30th International Biology Olympiad
SZEGED, HUNGARY

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Practical Exam 2. 實作二.

Blood Sugar Measurement 血糖的測定

Molecular Biology 分子生物學

16^{th} July 2019

COUNTRY
LANGUAGE 語言

Practical Exam 2. 實作 2.

General instructions 一般說明

This exam consists of two subtasks: 本實作包括兩部分

- Subtask 1. A model of home blood glucose measurement (50 points)
- Subtask 2. Examination of knock-out mutant cell lines produced by CRISPR-Cas 9 method (50 points)
- 第一部分：血糖量測的模式 (50 分)
- 第二部分：檢測以 Crispr-Cas9 基因編輯方法製備的基因剔除的突變細胞株 (50 分)

1. **Please remember to attach your barcode sticker to all pieces of paper on the answer sheet.**
2. Write your answers in the separate answer sheet provided. **Only answers given in the answer sheet will be considered**.
3. Ensure you received all necessary materials and equipment listed on the next page. If any items are missing indicate this by raising your red card within 10 minutes following the start of the exam.
4. During experiments ensure all materials and equipment is handled properly. Any spilled solutions or broken equipment will not be replaced.
5. Stop answering and put down your pencil immediately when the bell rings signalling the end of the exam.
6. No paper, materials or equipment should be taken out of the laboratory.

1. 記得把你的識別碼貼在答案紙的每一頁上。
2. 將答案寫在所提供的答案卷上，**只有寫在答案卷上的答案才計分**。
3. 確認你有下一頁所列出的所有必需材料與儀器，如有任何缺少者，請在考試 10 分鐘內舉起紅卡。
4. 在實驗過程中，確認所有材料與儀器操作正確，任何漏失或損壞的儀器將不會更換。
5. 當實驗結束鈴聲響起，停止作答並立即放下筆。
6. 不得從實驗室中取走紙張，材料或設備。

CAUTION: The experiment deals with materials that are fragile and sharp. Exercise caution when handling them.
注意：實驗材料易損毀且尖銳，應小心操作。

WEAR ALL SAFETY EQUIPMENT PROVIDED AT ALL TIMES OTHERWISE THE LAB ASSISTANT WILL ASK YOU TO LEAVE THE ROOM.
實驗中，全程穿戴所提供的所有安全配備，否則實驗助理將會要求你離開實驗室。

Materials and Equipment 材料與儀器

- Eppendorf tubes
- 10 µL micropipette
- 200 µL micropipette
- 1000 µL micropipette
- micropipette tips
- permanent marker
- calculator

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- red signaling card
- yellow signaling card
- tissues
- ACCU-CHEK Active blood glucose meter
- Test strip container with 15 test strips –keep closed to prevent damage to the strips
- standard glucose solution in distilled water; label: G; 1 mL
- distilled water; label: W
- beaker with ice
- beaker with room temperature water
- thermometer
- 37 °C dry thermostat (one shared by 4 competitors)
- Microcentrifuge tube labelled ‘M’: size ladder marker
- Microcentrifuge tube labelled ‘C’: control which is the PCR product of the original gene
- Microcentrifuge tube labelled ‘P1’: PCR product of the sample taken from P1 type cells
- Microcentrifuge tube labelled ‘P2’: PCR product of the sample taken from P2 type cells
- Microcentrifuge tube labelled ‘E’: restriction endonuclease (PstI.) –held on ice!
- gel electrophoresis chamber
- electrophoresis gel

材料與儀器

- 微量離心管
- 10 µL 微量吸管
- 200 µL 微量吸管
- 1000 µL 微量吸管
- 微量吸管尖
- 永久簽字筆
- 計算機
- 紅卡
- 黃卡
- 擦拭紙
- ACCU-CHEK 血糖儀
- 含有 15 條試條紙的管子，隨時保持管子關閉，以免試紙受損。
- 以蒸餾水泡製的葡萄糖標準液，標示為 G；1 mL
- 蒸餾水，標示為 W
- 裝有冰塊的燒杯
- 裝有常溫水的燒杯
- 溫度計
- 37 °C 乾浴槽；(4 位選手共用)
- 裝有 DNA 梯度標誌的微量離心管；標示為” M”

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- 裝有原始基因之 PCR 產物；標示為”C”
- 標示為”P1” 的微量離心管：採樣自 P1 細胞的 PCR 產物
- 標示為”P2” 的微量離心管：採樣自 P2 細胞的 PCR 產物
- 標示為”E” 的微量離心管：限制酶 (PstI) 置於冰上
- 電泳槽
- 電泳凝膠

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SUBTASK 1. A MODEL OF HOME BLOOD GLUCOSE MEASUREMENT 在家中進行血糖測定

Glucose in the mammalian body is metabolized in a complex system which provides a balance (homeostasis) of highly regulated processes.

哺乳動物體內的葡萄糖以複雜的代謝系統運作以提供能進行高度調節的穩定平衡 (homeostasis) 狀況。

Sufficient levels of blood glucose are maintained hormonally and allosterically by the regulation of transporters and key enzymes of glucose metabolism.

通過賀爾蒙及酵素易位 (allosterically) 調節葡萄糖運輸蛋白及葡萄糖代謝相關主要酵素，可維持足夠量的血糖。

Metabolic diseases that result in a disturbance of these regulating mechanisms have severe consequences to the affected individuals and have become of economic significance on a global level.

導致血糖調節機制紊亂的代謝疾病對受影響的個體產生嚴重後果，並對全球經濟造成重大影響。

The most common of them all –diabetes mellitus –is a group of metabolic diseases that have chronic hyperglycemia (elevated blood glucose levels) in common as a result of disturbances in insulin secretion and/or the effects of insulin.

其中最常見的 - 糖尿病 - 是一組代謝性疾病，起因於胰島素分泌紊亂和/或胰島素效應問題所造成的慢性高血糖症（血糖量升高）。

For the management of diabetes, monitoring blood glucose levels is essential. Especially patients undergoing insulin therapy are required to measure their blood glucose levels on a daily basis, since the calculation of the correct doses of insulin is essential to avoid hypo- and hyperglycemia, both life-threatening situations. Correct insulin dosage and consistently sufficient blood glucose levels are also key elements for prognosis improvement.

對於糖尿病的管理，監測血糖量至關重要。特別是在接受胰島素治療的患者需要每天測量他們的血糖量，因為正確劑量的胰島素的計算對於避免低血糖和高血糖都是必不可少，這兩種情況都是危及生命的。正確的胰島素劑量和穩定足夠血糖量也是改善預後的關鍵因素。

In this practical exam, handheld glucose meters developed for home-use by diabetes patients will be used to simulate blood glucose measurement in model glucose solutions.

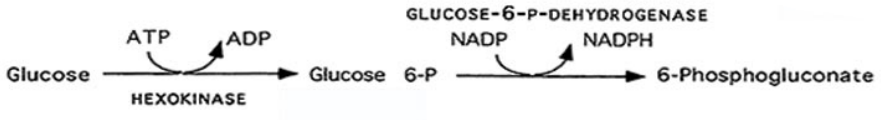
在本實作中，將以糖尿病患者使用的家庭用手持式血糖儀進行葡萄糖溶液量測以模擬血糖量。

Glucometers are used for home blood glucose monitoring by patients with diabetes mellitus and other diseases with a high risk for hypoglycemia.

血糖儀用於患有糖尿病和其他具有低血糖風險的疾病的患者家族的血糖監測。

The glucometer used for these determines blood glucose content with reflectance photometry using the hexokinase method:

這些量測血糖的血糖儀使用己糖激酶 (hexokinase) 法通過反射光度法測定血糖含量：



Since glucometers are calibrated using whole blood, calibration curves have to be prepared to obtain correct measurement results when using aqueous solutions.

由於血糖儀是使用全血校準的，因此在使用水溶液時必須準備校準曲線以獲得正確的測量結果。

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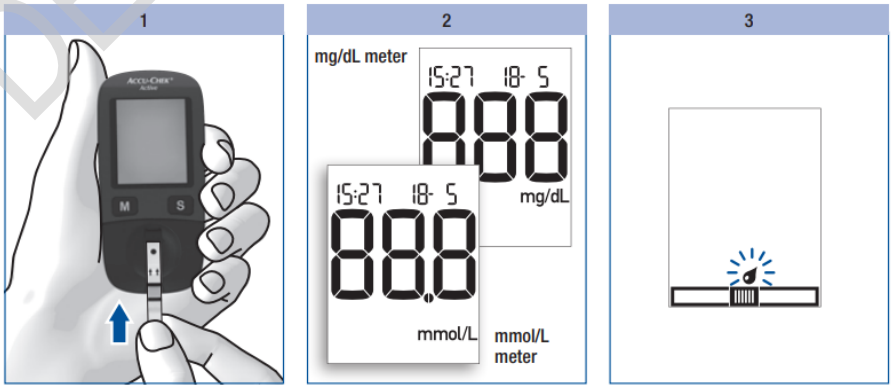
How to Measure Glucose Concentrations with the ACCU-CHEK Active Blood Glucose Meter (see figure below) 如何使用 ACCU-CHEK 活性血糖儀測量葡萄糖濃度（見下圖）

1. Take a test strip from the test strip container. Close the container immediately. 從試紙容器中取出試紙。立即關閉容器。
2. Hold the test strip so that the arrows printed on it and the green square face upwards 握住試紙，使印有箭頭，及綠色正方形的一端朝上
3. Slide the test strip into the test strip guide in the direction of the arrows until you feel it lock into place. (1) Do not bend the test strip. 沿箭頭方向將試紙滑入試紙槽上的箭頭方向，直到感覺到它鎖定到位。備註（1）不要彎曲試紙。
4. The test strip must lie flat on the measurement window cover. 試紙必須平放在測量窗蓋上。
5. The meter turns on and first performs a default display check (approx. 2 seconds). (2) 將血糖儀打開並進行預設顯示檢測（約 2 秒）(2)。
6. Following the display check, the test strip symbol and the flashing blood drop symbol appear on the display (3). 在顯示檢測完成後，試紙符號和閃爍的血滴符號會呈現在顯示器上（3）。
7. The meter is now ready to perform a blood glucose test. You have approx. 90 seconds to apply 3 µL of glucose solution onto the middle of the green square of the test strip. After this time, the meter turns itself off. If this happens, remove the strip and slide it in again. 血糖儀現在已準備好進行血糖測試。你有大約 90 秒時間可將 3µL 葡萄糖溶液滴加到試紙的綠色正方形的中間。超過此時間後，儀器會自動關閉。如果發生這種情況，請取下試紙並重新再將之滑入。
8. The test starts. The flashing hourglass symbol indicates that the test is in progress. 測試開始。閃爍的沙漏符號表示測試正在進行中。
9. The test is over in approximately 5 seconds. 測試在大約 5 秒內結束。
10. You can now mark the test result 您現在可以記錄測試結果。
11. After removing the test strip, the device will turn itself off. Discard any used test strips.

在移除試紙後，儀器將自行關閉。丟棄任何用過的試紙。

(If an error appears before the application of the liquid, remove the test strip and try again. If it doesn´t work the second time or you already applied the glucose solution, discard the test strip and use a new one.)

(如果在加入液體前出現錯誤訊號，請取下試紙並再試一次。如果第二次仍出現錯誤，或您已將葡萄糖溶液加入，則丟棄試紙並使用另一新的試紙。)



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Part 1. Glucometer Measurement of the Concentration of Model Glucose Solutions 第 1 部分。血糖儀測量模型葡萄糖溶液的濃度

First, you are going to be testing the accuracy of glucose measurement with the glucose meter on water-based model glucose solutions.

首先，您將使用血糖儀以測試葡萄糖水溶液中葡萄糖含量為模式，並測試血糖儀的準確性。

Procedure 程序

1. Prepare the following dilutions from the provided standard glucose solution in Eppendorf tubes using distilled water: 1:5; 1:2; 1:1; 2:1 (in a sample volume : diluent volume format) –300 µL of each. Label the tubes accordingly. 使用蒸餾水，從提供的標準葡萄糖溶液中在微量離心管中製備以下稀釋液：1：5；1：2；1：1；2：1（樣品體積：稀釋劑體積）- 每種 300µL。在相應地離心管上標記。
2. Measure the glucose content of the solutions. 測量溶液的葡萄糖含量。

Q.2.1.1 Fill in the table on the answer sheet. 將答題紙上的表格填好。
Table Legend: 表格說明：
D –Dilution (standard volume : total volume)
D - 稀釋（標準體積：總體積）
Vs–added volume of standard solution
V_S - 加入的標準溶液
VH2O –added volume of distilled water
V_{H_2O} - 加入的蒸餾水量
MC –Measured glucose concentration, one decimal place accuracy
MC - 測量的葡萄糖濃度，小數點後一位精準度

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Dependence of Blood Glucose Measurement on Sample Temperature 血糖測量對樣本溫度的依賴性

Glucose concentration values measured by some glucometers are affected by temperature. To confirm or refute this, you will measure the glucose concentration of the solution previously diluted 1:2 at different temperatures.

有些血糖儀測量的葡萄糖濃度值會受溫度影響。為了確認或反駁這一點，您將測量在不同溫度下以 1：2 稀釋的葡萄糖溶液的濃度。

Procedure 實驗步驟

1. Label 4 Eppendorf tubes with the respective temperature. You are going to measure the glucose concentrations at 0 °C, 10 °C, room temperature (RT) and 37 °C. **On the tube for 37 °C, also add your competitor´s ID number legibly.** 將 4 個微量離心管標記好各種溫度。您將在 0°C，10°C，室溫（RT）和 37°C 下測量葡萄糖濃度。**在 37°C 的管子上，還要清楚地加標上參賽者的編號。**
2. To each tube, add 50 µL of the 1:2 diluted glucose solution. 向每個管中各加入 50µL 的 1：2 稀釋的葡萄糖溶液。
3. Move the solutions to the respective temperatures: 0 °C in ice water, 10-15 °C by cooling room temperature water with ice and controlling the temperature with a thermometer, room temperature left on the rack and 37 °C in the dry thermostat. Leave the solutions to equilibrate for at least 5 minutes. You´ll have to pay attention to keep the 10-15 °C beaker at the right temperature range. 將溶液移至各自的溫度：冰水 0°C，將室溫水用冰冷卻至 10-15°C，用溫度計控制溫度，將室溫管子置於管架上，乾浴槽溫度設為 37°C。讓溶液平衡至少 5 分鐘。您必須**注意**將 10-15°C 的燒杯保持在合適的溫度範圍內。
4. After at least 5 minutes, measure the glucose concentrations with the glucometer. You have to work quickly at this point, not to let the samples differ too much from the required temperature. 至少 5 分鐘後，用血糖儀測量葡萄糖濃度。此時您必須快速工作，不要讓樣品與所需溫度相差太大。

Q.2.1.2 Record the results of your measurements in the table on the answer sheet.
在答題紙上的表格中記錄您的測量結果。
Table Legend: 表格說明：
T –solution temperature RT –room temperature MC –Measured glucose concentration, one decimal place accuracy T - 溶液溫度 RT - 室溫 MC - 測量的葡萄糖濃度，小數點後一位精準度

When you´re finished, signal with the flag. For Part 2, an assistant will hand you the results of related experiments, which you´ll have to analyze. Your own measurement results will be taped over with translucent tape so beyond this point, no more changes on part 1 answers will be possible.

當您完成後，**使用小旗子通知助教**。在第 2 部分，助理將提供您另一相關實驗的結果，您必須對其進行分析。您自己的測量結果將被用半透明膠帶覆蓋，因此在**此之後，第 1 部分的答案將無法再進行更改。**

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Part 2. Preparation of a Calibration Curve Based on Pre-Acquired Results 第 2 部分。先針對已獲得 (Pre-Acquired) 的結果製備校正曲線

Presented are the results of several glucometer measurements of glucose concentrations in aqueous glucose solutions analogous to the measurements you carried out in Part 1 此處提供的數據為用幾種血糖儀量測葡萄糖水溶液中葡萄糖濃度之結果，類似於您在第 1 部分中進行的測量實驗結果

SC [mmol/L]	MC [mmol/L]
4.0	4.5
6.0	6.4
8.3	8.0
9.9	9.6
13.6	13.3
16.0	15.0

Legend: SC –standard glucose concentration. MC –glucose concentration measured by glucometer

說明：SC - 標準葡萄糖濃度。MC-用血糖儀測量得之葡萄糖濃度

Q.2.1.3 Plot the measured concentrations (y) against the standard concentration (x) in Graph 1 on the answer sheet as a scatter plot. Use small but defined dots, less than 2 mm of diameter. Pay attention to the correct labeling of the axes; use the abbreviations in the table above. 將測量濃度（y）與答案紙上圖 1 中的標準濃度（x）繪製成點狀圖。使用小但明確的點，直徑要小於 2 毫米。注意在軸上標示正確的標記; 並使用上表中所示的縮寫。

Q.2.1.4 Applying simple linear regression, calculate the regression equation for the best fitting line using the least square method. Calculate at 2 decimals accuracy. Use the equation below. Make sure to include the calculated values in the table on the answer sheet. The grey columns are provided for convenient calculation and will not be marked.
應用簡單線性回歸，使用最小平方法計算最適線的回歸方程。計算小數點後 2 位的精準度。使用下面的等式。使用下面的方程式, 確記使用在答案紙上表格中所記的計算值。所提供的灰色列是為了方便計算，不會被記分。
Table Legend: SC –standard glucose concentration MC –glucose concentration measured by glucometer n –number of measurement
表格說明： SC - 標準葡萄糖 濃度 MC-用血糖儀測量得之葡萄糖濃度 n - 測量次數

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Q.2.1.5 Draw your calculated line in Graph 1 according to the completed formula below.
根據下面的完整公式在圖 1 中繪製所計算出的線。
Regression equation: $y = a + bx$
回歸方程式：$y = a + bx$

$$s_x = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

$$s_y = \sqrt{\frac{\sum(y - \bar{y})^2}{n - 1}}$$

$$r = \frac{((x - \bar{x})(y - \bar{y}))}{\sqrt{\sum(x - \bar{x})^2 \sum(y - \bar{y})^2}}$$

$$b = r\frac{s_y}{s_x}$$

$$a = \bar{y} - b\bar{x}$$

其中 n = 測量次數

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Comparison of Blood Glucometer Readings at Different Temperatures 不同溫度下血糖儀量測讀值的比較

S	L15	R30
1	5.4	6.7
2	4.2	5.2
3	6.0	6.8
4	4.4	6.2
5	5.7	9.4
6	4.6	5.5
7	5.1	8.7
8	5.9	8.6
9	3.7	2.6
10	4.4	4.4
11	4.8	7.0
12	5.6	8.9
13	3.9	5.7
14	4.3	6.4
15	4.5	5.4

Legend: n–subject number. L15 –glucose meter reading at 15 °C. R30 –glucose meter reading at 30 °C

說明：n- 測試編號。 L15 - 血糖儀在 15°C 讀數。 R30 - 血糖儀在 30°C 讀數

Q.2.1.6 On the answer sheet, fill in the table with values of 2 decimals precision (the grey columns are provided for convenient calculation and will not be marked).
在答題卷上，以小數點下 2 位精準度值填寫表格（提供灰色列以方便計算，不會被記分）。
Table Legend:
D –the difference (L15 - R30) for each test subject;
n –number of test subjects;
df –degrees of freedom
表格說明：
D - 每個受測試樣品的差異（L15-R30）；
n - 測試樣品的數目;
df - 自由度

Q.2.1.7 Perform a paired Student´s t test to determine if there´s a difference between the readings at different temperatures at a significance level of α=0.001. Indicate your calculated t value（t_{stat}）and critical t value（t_{crit}）according to the t value distribution table, with 2 decimals precision.(See formula below) 進行配對學生 t 檢測 (Student´s t test)，以確定在不同溫度下讀值是否存在顯著性差異 $\alpha = 0.001$ 。根據 t 值分佈表以呈現您計算的 t 值（t_{stat}）和臨界 t 值（t_{crit}），精準度在小數點下 2 位。
(請參考下列公式)

Decide whether the following statements are true (T) or false (F) considering your calculations. Place one X per statement in one of the boxes in the table on the answer sheet.

參考您的計算，決定以下陳述是真（T）還是假（F）。在答案紙上的表格的框框中以 X 標記出您的答案。

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Q.2.1.8 The null hypothesis assumes that the true mean difference between the 15 °C and 30 °C measurements is zero.
虛無假說 (null hypothesis) 假設 15°C 和 30°C 測量值之間的真實平均差值為零。

Q.2.1.9 The value of t_{stat} is lower than the value of t_{crit}, therefore the null hypothesis cannot be rejected.
t_{stat} 值低於 t_{crit} 值時則虛無假說成立。

$$t = \frac{\sum D}{\sqrt{\frac{n\sum D^2 - (\sum D)^2}{n-1}}}$$

Legend: D –the difference (L15 - R30) for each test subject; n –number of test subjects; tstat –t value of the statistics; tcrit –critical value of t according to the t distribution table
說明：D - 每個受測樣品的差異（L15 - R30）; n - 測試樣品的數量; tstat - t 統計值; tcrit - 根據 t 分佈表的 t 的臨界值

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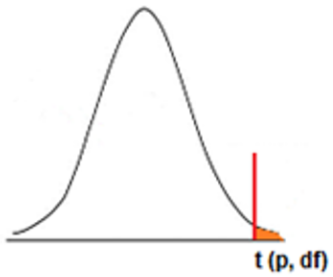
右側 t 分佈



df/p	0.40	0.25	0.10	0.05	0.025	0.01	0.005	0.0005
1	0.324920	1.000000	3.077684	6.313752	12.70620	31.82052	63.65674	636.6192
2	0.288675	0.816497	1.885618	2.919986	4.30265	6.96456	9.92484	31.5991
3	0.276671	0.764892	1.637744	2.353363	3.18245	4.54070	5.84091	12.9240
4	0.270722	0.740697	1.533206	2.131847	2.77645	3.74695	4.60409	8.6103
5	0.267181	0.726687	1.475884	2.015048	2.57058	3.36493	4.03214	6.8688
6	0.264835	0.717558	1.439756	1.943180	2.44691	3.14267	3.70743	5.9588
7	0.263167	0.711142	1.414924	1.894579	2.36462	2.99795	3.49948	5.4079
8	0.261921	0.706387	1.396815	1.859548	2.30600	2.89646	3.35539	5.0413
9	0.260955	0.702722	1.383029	1.833113	2.26216	2.82144	3.24984	4.7809
10	0.260185	0.699812	1.372184	1.812461	2.22814	2.76377	3.16927	4.5869
11	0.259556	0.697445	1.363430	1.795885	2.20099	2.71808	3.10581	4.4370
12	0.259033	0.695483	1.356217	1.782288	2.17881	2.68100	3.05454	4.3178
13	0.258591	0.693829	1.350171	1.770933	2.16037	2.65031	3.01228	4.2208
14	0.258213	0.692417	1.345030	1.761310	2.14479	2.62449	2.97684	4.1405
15	0.257885	0.691197	1.340606	1.753050	2.13145	2.60248	2.94671	4.0728
16	0.257599	0.690132	1.336757	1.745884	2.11991	2.58349	2.92078	4.0150
17	0.257347	0.689195	1.333379	1.739607	2.10982	2.56693	2.89823	3.9651
18	0.257123	0.688364	1.330391	1.734064	2.10092	2.55238	2.87844	3.9216
19	0.256923	0.687621	1.327728	1.729133	2.09302	2.53948	2.86093	3.8834
20	0.256743	0.686954	1.325341	1.724718	2.08596	2.52798	2.84534	3.8495
21	0.256580	0.686352	1.323188	1.720743	2.07961	2.51765	2.83136	3.8193
22	0.256432	0.685805	1.321237	1.717144	2.07387	2.50832	2.81876	3.7921
23	0.256297	0.685306	1.319460	1.713872	2.06866	2.49987	2.80734	3.7676
24	0.256173	0.684850	1.317836	1.710882	2.06390	2.49216	2.79694	3.7454
25	0.256060	0.684430	1.316345	1.708141	2.05954	2.48511	2.78744	3.7251
26	0.255955	0.684043	1.314972	1.705618	2.05553	2.47863	2.77871	3.7066
27	0.255858	0.683685	1.313703	1.703288	2.05183	2.47266	2.77068	3.6896
28	0.255768	0.683353	1.312527	1.701131	2.04841	2.46714	2.76326	3.6739
29	0.255684	0.683044	1.311434	1.699127	2.04523	2.46202	2.75639	3.6594
30	0.255605	0.682756	1.310415	1.697261	2.04227	2.45726	2.75000	3.6460
∞	0.253347	0.674490	1.281552	1.644854	1.95996	2.32635	2.57583	3.2905

PREPARATION PRINT

SUBTASK 2. EXAMINATION OF KNOCK-OUT MUTANT CELL LINES PRODUCED BY CRISPR-CAS9 METHOD 子任務 2. CRISPR-CAS9 方法產生的基因剔除突變細胞系的檢測

In this practical task you will examine constructs created with a modern gene-editing procedure, the CRISPR-Cas9 technique. With this technique, we can ‘knock out’ (KO) genes much more efficiently and quickly relative to the previously used homologous recombination technique.

在這個實作中，您將檢查使用現代基因編輯 CRISPR-Cas9 技術所建構的突變體。使用這種技術，相對於先前使用的同源重組技術，我們可以更有效和快速地“剔除”（KO）基因。

This made it easier than ever to investigate the effect of the lack of a gene selected by us. To facilitate the workflow even further, an antibiotic resistance gene may also be used which is incorporated into our target gene. This not only renders the target gene non-functional, but also provides an easy means of selecting successfully mutated cells by adding the appropriate antibiotic to the growth medium

這使我們比以往更容易探究當我們選擇的基因缺乏時所造成的影響。為了進一步促進工作流程，可將抗生素抗藥性基因先植入到我們選定的標的基因中。這不僅使標的基因失去功能，而且還提供了在生長培養基中添加適當的抗生素來篩選編輯成功的突變細胞之簡易方法。

Part 1. The theory of PCR based gene insertion detection 第 1 部分 以 PCR 方法檢測基因插入的原理

The following task describes the testing of CRISPR induced mutant cell lines using a PCR and restriction digest based method. Assume that all of the experiments are carried out on diploid mammalian cells.

以下任務使用 PCR 和限制酶切割方法檢測 CRISPR 誘導的突變細胞系。假設所有實驗都在二倍體哺乳動物細胞中進行。

Using our CRISPR system, we insert the antibiotic resistance gene (PURO) that codes for an enzyme cleaving the antibiotic puromycin. Due to the characteristics of this method, the PURO gene may be inserted both ways.

在我們的 CRISPR 系統中, 我們將編碼切割抗生素嘌呤黴素 (puromycin) 酶的抗藥性基因（PURO）插入。由於該方法的特徵，PURO 基因可以兩種型式插入。

In many cases –not discussed in detail here –the orientation of insertion has a significant impact on the outcome of the entire experiment, so as a rule, the orientation of insertion should be checked.

使用我們的 CRISPR 系統，在許多情況下 - 這裡不會詳細討論 - 插入方向對整個實驗的結果有顯著影響，因此通常應檢查其插入方向。

In order to achieve a complete KO we should also determine whether the resistance gene has been inserted into only one or both chromosomes, since a non-functional KO mutant can only expected in the latter case.

為了獲得完整的剔除突變體，我們還應該確定抗性基因是否僅插入到一個或兩個染色體中，因為非功能性基因剔除突變體只能在後一種情況下獲得。

Last but not least, it should also be checked that the PURO gene is inserted into the desired target area or if it is at a different site. Some of these different scenarios are illustrated in Fig. 1. Please study this figure before proceeding.

最後還應檢查 PURO 基因是否插入到標的基因所在的區域, 或插到不同的位點。其中一些不同的情況呈現如圖 1 所示。請在進行下去前先研讀此圖 1。

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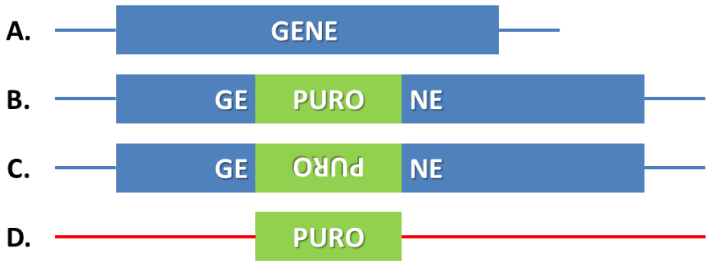

Fig. 1. A.: target gene; B.: PURO insertion in forward orientation; C.: PURO insertion in reverse orientation; D.: PURO insertion into the genome at a site other than the target gene.

圖 1. A：標的基因; B：PURO 基因以前向 (forward direction) 方式插入; C：PURO 基因以反向插入; D：PURO 基因插入到標的基因以外的基因組位點中。

To examine the above insertions, a PCR reaction is designed using three kind of primers. We design primers to match both ends of the original gene, and a third primer to match the end of the PURO gene opposite the promoter region (i.e., it is complementary to the 3' end).

為了檢測區分上述各種插入情形，我們設計三種引子, 兩種分別與各原基因的兩端配對, 第三種引子能與 PURO 基因啟動子區域相對的 PURO 基因的末端 (即其與基因的 3' 末端) 配對互補。

Then we examine the products obtained in a normal PCR reaction. It is a well-known phenomenon that in the case of competitive primers appearing during a PCR reaction, the amplification of shorter DNA region is expected to be predominant. In the following simplified gel images the less intense bands are not indicated.

接下來我們檢查正常 PCR 反應中獲得的產物。眾所周知的現象是在 PCR 反應中有競爭性引子存在的情況下，預期主要擴增產物為較短的 DNA 產物。下圖為簡化的凝膠圖 像，其中較不強的條帶並未顯示出。

Q.2.2.1 Match the lanes (1-5) of the gel image of PCR products (Fig. 2) with the schematics of potential genomic insertions (Fig. 3, A-F) with an X in your answer sheet.
將 PCR 產物的凝膠電泳圖像（圖 2）的泳 道（1-5）與可能的基因組插入（圖 3，A-F）示意圖相匹配，並在答題紙中以 **X 標註.**

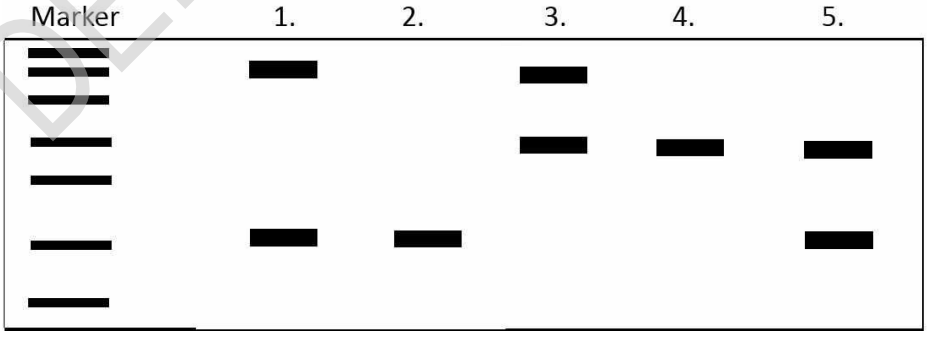

Fig. 2. Image of gel electrophoresis of PCR products

圖 2. PCR 產物的凝膠電泳圖像

DELEGATION PRINT

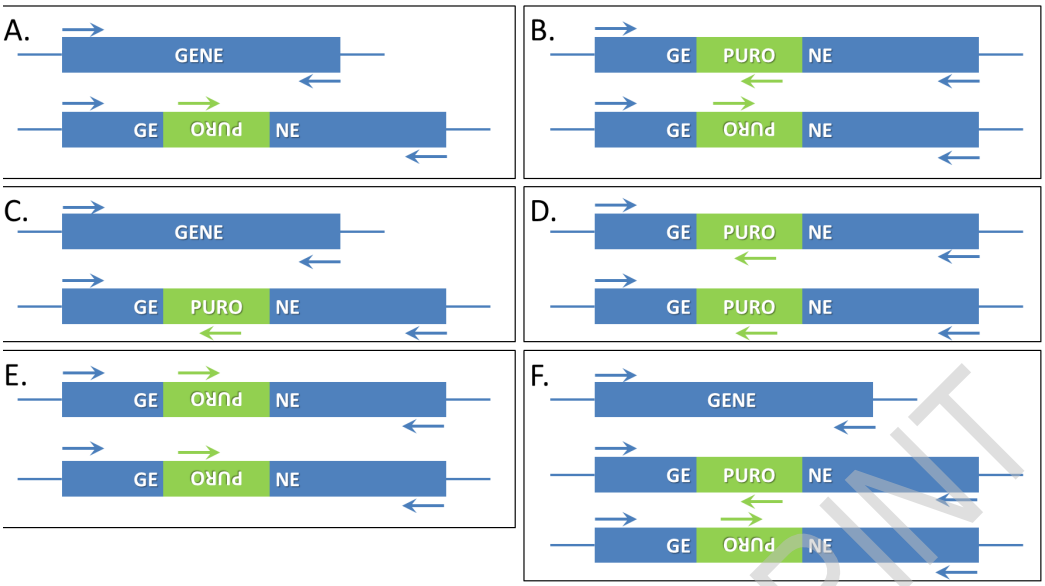

Fig. 3. Possible insertion scenarios

圖 3. 可能的基因插入方案

Now that you understand how to check in general the asymmetrical insertion characteristics of the PURO gene in diploid cells using a PCR reaction, you may face another issue in relation to research work.

既然您已了解如何使用 PCR 反應檢查基因在二倍體細胞中的不對稱插入特徵，您可能會面臨與研究工作相關的另一個問題。

Sometimes it occurs that –again for reasons not described here in detail –the PURO gene has to be symmetrically inserted in the middle region of the target gene.

有時它會發生 PURO 基因對稱插入標的基因的中間區域，其原因在此處未詳細描述。

Suppose that the 500-bp long PURO gene is to be inserted into the center of a 1400-bp gene. It is obvious that in this case the orientation of insertion cannot be detected by the above simple 3-primer PCR reaction, because the sizes of the resulting products are the same,

假設將 500bp 長的 PURO 基因插入 1400bp 基因的中心。顯然，在這種情況下，通過上述簡單的 3-引子 PCR 反應不能區分插入的方向，因為無論插入方向如何，所得產物的大小都是相同的。

Considering that in this size range, a difference of 100 bp is hardly visible on gels with such resolution, the above statement can be extended to cases where insertion takes place near the cent of the target.

考慮到在這個尺寸範圍內，在具有這種分辨率的凝膠上幾乎無法分辨 100bp 差異的片段，當基因插入在目標基因中心附近時 也會出現無法區分的情況。

In some fortunate cases, however, both the target gene and the PURO gene already contain a restriction enzyme cleavage site in an asymmetrical position. In this case, after the restriction cleavage of the PCR product prepared using 2 primers complementary to the ends of the target gene, the orientation of insertion can still be determined. Study Fig. 4 in the given model system.

然而，在一些幸運的情況下，當標的基因和 PURO 基因都具有不對稱限制酶切割位點時。在這種情況下，可使用與標的基因的兩端互補的 2 個引子進行製備 PCR 產物，經限制酶切割後，仍可區分並確定基因插入的方向。請探究圖 4 所給的模式系統。

DELEGATION PRINT

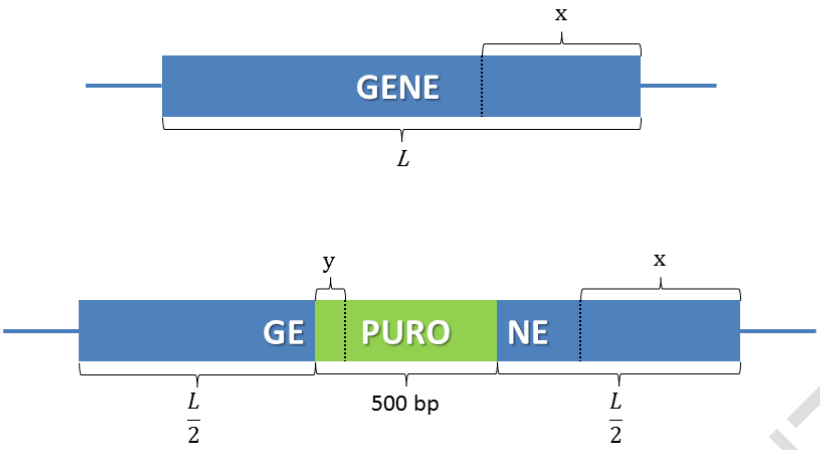

Fig. 4 Schematic of the restriction based insertion mapping. L = length of the target gene in base pairs, x = distance of the restriction site from the end in the target gene (in bp), y = distance of the restriction site from the end in the PURO gene.

圖 4 限制酶切割的基因插入圖譜分析示意圖。L = 標的基因的長度 (以鹼基對計)，x = 標的基因中限制酶切割位點與基因末端的距離 (以鹼基對計)，y = 限制酶切割位點與 PURO 基因末端的距離。

	L	x	y
A	1400	500	100
B	1000	500	100
C	1400		
D	1000		

Table 1. L = Length of the target gene in base pairs (bp). x = distance of the restriction site from the end in the target gene (bp), y = distance of the restriction site from the end in the PURO gene (bp).

表 1. L = 標的基因的鹼基對長度（bp）。x = 限制酶切割位點與標的基因末端的距離（bp)，y = 限制酶切割位點與 PURO 基因末端的距離（bp）。

In Table 1 you can see different symmetrical insertion scenarios as shown in Fig. 4. Suppose that the products are amplified with primers complementing the ends of the target gene and then fully cleaved with restriction enzyme E.

在表 1 中，您可以看到如圖 4 所示不同的對稱性基因插入的情況。假設用與標的基因末端配對的引子擴增產物，該產物再用限制酶 E 進行完全切割。

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Q.2.2.2 A - How many DNA bands are visible during electrophoresis after enzymatic digestion when the PURO gene is inserted in the same orientation as the target gene in Case A Table 1.?
如在表 1 的 A 中，當 PURO 基因以與標的基因相同的方向插入時，限制酶消化後電泳過程中可見到多少條 DNA?
B - How many DNA bands are visible during electrophoresis after enzymatic digestion when the PURO gene is inserted in the opposite orientation as the target gene?
當 PURO 基因以與標的基因相反的方向插入時，限制酶切割後電泳過程中可見多少 DNA 條帶？
C - How many bp is the size of the longest DNA band after enzymatic digestion when the PURO gene is inserted in the opposite orientation as the target gene?
當 PURO 基因以與標的基因相反的方向插入時，限制酶切割後最長 DNA 條帶的大小是多少 bp？
D - How many bp is the size of the shortest DNA band after enzymatic digestion when the PURO gene is inserted in the opposite orientation as the target gene?
當 PURO 基因以與標的基因相反的方向插入時，限制酶切割後最短 DNA 條帶的大小是多少 bp？

Q.2.2.3 Indicate the letter of the scenario (A-D) with an X in which not even the restriction digestion method helps to determine the orientation of insertion.
以 **X 標示**出無法以限制酶切割方法來確定其基因插入的方向性的 A-D 字母所代表的各種基因插入型態。

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Part 2. Restriction mapping of gene insertion 第 2 部分基因插入的限制酶圖譜製備譜

Now that you understand how this procedure works, you will examine an actual gene insertion process. In the current practical, we attempted to insert the PURO gene with a length of 500 bp in the centre of a 1000-bp long target gene (for clarity’s sake, precisely after the 500th base pair) using the CRISPR technique.

現在您已了解此過程如何運作，您將檢測實際基因插入程序之結果。在目前的實際應用中，我們嘗試使用 CRISPR 技術將長度為 500bp 的 PURO 基因插入到 1000bp 長的標的基因的中心（為了清楚起見，恰好在第 500 鹼基對之後）。

It is also known that the value of X also shown in Fig. 4 is 300 bp, whereas the value of Y is 100 bp.

已知圖 4 中也顯示的 X 值為 300 bp，而 Y 的值為 100 bp。

The researchers carried out the insertion by CRISPR and selected five genetically stable, puromycin-resistant cell cultures (P1 to P5). Subsequently, DNA from each culture was isolated, and the DNA region of interest was amplified using a primer pair complementary to both ends of the target gene.

研究人員通過 CRISPR 進行插入並篩選了 5 種遺傳穩定的嘌呤黴素抗藥性細胞（P1 至 P5）。隨後，並分離各種抗藥性細胞的 DNA，並使用與標的基因兩端互補的引子進行擴增其間的標的基因 DNA 片段。

Of the five experimental products, you will examine two: a completely non-functional KO mutant (P1) and a mutant with partial loss of function (P2), in which RNA of normal length is transcribed from the target gene at a rate of approx. 50% in the cells. The goal of your examination is to carry out enzyme restriction cleavage with the obtained products and to separate the digestion products by gel electrophoresis.

在五個實驗產品中，您將檢測兩個突變體：其中一個為完全無功能的基因剔除突變體（P1）和另一個功能部分喪失的突變體（P2），在 P2 中，正常長度的 RNA 以大約 50% 的速率從標的基因轉錄出。您的檢查目標是使用已獲得的產物進行限制酶切割，並進行凝膠電泳分析限制酶切割的產物。

Procedure: **程序：**

IMPORTANT! The samples for the DNA analysis are provided in coloured microcentrifuge tubes, in a storage box.
重要！用於 DNA 分析的樣品在有色微量離心管中，並置放在儲存盒中。

- Microcentrifuge tube labelled ‘M’: size ladder marker 標記為“M”的微量離心管內為 DNA ladder marker 樣品
- Microcentrifuge tube labelled ‘C’: control which is the PCR product of the original gene 標記為“C”的微量離心管：控制組，為原始基因的 PCR 產物
- Microcentrifuge tube labelled ‘P1’: PCR product of the sample taken from P1 type cells 標記為“P1”的微量離心管：為 P1 細胞突變體的 DNA 樣品經 PCR 的產物
- Microcentrifuge tube labelled ‘P2’: PCR product of the sample taken from P2 type cells 標記為“P2”的微量離心管：取自 P2 細胞突變體的 DNA 樣品之 PCR 產物
- Microcentrifuge tube labelled ‘E’: restriction endonuclease (PstI.) –held on ice! 標記為’E’ 的微量離心管：限制性核酸內切酶（PstI 。）- 保持在冰上！

1. The DNA samples already contain the loading dye, which does not interfere with the enzyme activity. DNA 樣本中已含有不會干擾酶活性的注膠染劑。

2. Add 2 µl of ‘E’ to tubes ‘P1’ and ‘P2’ using the 2–20 µl micropipette respectively. Set the pipette to 18 µl and mix the contents of the tube by aspirating and releasing it with the pipette. 以 2-20 μl 的微量吸管加 2 μl 的’E’ 到’P1’ 和’P2’ 管中。將吸管調至 18 μl 並將管內混合物吸取並釋放方式充分混合。

3. Clearly label your tube with the abbreviation of the name of country. 在你的管子上清楚標上您國家名稱的縮寫。

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4. Place the tubes in the designated dry thermostat (intended for shared use), which is already set at the digestion temperature of 37°C, for at least 15 minutes (no need to be accurate to the second). The accuracy of the wall clock in the room is sufficient. 將試管放入指定的乾浴槽中（共用儀器），該乾浴槽已經設定在 37°C 的切割溫度下至少 15 分鐘（無需精確到秒）。牆上掛鐘的準確性是足夠的。

5. At the end of the incubation period, remove tubes ‘P1’ and ‘P2’ from the dry thermostat and place them on ice for approx. 1 minute, then put them back in the tube storage box. 在作用結束時，從乾浴槽中取出管’ P1’ 和’ P2’ ，並將它們置於冰上約 1 分鐘。然後將它們放回管子儲存盒中。

6. Remove the orange lid of the electrophoresis equipment. Check the buffer level in the equipment. The buffer should cover the gel completely. If this is not the case, pour some buffer into the gel tank. You can ask the lab assistant for help by raising your red card. 取下電泳儀器的橙色蓋子。檢查儀器中的緩衝液含量。緩衝液應完全覆蓋到凝膠上。如果不是這種情況，請補充足夠的緩衝液。您可以舉高紅牌向實驗室助理尋求幫助。

7. Transfer 18 µl into each well of the gel as follows: 在凝膠的樣品槽中注入 18 μl 的樣品：(如下)

Well 1: M 樣品槽 1：M

Well 2: C 樣品槽 2：C

Well 3: P1 樣品槽 3: P1

Well 4: P2 樣品槽 4：P2

8. Put the orange lid back on the electrophoresis equipment and inform the lab assistant by raising your yellow card.

將橙色蓋子放回電泳儀器上，然後舉起**黃卡**知會實驗助理。

Q.2.2.4 Your lab assistant will note the time you started your gel (START). Do not write in that field. 您的實驗助教會記下您開始凝膠電泳的時間（START）。不要在該區域寫字。

9. Get the gel running with the help of the lab assistant. You can turn off the equipment with the power switch.

在實驗助教的協助下進行凝膠電泳。您可以將儀器電源開關關閉。

10. During the run, you can check the DNA bands by turning on the lights. 在電泳運行中，您可以打開燈來檢查 DNA 條帶。

11. Run the gel for 20 minutes, gels run for longer or shorter times will get partial marks. When finished, call for the lab assistant immediately who will take a picture of your gel. 凝膠電泳進行 20 分鐘，如凝膠電泳進行時間過長或過短將只得到部分分數。當您完成電泳後，請立即通知實驗助理來為您的凝膠拍照。

12. Signal to the lab assistant by raising your **yellow card**.

舉高你的**黃牌通知**實驗助教。

Q.2.2.4 Your lab assistant will note the time at the end of your gel run (END) and photograph your gel. Your gel running time (TIME) will be calculated based on your start and end times. Do not write in these fields.
您的實驗助教將記錄凝膠電泳運行的結束時時間（END）並為凝膠拍照。將根據您開始和結束電泳時間計算您的凝膠運行時間（TIME）。請不要在這些區域寫字。

Q.2.2.5 Enter the three-digit number located on the gel tray on the answer sheet (CODE). Your score will be identified based on this.
在答案卷上寫下凝膠托盤上方的三位數字（CODE）。您的分數將以此來確認。

If you have entered the number on the sheet and the picture has also been taken, you will receive a virtual gel image from the lab assistant. Use this image to answer the following question. Lane C is the control, non-digested sample. Lanes (P1-P5) are five puromycin resistant cell lines.

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如果您已在答案卷上輸入了數字並且也拍攝了照片，您將從實驗室助理處收到**虛擬凝膠圖像**。**使用此圖片來回答以下問題。**泳道 C 是對照組未切割的樣品。泳道（P1-P5）是五種原始的嘌呤黴素抗藥性細胞系。

Q.2.2.6 Indicate with an X in your answer sheet all type(s) of PURO insertions, as seen in Fig. 1 (A-D), found in samples P1-P5 on the gel image you received.
在您的答案卷上用 **X 標**示出您收到的膠體影像中 P1-P5 樣品所具有的**如圖 1** 中（A-D）所示的 PURO 插入的所有類型。

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