



## 基因剔除(剔入)細胞株核心實驗室 Gene Knockout/in Cell Line Modeling Core

本期編輯：基因剔除(剔入)細胞株核心實驗室 黃呈彥

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本期目錄：基因剔除(剔入)細胞株核心實驗室簡介

基因剔除(剔入)實驗方法介紹

靶位設計 基因轉殖 活性測試



# 基因剔除(剔入)細胞株核心實驗室簡介

為協助醫學校區學術研究，醫學院研究發展分處第一共同研究室在2014年4月於人類疾病模式生物中心(Human disease modeling center)底下增設基因剔除(剔入)細胞株核心實驗室(Gene Knockout/in Cell Line Modeling Core)，由黃呈彥博士負責相關業務，目前正在進行四間實驗室(五個基因)委託案。服務與收費方式詳見醫學院研發分處第一共研網頁：

<http://rd.mc.ntu.edu.tw/bomrd/ntu2/firstcore6.asp>

設置地點：醫學院基礎醫學大樓R1448, R1442 & R1439

開放時間：週一至週五 9:00~17:00

使用資格：本校各科系所實驗室或計畫及附設醫院各科部負責人為主

服務項目：基因剔除(剔入)靶位設計

載體構築

基因剔除(剔入)活性測試

基因剔除(剔入)細胞株篩選

聯絡方式：電子郵件 [cyh0729@ntu.edu.tw](mailto:cyh0729@ntu.edu.tw)

分機 88930或88507

(請多利用電子郵件聯絡，謝謝。)



# 申請方式與服務流程

於第一共研網頁下載委託調查表，  
傳送電子郵件予黃呈彥博士

靶位設計後預約面談並填寫申請表

載體構築

細胞轉殖測試與基因剔除(剔入)活性測試

基因剔除(剔入)細胞株篩選

# 基因剔除(剔入)實驗方法介紹

## 靶位設計

利用E-TALEN(<http://www.e-talen.org/E-TALEN/>)或E-CRISPR(<http://e-crisp-test.dkfz.de/E-CRISPR/>)幫助尋找合適位點，以下以E-CRISPR為例介紹：

進入網頁後按De-novo進入設計頁面

**E-CRISPR**  
Design of CRISPR constructs

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De-novo Evaluation Database Forum Help Links

E-CRISPR is a software tool to design and evaluate target sites for use with the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) system.

The web application uses fast algorithms to identify sgRNA target sequences in any nucleotide sequence for use in CRISPR/Cas mediated genome editing. E-CRISPR analyzes target specificity of the putative designs and assesses their genomic context (e.g. exons, transcripts, CpG islands). The design process incorporates different options of how CRISPR constructs can be used in experimental applications.

**Recent Updates:**

- 26 May 2014 We are happy to announce a further major update to our E-CRISPR web service. Many new organisms have been added together with big changes in the web front end. Hence you will find the new forum and many other new things here in the new BETA version 3.1.
- 14 April 2014 In this minor update different default values for de-novo sgRNA design have been implemented, allowing for more designs to be found.
- 01 April 2014 The following organisms have been added to E-CRISPR:  
Zea mays  
Ustilago hordei  
Toxoplasma gondii ME49  
Gasterosteus aculeatus  
Populus trichocarpa
- 20 March 2014 A new version of E-CRISPR has been released (version 3.0). It includes more off-target search options and we implemented speed improvements to enable the design of sgRNAs against up to 200 genes in parallel.

**De-NOVO**

```
>seq
ACTGATTATTGGGAGTCGATCGTAGTCGATTAGCTAGCATAATAGCGTACTGAT
TGGCTATGCTGAGCT
```

**EVALUATION**

```
>seq1
ACCGATATCGATGCTGGATATCGATACATCGATGTAGCTAGCTGATC
>seq2
TTAGGGACTGACCGGAGTATAGCGGATTA CGGGCGATTACGGCGATTA
```

The diagram illustrates the workflow from a DNA sequence to the evaluation of target sites. The 'De-NOVO' step shows a sequence with a red arrow pointing to a target site. The 'EVALUATION' step shows the same sequence with a red arrow pointing to a target site and a purple arrow pointing to a target site, indicating the evaluation of different target sites.

首先選擇物種，之後可於FASTA Reset空格輸入基因的 ENSEMBL accession numbers或直接將序列貼入，第一行需輸入 (>基因名稱)。第3步先選擇Strict設定較為嚴苛條件，之後可按第4步 Search鍵開始或點選Display advanced options做進階設定。

## E-CRISP

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De-novo Evaluation Database

Forum Help

Links

### 1. Select organism:

Homo sapiens sapiens (Human, GRCh37.75) [HELP]

### 2. Enter target sequence:

FASTA format sequence(s)

FASTA example [HELP]

```
>TBK
AGCGAGCTCGGCCGCCGCGGAGTACGCACGCGCAGGCGCGCCCCGC
ACTCGGTCACGTGCTCCGTGGCCCGGGCTGGCGAAGCCGGAAGTAG
CCTGGGGCGCGAGAAGGCCCGGGAGCCGCGGGCTGTACGCGCGGA
CACTCGCGGGGTAAAGAGGGGCGGGCGAGGCCGGTGGGGCGGACG
```

Alternatively upload a file in FASTA format or a new line separated list of gene symbols and/or accession numbers

選擇檔案 未選擇檔案

[HELP]

Clear

### 3. Choose parameter presets:

Relaxed Medium **Strict** Single design

選擇Single design (CRISPR Wt)或  
Paired design (CRISPR D10A)

### 4. Start web application:

Search

Start sgRNA analysis

Display advanced options

## 進階設定

[click here to change CRISPR properties](#)

17-20的長度為佳

bp Minimum guide RNA length after PAM  
[HELP]

bp Maximum guide RNA length after PAM  
[HELP]

% < G <  %

% < A <  %

% < T <  %

% < C <  %

bp 3' flanking sequence length [HELP]

bp 5' flanking sequence length [HELP]

bp Expected flank size (for restriction site analysis)

bp Tagging window downstream of the codon

bp Tagging window upstream of the codon

Number of coding exons downstream the start codon for K.O.

bp Minimal spacer length for paired designs

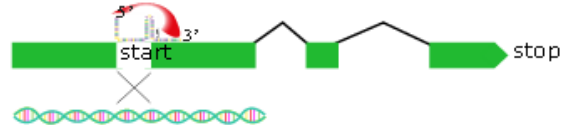
bp Maximum spacer length for paired designs

5' preceding Base requirement

K.O.:



N-Term:



C-Term:



在第3步驟選擇**Strict**則第6步會自動全選，主要是第7步驟選擇排除 off-targets 條件，可選Bowtie或Bowtie2做比對，也可利用右方排除 puromycin 或GFP 等等篩選標誌基因被剔除的機會。

#### 6. Gene annotation filtering:

Exclude non-gene hits [HELP]

Exclude non-exon hits [HELP]

Exclude hits outside of coding sequences

Exclude hits in CpG islands [HELP]

Transcript specificity [HELP]

Exon specificity [HELP]

Retrieve and save a recombination donor matrix

Assess restriction sites of the whole sequence [HELP]

Restriction enzyme library [HELP]

#### 7. Off-target analysis:

select bowtie version

select off-target database

Exclude designs with more than X off-targets

Number of 5' mismatch positions

Tolerated edit distance to the target sequence

Bowtie2 pre-sets

Select to check for secondary off-targets [HELP]

Please choose the targets to test and/or paste a custom sequence into the text area below.

The pasted sequence should be FASTA format.

[HELP]

全部設定完成後，按下方start鍵。

#### 8. Output:

Maximum number of results per exon

- Produce a GFF formatted output file
- Create an image showing genomic context
- Output the result table to the browser window
- Produce additional information for the Matchstring (Warning: This option might take a while!)

Start

This is a beta version. If you have any questions or find potential bugs please do not hesitate to contact us.  
Go to original E-CRISP

等待中.....

## E-CRISP

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De-novo

Evaluation

E-TALEN

E-RNAi

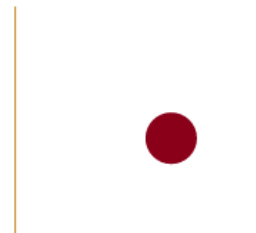
GenomeRNAi

Help About

While you are waiting, why don't you visit E-RNAi or E-TALEN?

Did you know that:

The **nails** on long fingers grow faster than short fingers; and fingernails grow four times faster than toenails.



This page will be automatically updated every 28 seconds until search is done.



設計完成後，可能沒有合適靶位，此時可以回到第3步驟選擇較不嚴苛的條件(Medium或Relaxed)再試一次，有可能會出現多個off-target位點的結果。

## E-CRISP

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E-RNAi
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[Download a tabular report for all query sequences together](#)

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**Query name:** TBK **Query length:** 483 **Query location:** ::0::0

Total number of possible designs = 174  
 Number of successful designs = 4  
 Number of designs that hit a specific target = 71  
 Number of designs excluded because they hit multiple targets or none = 37  
 Number of designs excluded because their nucleotide composition was too invariable or contained TTTT = 3  
 Number of designs excluded because the maximum of designs per exon was exceeded = 67

Name	Nucleotide sequence	Ranking	Target	Matchstring	Number of Hits
TBK_6_96	TTCTTCCCCCTTATCG TGAGG	109.52	ENSG00000058668::A TP2B4	<a href="#">Matchstring Info</a>	1
TBK_28_384	AACTAGCACCCTAGG TAGG	100	5	<a href="#">Matchstring Info</a>	2
TBK_28_384	AACTAGCACCCTAGG TAGG	100	ENSG00000145675::PI K3R1	<a href="#">Matchstring Info</a>	2
TBK_21_384	AGTTTCTGAGTTTACA GAATGG	87.27	8	<a href="#">Matchstring Info</a>	3
TBK_21_384	AGTTTCTGAGTTTACA GAATGG	87.27	ENSG00000175497::D PP10	<a href="#">Matchstring Info</a>	3
TBK_21_384	AGTTTCTGAGTTTACA GAATGG	87.27	1	<a href="#">Matchstring Info</a>	3
TBK_1_0	AAACACTATCTCCAGA GGG	70	11	<a href="#">Matchstring Info</a>	5

[Download results as zipped folder](#)

## 基因轉殖

靶位設計完成後，仍然需要進行人工判讀以及篩選，一般要讓基因不表現，靶位設計越接近start codon越好，也可以設計在此基因的重要區域(domain)使其失去應有的功能。

設計完成即可進行載體構築，TALEN以及CRISPR -D10A因為是一組靶位的設計，因此需要同時構築兩個質體，而CRISPR -Wt則只需要構築一個質體。在進行細胞轉殖時，轉入一個質體會比兩個質體共轉容易的多，若是細胞本身轉殖效率不高，共轉的成功率將極低，一般轉殖效率要大於50%為佳。除此之外，載體上若帶有篩選標誌基因，如puromycin可進行藥物篩選，或是GFP螢光蛋白可以將表現螢光的細胞分選出來，都可以提高基因轉殖成功率。

當我們進行TALEN或是CRISPR實驗時，要注意細胞轉殖成功並不代表內切酶發揮作用，由於送進去表達的TALEN或是CRISPR必須辨認並結合細胞染色體上的特定位點，不同的位點上可能存在結構或是特殊修飾而影響結合，因此在進行單株細胞篩選之前，必須先確定所送入的TALEN或是CRISPR具有活性並測試其突變效率高低，以下介紹幾種活性測試方法：

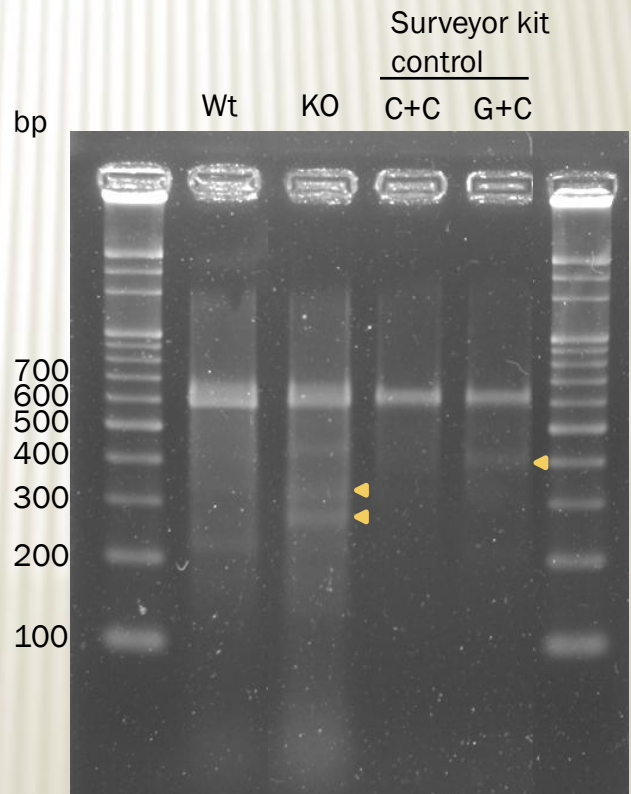
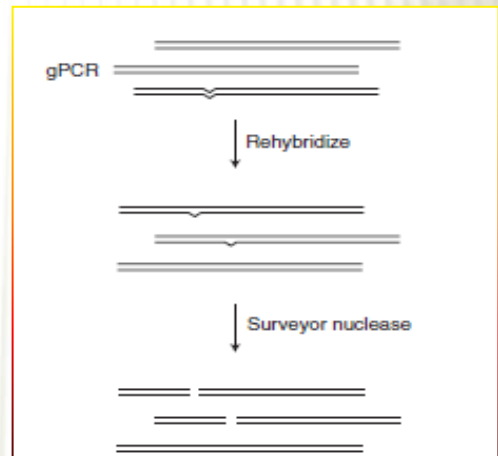
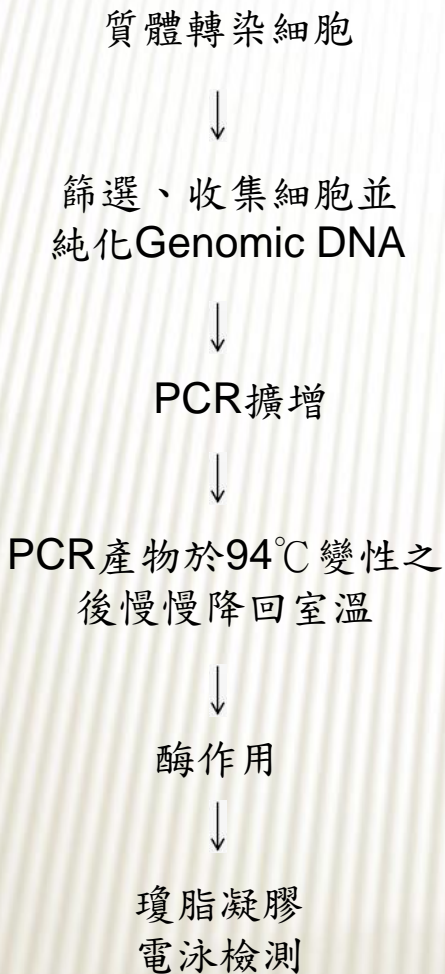
# 基因剔除(剔入)實驗方法介紹

## 活性檢測

### 方法一、T7E1 or Surveyor assay

原理：T7 endonuclease 1 或 Surveyor Nuclease 辨認並切割不完美黏合的DNA。

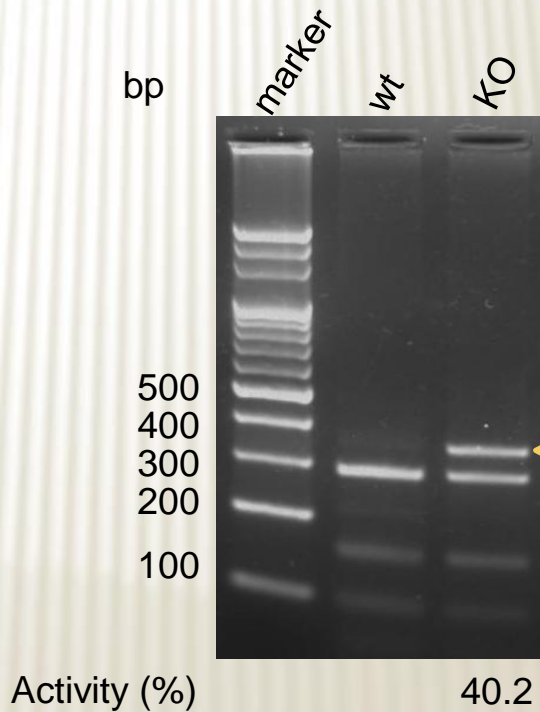
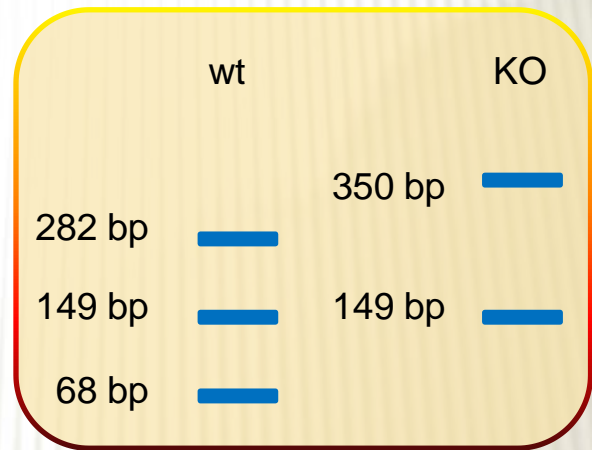
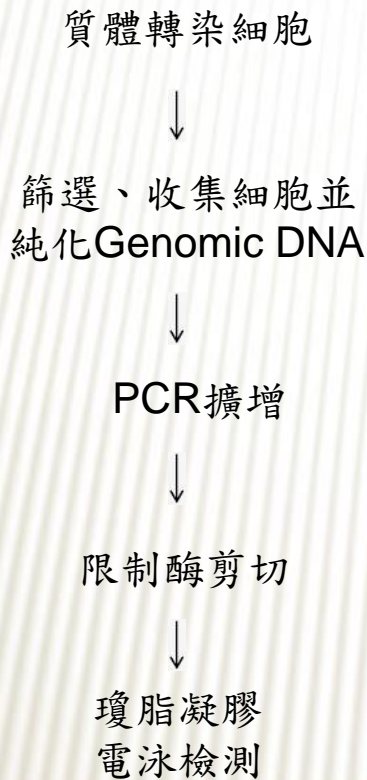
方法：



## 方法二、Restriction Enzyme (RE) digestion assay

原理：因TALEN或CRISPR作用後導致特定限制酶切點消失

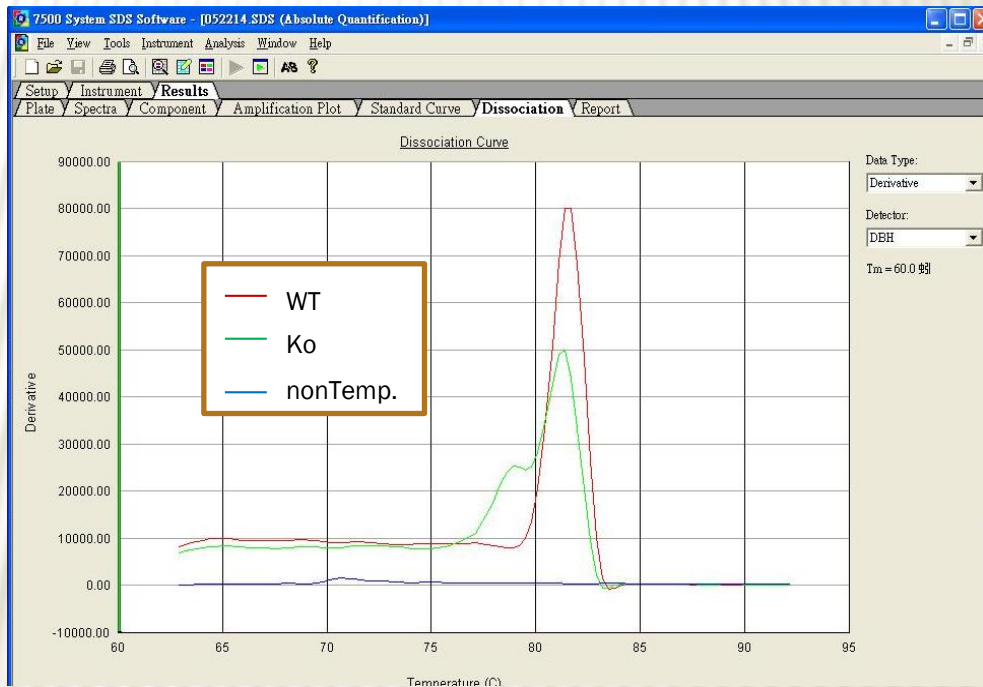
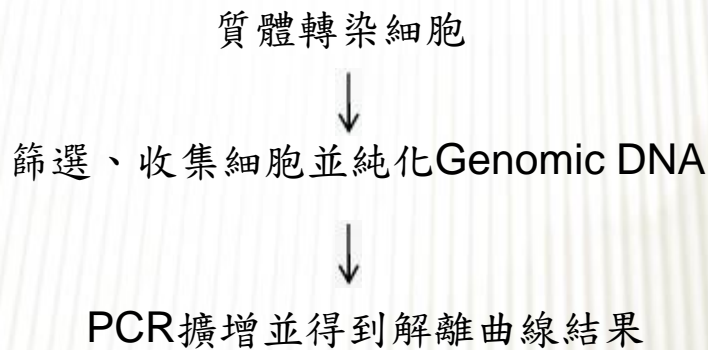
方法：



## 方法三、qPCR Dissociation assay or HRM

原理：因TALEN或CRISPR作用後導致序列突變，而影響 $T_m$ 值

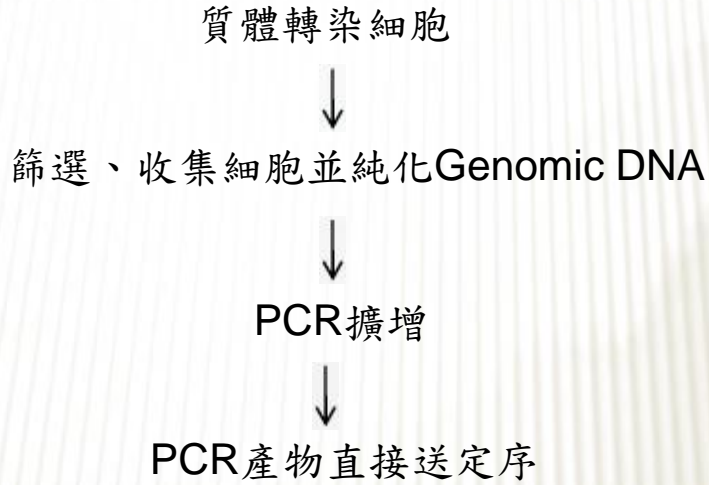
方法：



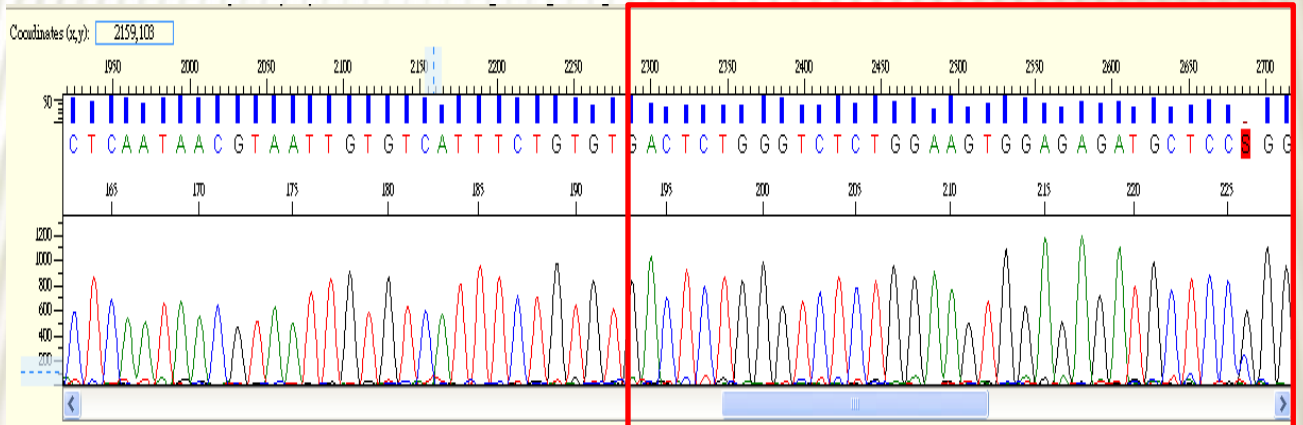
附註：第一共研目前有qPCR機器，並無HRM機器。

## 方法四、sequencing assay

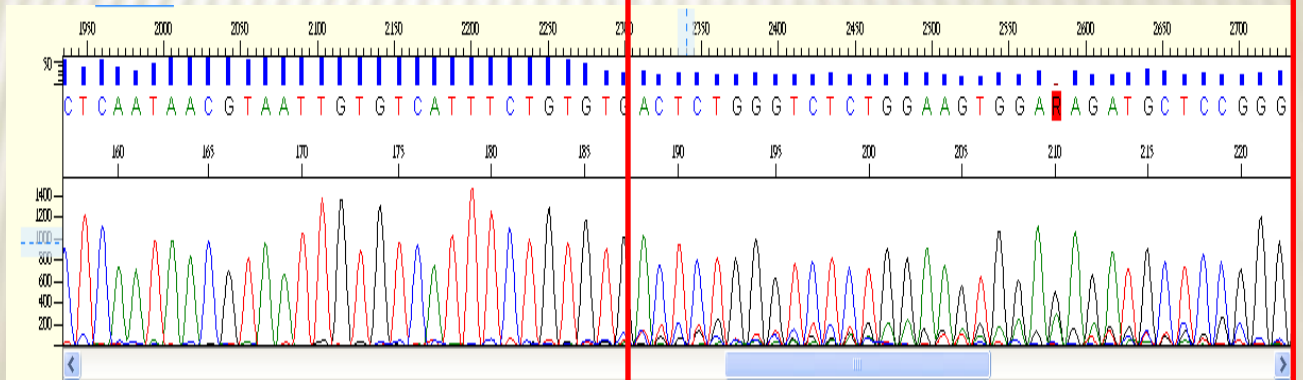
方法A：



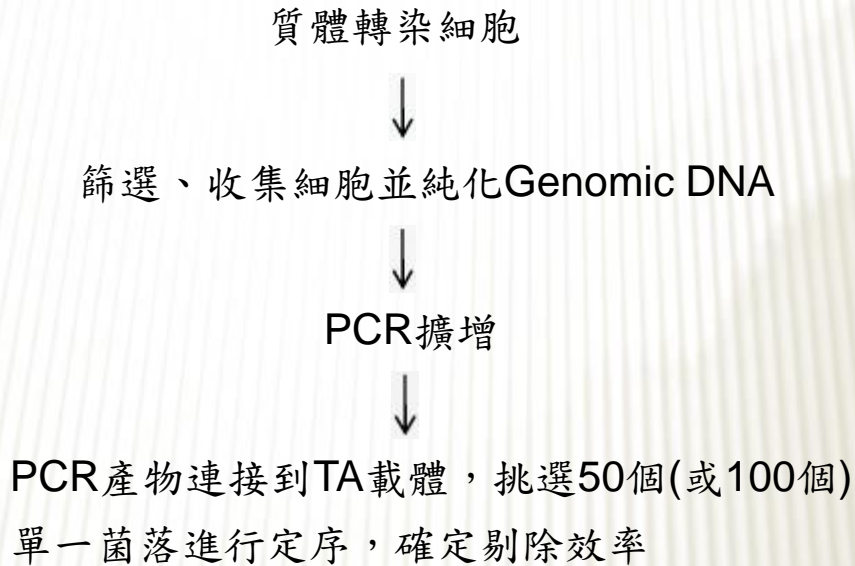
wt



Ko



方法B：



目前較為常用的方法為T7 E1分析法，因為設計靶位時不需存在限制酶切點，同時可量化突變效率，至於利用qPCR分析解離曲線或是HRM的方法其優點是經濟快速，但是無法量化突變效率，最佳應用方式是在進行單株細胞篩選時可進行初步快篩。至於定序的方法A在進行突變效率分析時只能依照分析圖上基因突變區圖形紊亂程度做定性分析，無法量化，但是運用在單一細胞株篩選時可以直接將序列進行解讀為其優點。至於方法B則過於耗時費力，適用於最後確定單一細胞株的基因序列。

# 相關生技公司網站連結

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## 冷泉港生物科技

<http://www.fjbio.com.tw/index.php?do=prod&toppid=195&pid=197&id=352>

## 力鈞生物科技

<http://www.zgenebio.com.tw/>

## 威新生物科技

<https://www.facebook.com/wethinkbio>

## 圖爾思生物科技

<http://www.toolsbiotech.com/>

## Sigma-Aldrich

[http://www.sigmaaldrich.com/catalog/product/sigma/crispr?lang=en&region=TW&cm\\_guid=1--1000000000000000038491--50835121278&cm\\_mmc=Google\\_Biosilencing--CRISPR--CRISPR--sigma%20crispr\\_Phase\\_50835121278](http://www.sigmaaldrich.com/catalog/product/sigma/crispr?lang=en&region=TW&cm_guid=1--1000000000000000038491--50835121278&cm_mmc=Google_Biosilencing--CRISPR--CRISPR--sigma%20crispr_Phase_50835121278)

## lifetechnologies

[http://www.lifetechnologies.com/tw/zt/home/life-science/cloning/gene-synthesis/geneart-precision-tals/geneart-crispr.html?s\\_kwcid=AL!3652!3!43210957038!b!!g!!+crispr&ef\\_id=U1c6-wAAPaivQiT:20140728043100:s](http://www.lifetechnologies.com/tw/zt/home/life-science/cloning/gene-synthesis/geneart-precision-tals/geneart-crispr.html?s_kwcid=AL!3652!3!43210957038!b!!g!!+crispr&ef_id=U1c6-wAAPaivQiT:20140728043100:s)

## System Bioscience

<http://www.systembio.com/cas9?gclid=CIGJ4IGM778CFVh6vQodoxsArA>

## ASC: Applied StemCell

<https://www.appliedstemcell.com/services/cell-line-models/cell-line-modification/>

## Cellectis bioresearch

<http://www.cellectis-bioresearch.com/content/custom-talen%E2%84%A2>

## Haplogen genomics

<https://www.haplogen-genomics.com/crispr?gclid=CJHG5dWa578CFZQAvAodA4MAkw>