週一及三早上9:00前	收單當日製作, 3-4個工作天後取貨。
宅配寄送	※ 每週一及三早上9:00前	收單當日製作, 每週四及隔週一出貨, 預計5-8個工作天到貨。

Showing 1 to 10 of 10

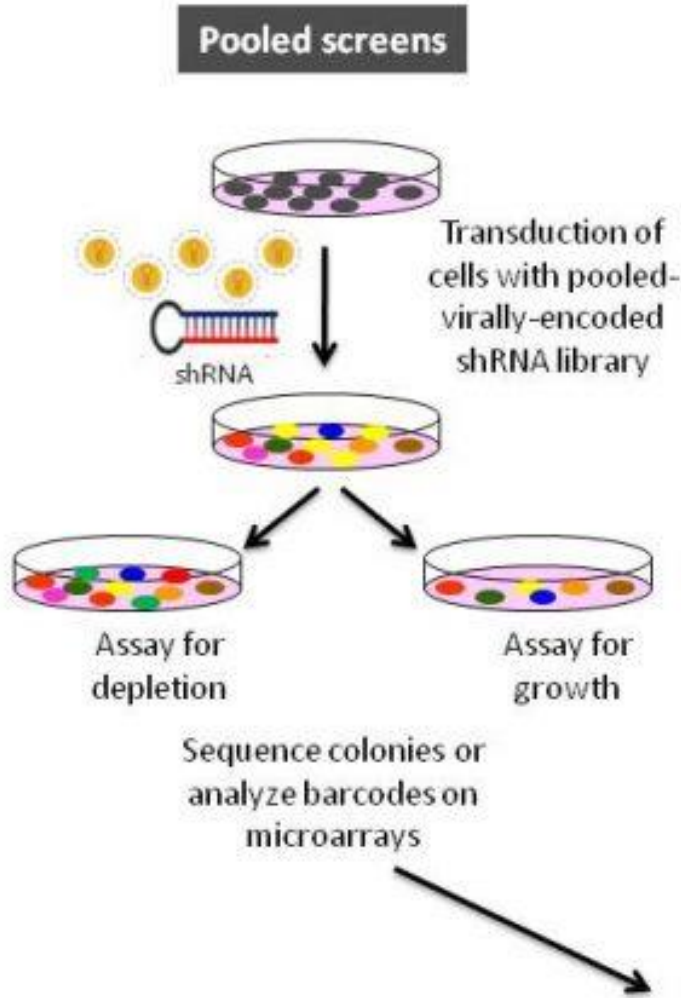
<<< 1 >>>



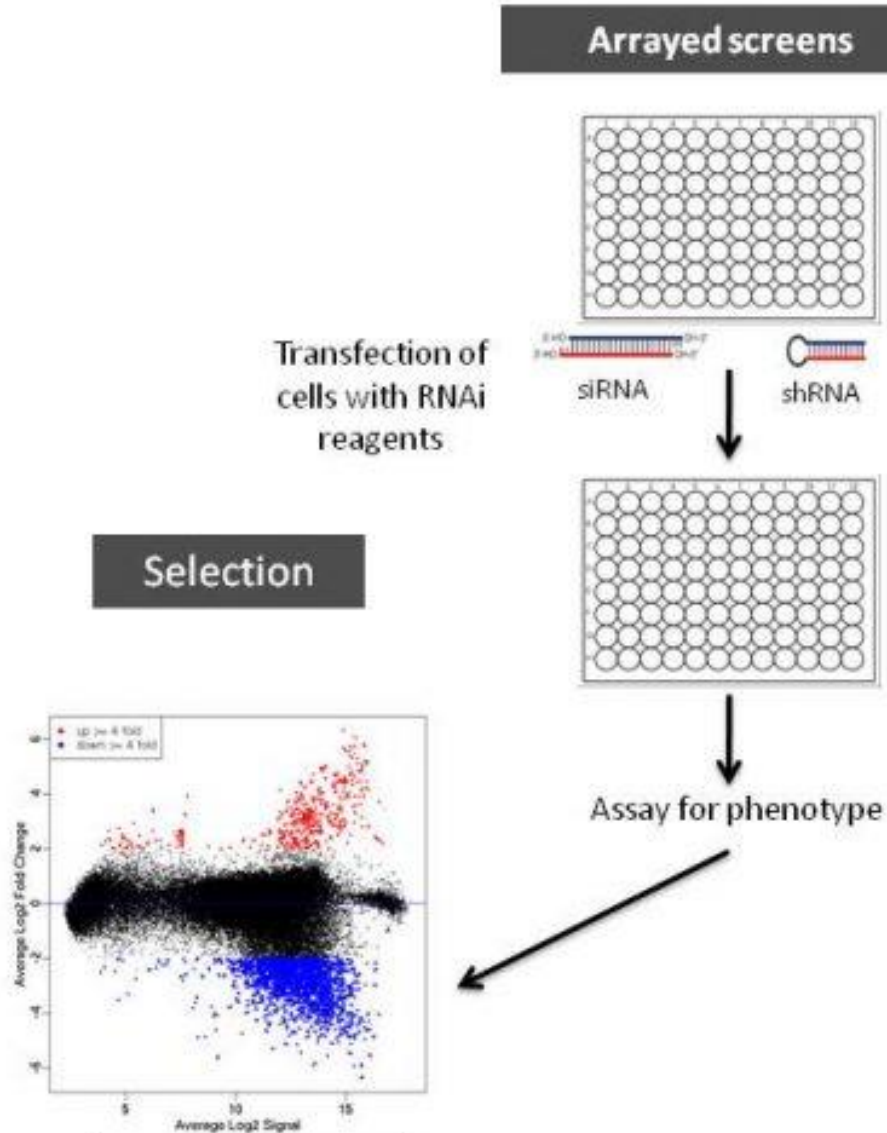
# 4. RNAi篩選

產業應用：1. 鑑定藥物作用機轉；2. 抗藥性基因；3. 抗癌基因標的；4. 抗病毒基因  
5. 支援抗體藥物開發工作；6. 其它遺傳篩選應用 (基因標的鑑定工作)

## pooled RNAi screen



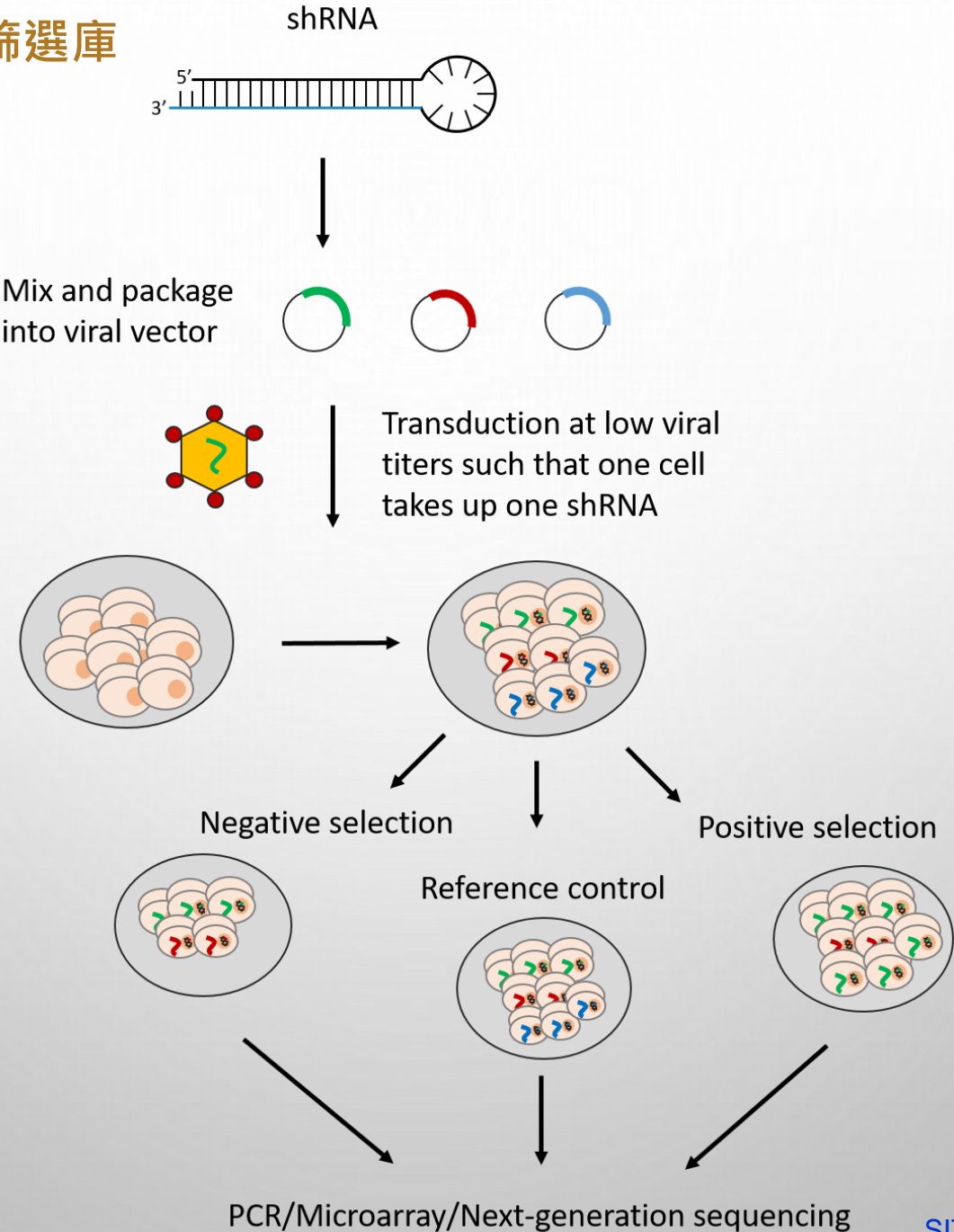
## arrayed RNAi screen



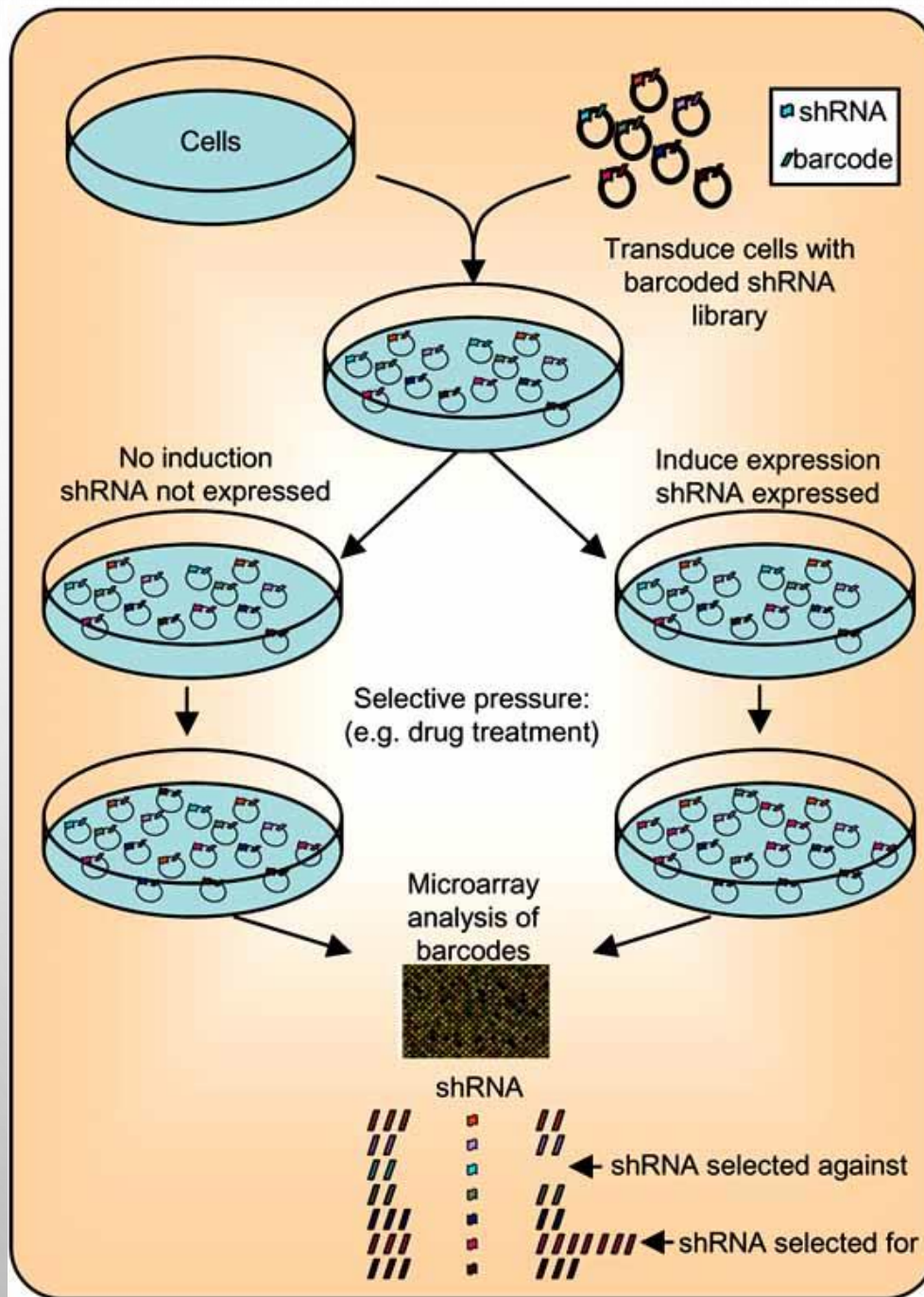
Data analysis and "hit"

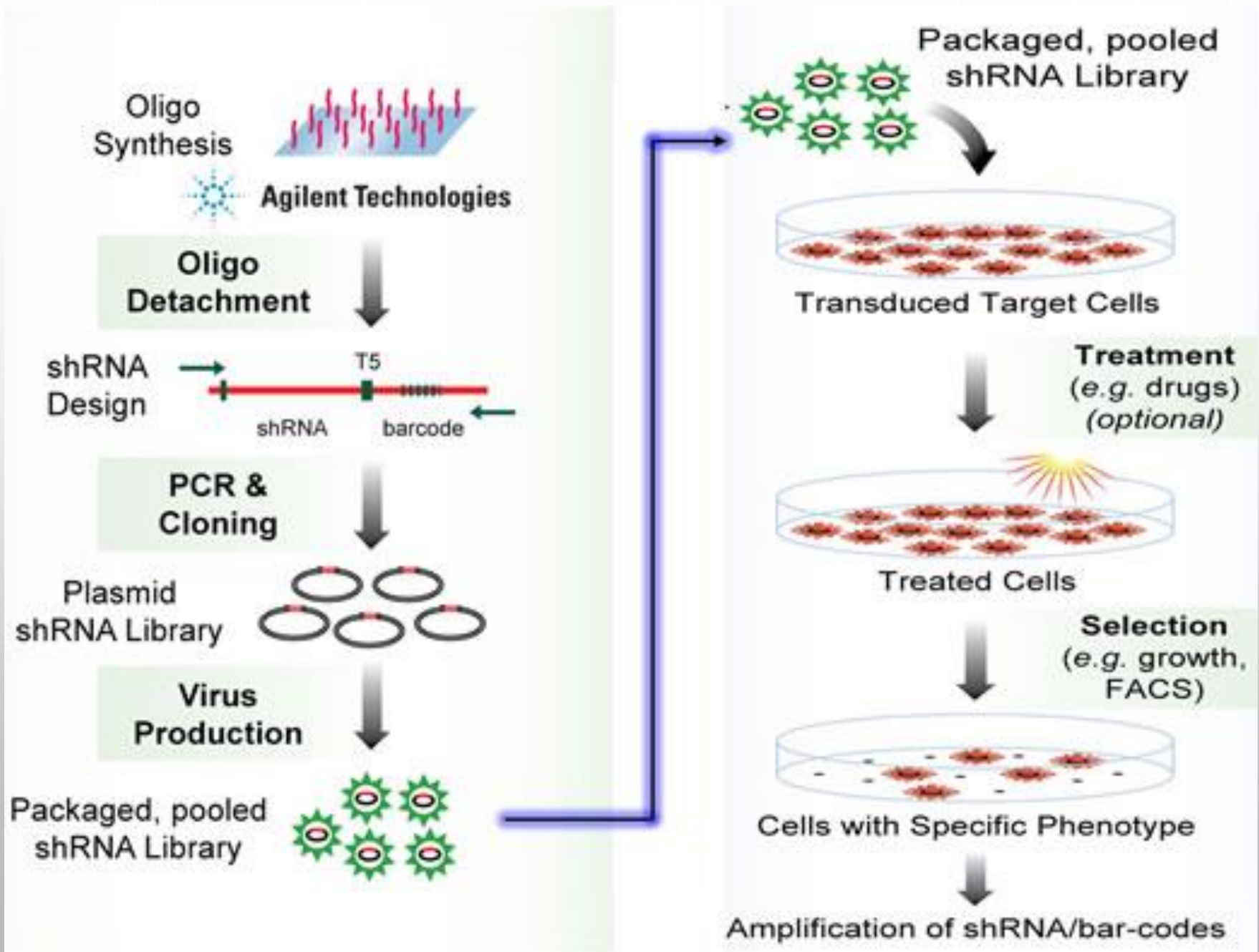


# (a) Pooled RNAi 篩選庫

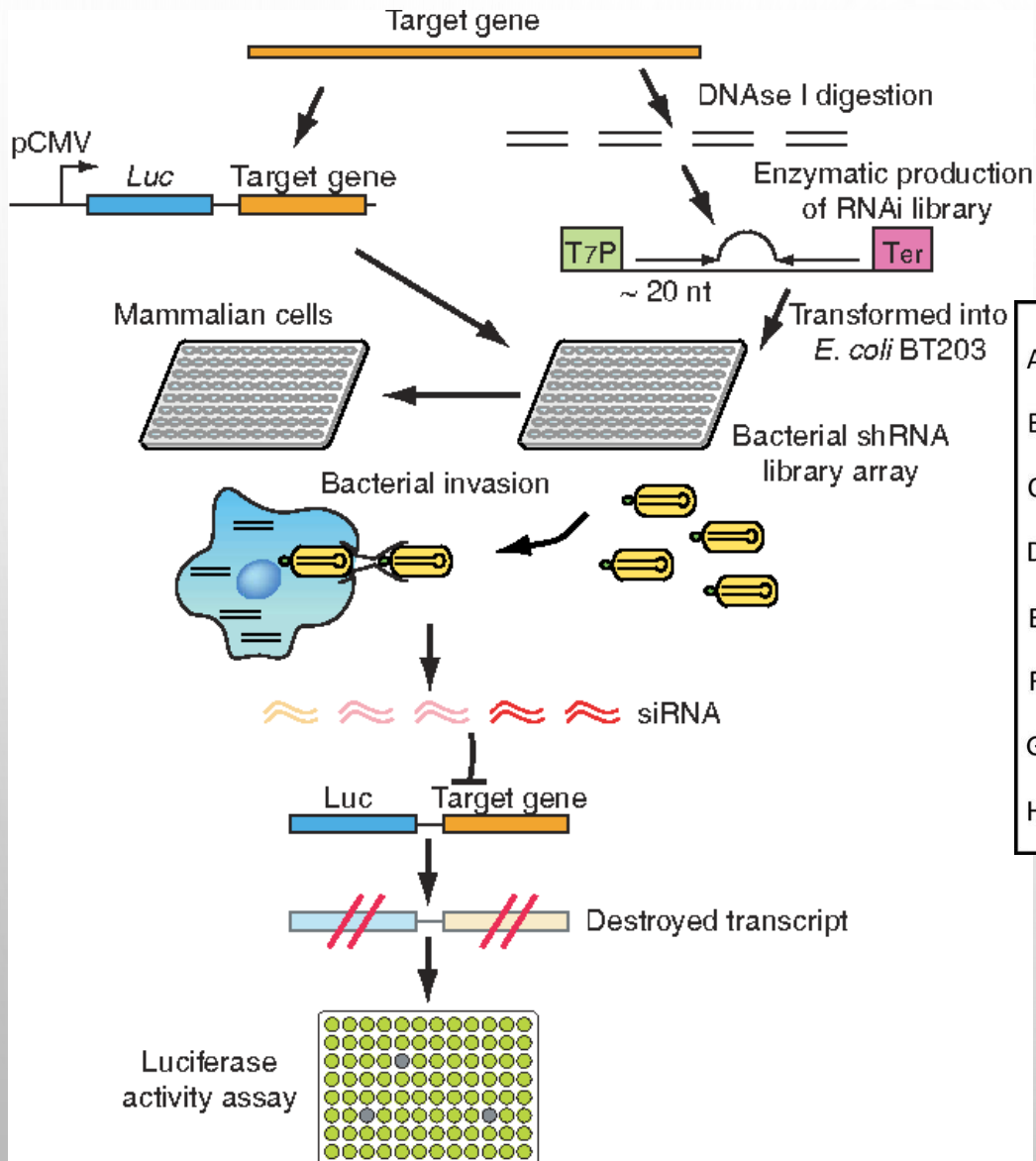








## (b) arrayed RNAi 篩選庫

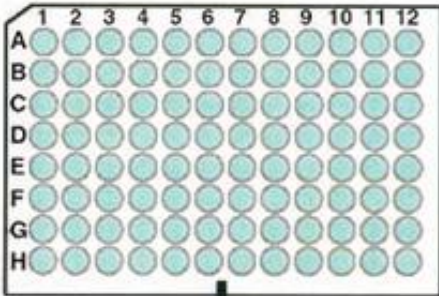


arrayed RNAi 篩選庫由單獨的 siRNA 或 shRNA 試劑組成，這些試劑針對不同的基因並放置於多孔板的每個孔中

	1	2	3	4	5	6	7	8	9	10	11	12
A	NaCl	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	Positive control
B	NaCl	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	Positive control
C	NaCl	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	Positive control
D	NaCl	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	Positive control
E	negative control	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	negative control
F	negative control	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	negative control
G	negative control	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	negative control
H	negative control	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	siRNA library	negative control

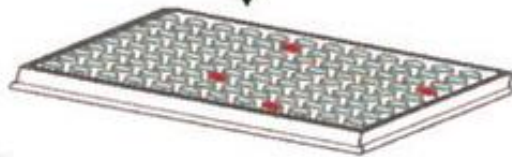


siRNAs

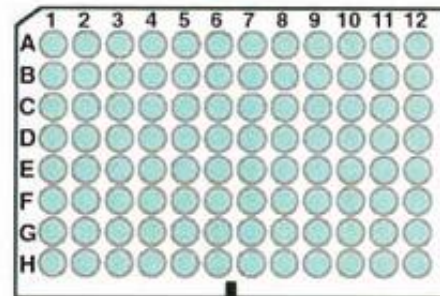


+/- compound

Transfect cell lines  
HEK, HeLa, SKO3, etc.

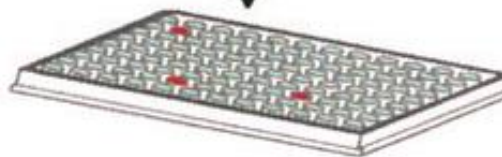


shRNA plasmids

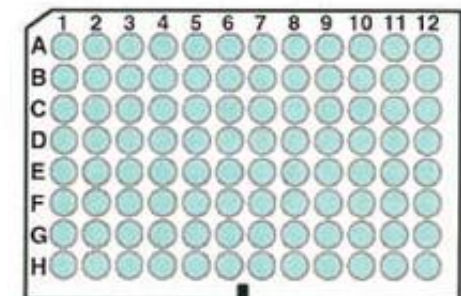


+/- compound

Transfect cell lines  
HEK, HeLa, SKO3, etc.

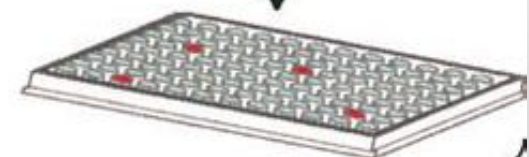


Viral particles



+/- compound

Infect hard to  
transfect cells



### Score phenotypes from cell based assays

- ◆ Luminescent reporter
- ◆ Cell viability
- ◆ High content imaging
- ◆ Fluorescent reporter
- ◆ ELISA
- ◆ FRET
- ◆ Barcode PCR

### Oncology specific cell based assays

- ◆ Wound healing
- ◆ Proliferation
- ◆ Caspase activation
- ◆ Colony formation
- ◆ 3D Coculture
- ◆ Invasion assay
- ◆ Angiogenesis assay

## RNA interference of HIV replication

Miguel Angel Martinez, Bonaventura Clotet and José A. Esté

Double-stranded RNA-mediated interference (RNAi) induces sequence-specific post-transcriptional gene silencing and has emerged as a powerful tool to silence gene expression in multiple organisms. In mammalian cells, duplexes of 21 nucleotide RNAs, known as short-interfering RNAs (siRNAs), efficiently inhibit gene expression. Recent research demonstrates the general use of siRNAs to specifically inhibit HIV-1 replication by targeting viral or cellular genes. Importantly, RNAi opens a new avenue for gene-based therapeutics.

siRNA可以將HIV水準  
降低30-50倍!!!



## **Inhibition of Retroviral Pathogenesis by RNA Interference**

.....

## **Short interfering RNA confers intracellular antiviral immunity in human cells**

**Leonid Gitlin<sup>1\*</sup>, Sveta Karelsky<sup>1\*</sup> & Raul Andino<sup>1\*</sup>**

## **A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference**

Douglas A. Rubinson<sup>1\*</sup>, Christopher P. Dillon<sup>1,2\*</sup>, Adam V. Kwiakowski<sup>1</sup>, Claudia Sievers<sup>1,2,3</sup>, Lili Yang<sup>4</sup>,  
Johnny Kopinja<sup>5</sup>, Mingdi Zhang<sup>5</sup>, Michael T. McManus<sup>1,2</sup>, Frank B. Gertler<sup>1</sup>, Martin L. Scott<sup>5</sup> & Luk Van Parijs<sup>1,2</sup>

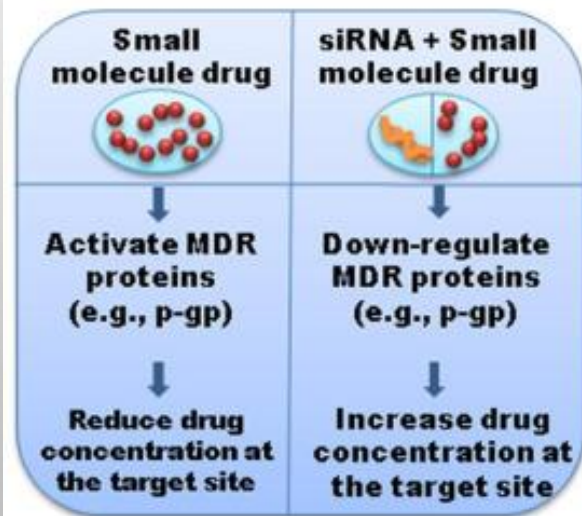
*\*These authors contributed equally to this work.*

## **Blocking oncogenes in malignant cells by RNA interference— New hope for a highly specific cancer treatment?**

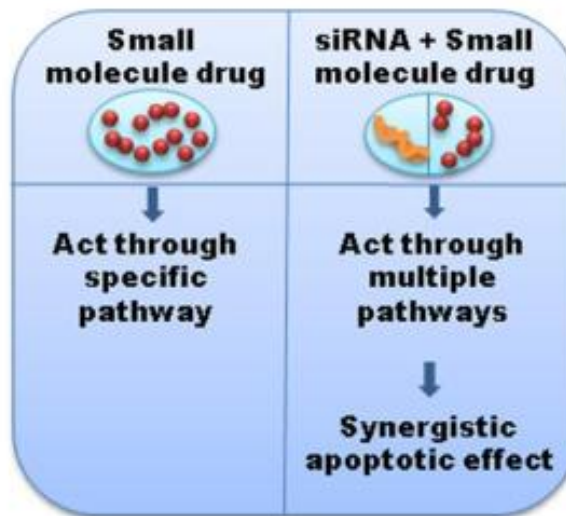
A little more than one year after the first demonstration that silencing of endogenous human genes is possible in cell culture, the new tool of RNA interference (RNAi) enters the field of tumor therapy.

# Advantages of co-delivery of siRNA and small molecule anticancer drug

## Overcome multidrug resistance



## Generate synergistic apoptotic effect



## Reduce toxicity

