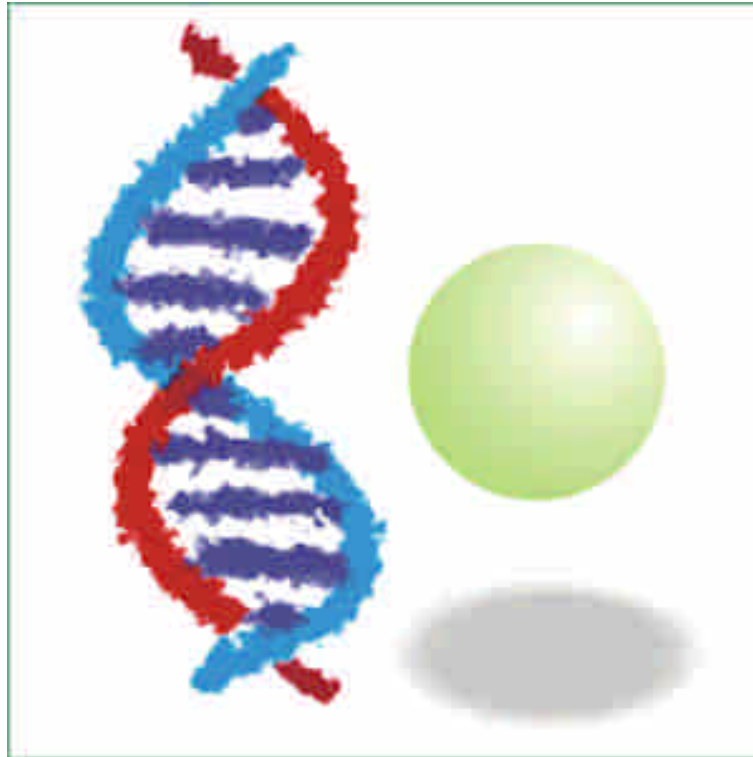


BST
生化科技系

BCX



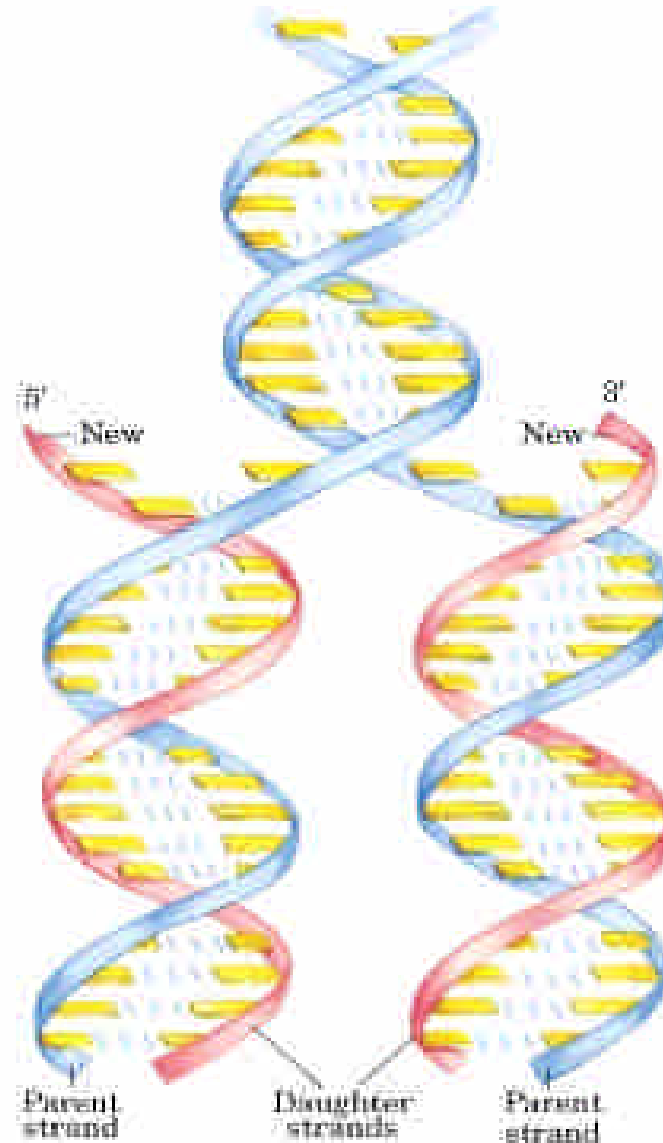
N2

生物化學實驗

Polymerase Chain Reaction

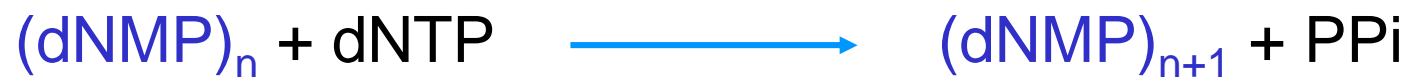
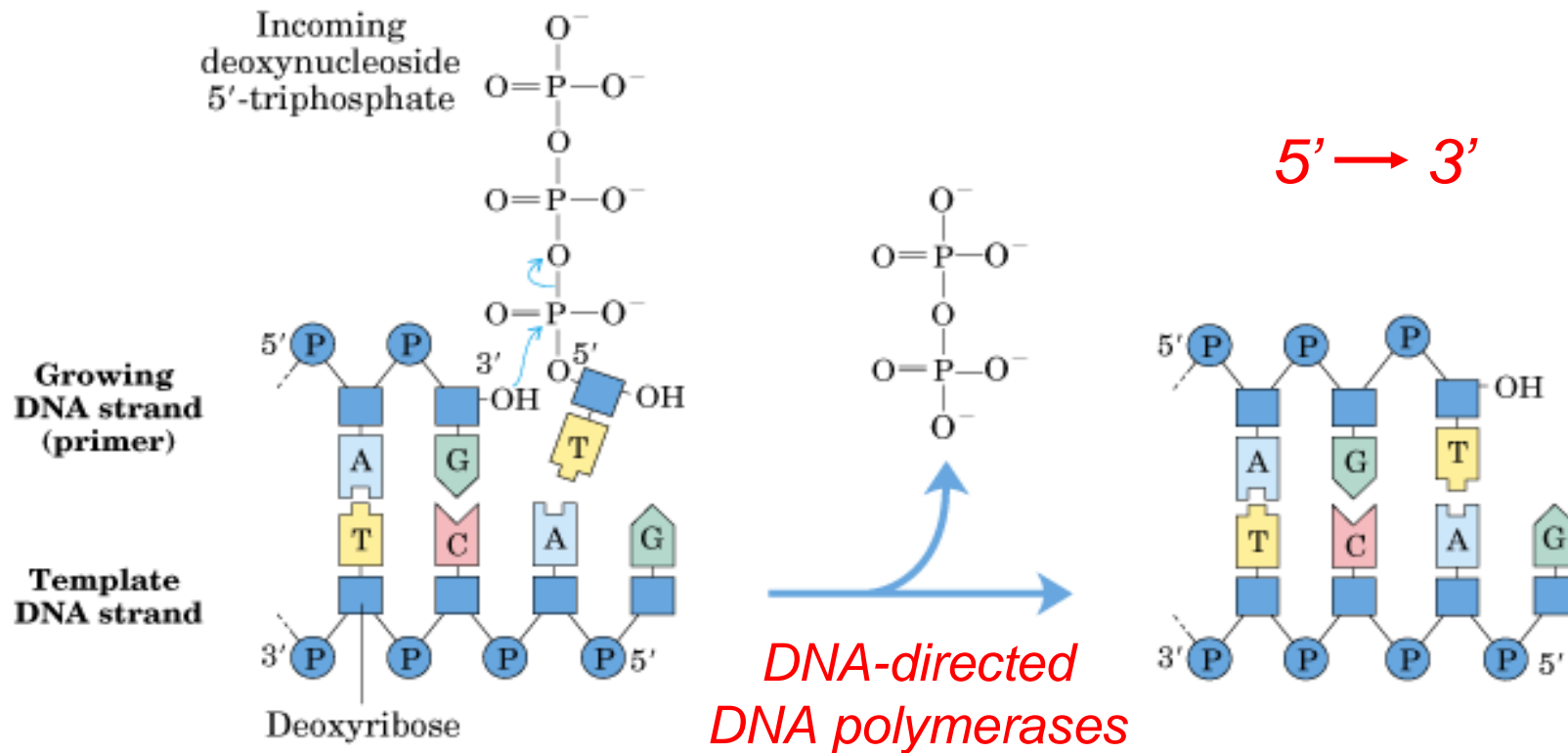
■ 細胞中 DNA 的複製模式：

Semiconservative replication

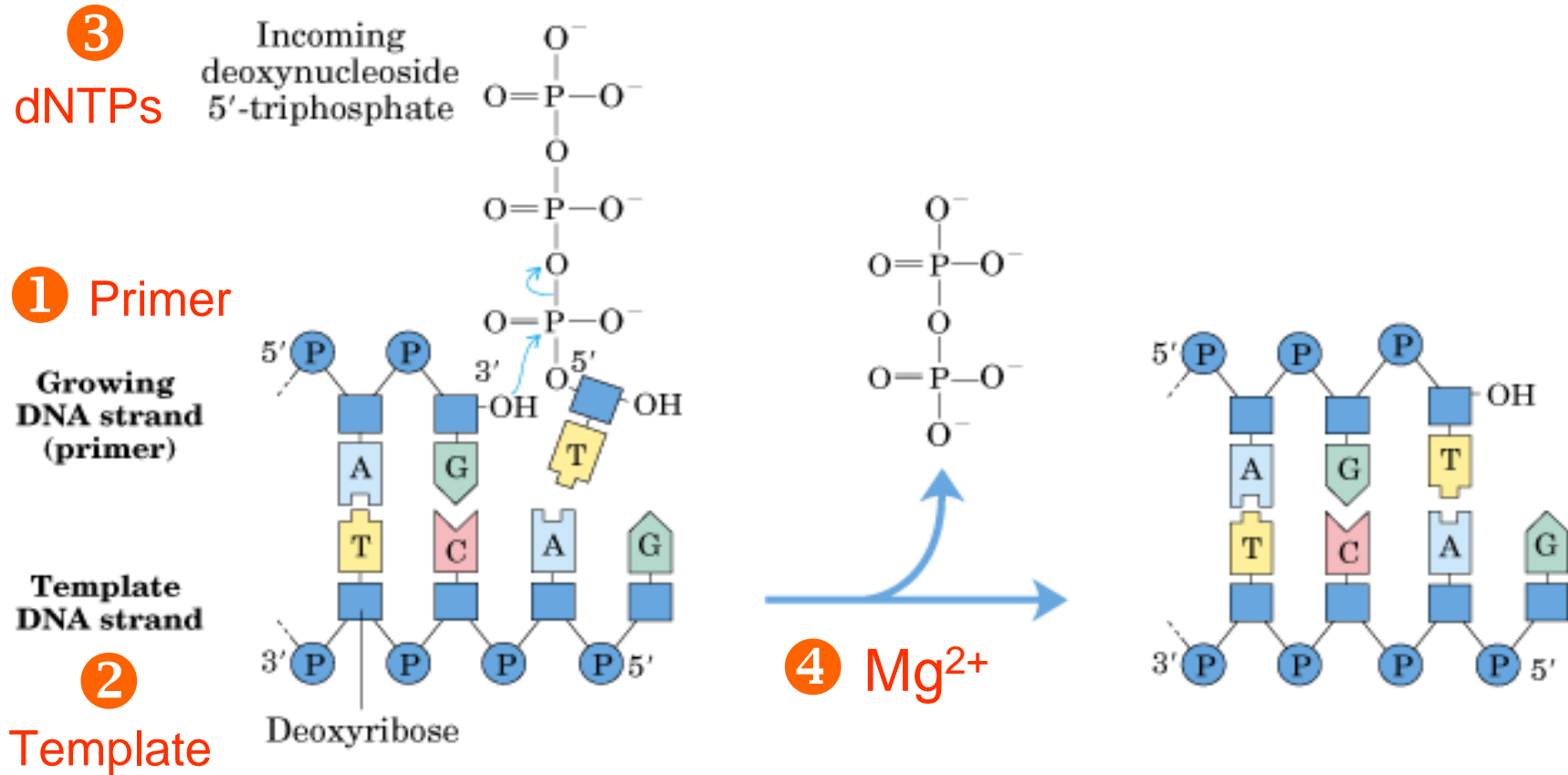


圖引用自：Nelson, D. L. and Cox, M. M. (2000) *Lehninger Principles of Biochemistry*. 3rd Ed., Worth Publishers. Fig. 10-17

■ DNA polymerase 催化細胞中 DNA 的複製：



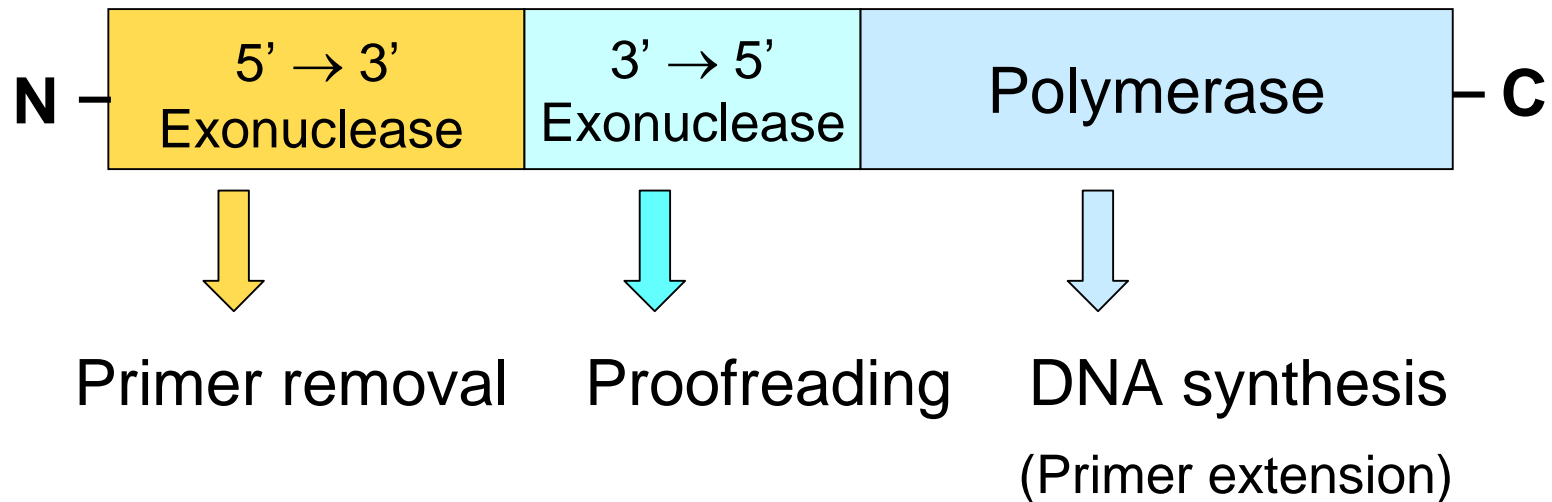
DNA-directed DNA polymerase 反應需求：



圖引用自：Nelson, D. L. and Cox, M. M. (2000) Lehninger Principles of Biochemistry. 3rd Ed., Worth Publishers. Fig. 25-5

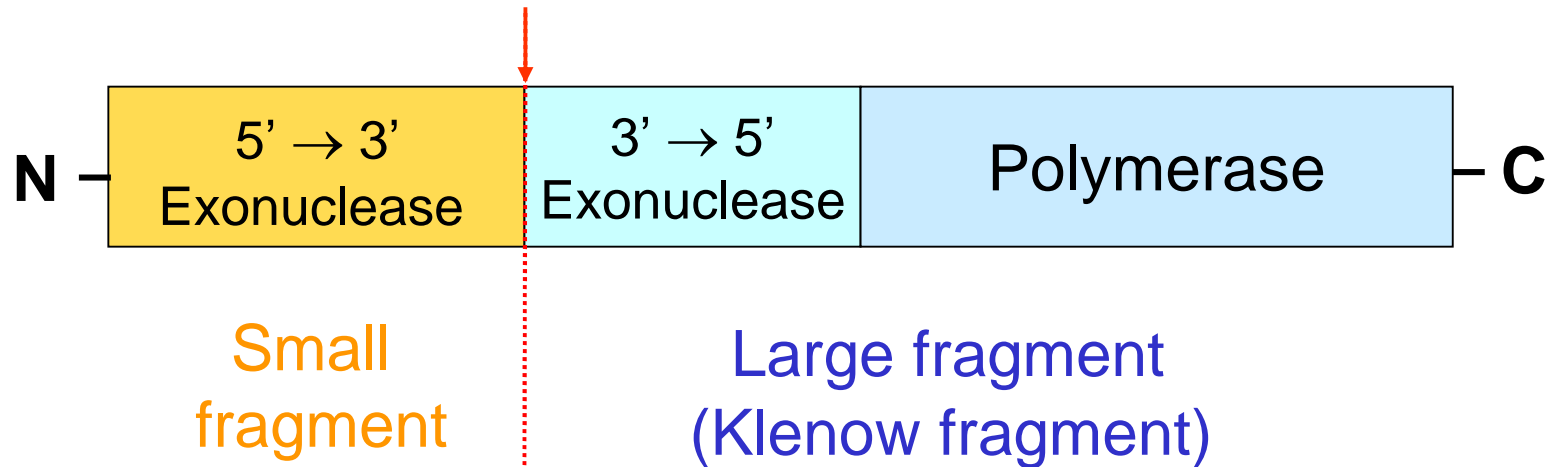
■ DNA polymerase I of *E. coli* :

- ▶ 第一個被發現的 DNA polymerase
- ▶ 103 kD, 928 amino acid residues



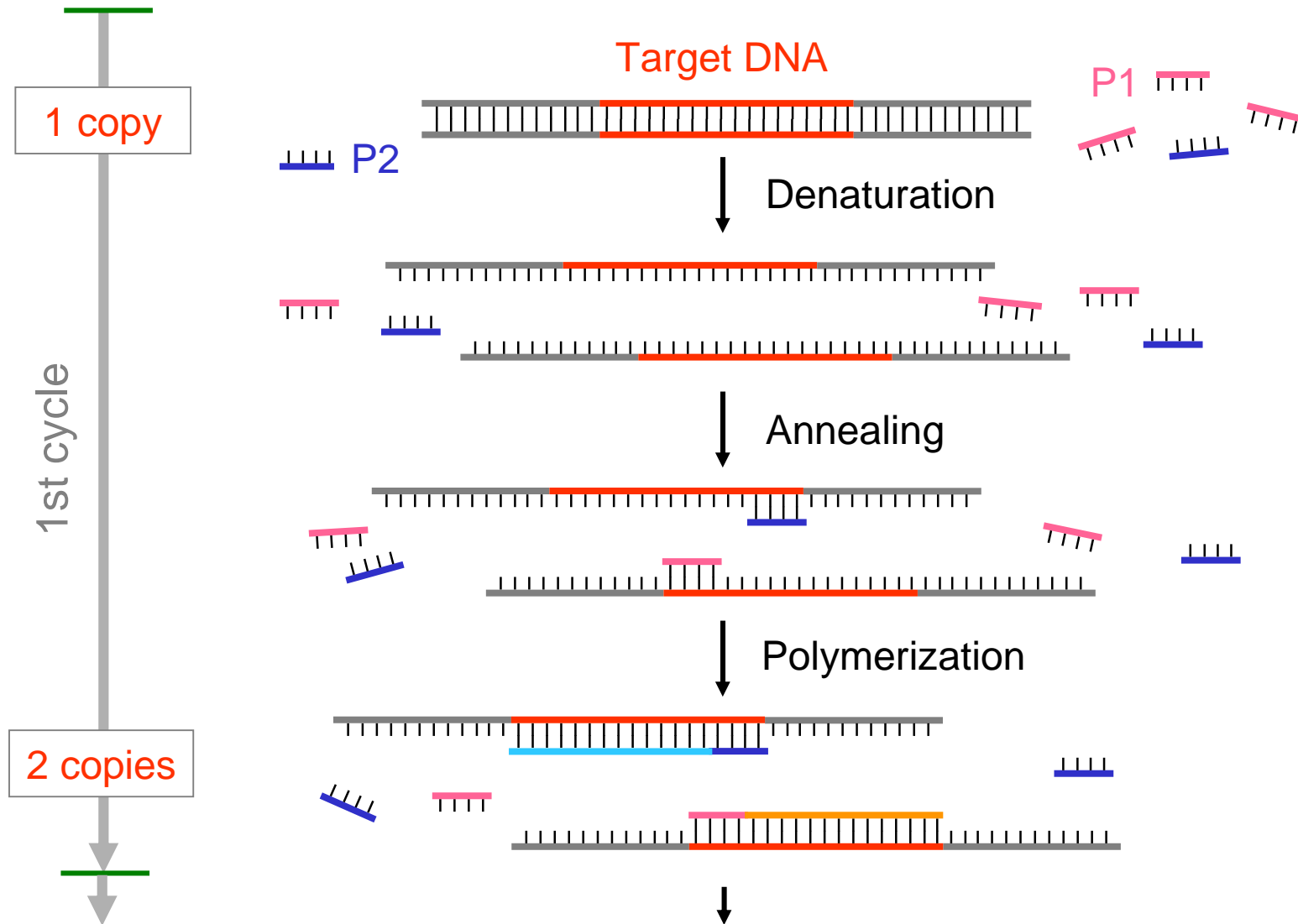
Large fragment of DNA polymerase I:

- Proteolytic enzyme 可將 DNA polymerase I 切成兩段

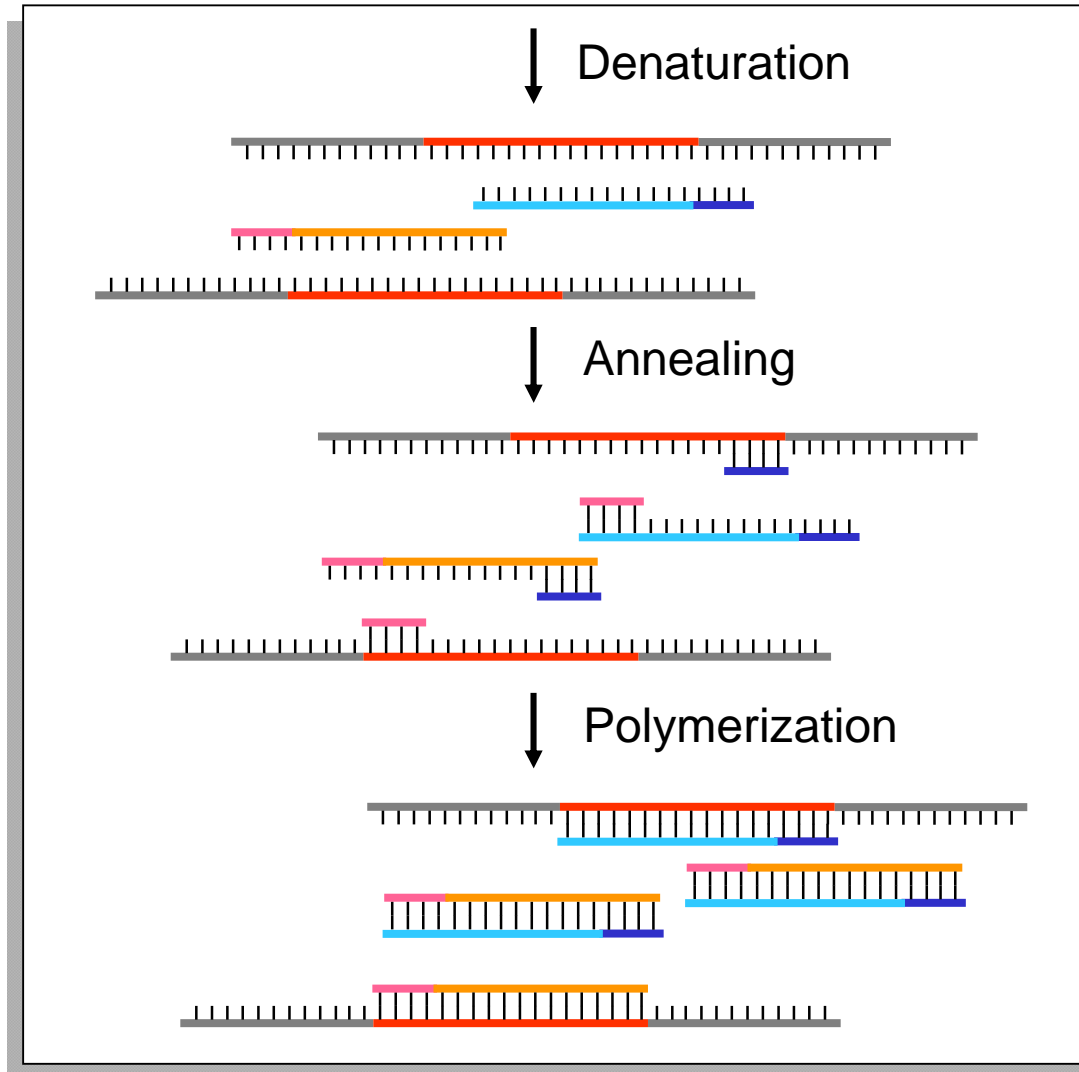


- Klenow fragment 在分子生物實驗中的應用極多，例如，修補 DNA 末端、放射線核酸探針標幟... 等等。
- 最初的 polymerase chain reaction 反應即是利用 Klenow enzyme 進行。

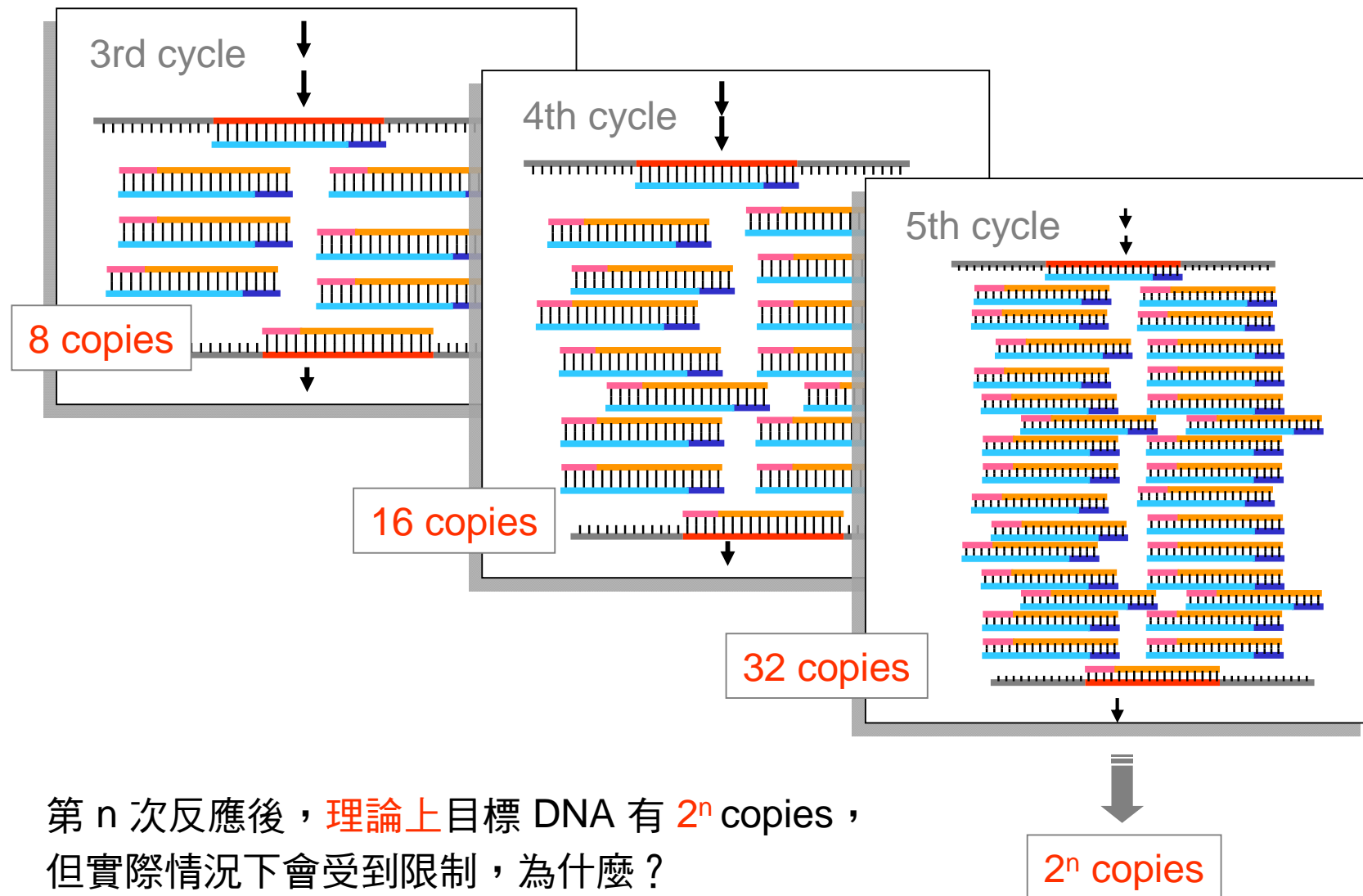
■ Polymerase chain reaction:



■ Polymerase chain reaction:



■ Polymerase chain reaction:



第 n 次反應後，理論上目標 DNA 有 2^n copies，
但實際情況下會受到限制，為什麼？

■ Experimental approach: Standard PCR



Primer design, Preparation of template DNA



Determination of experimental parameters



PCR



Analysis of products



Purification of products



Subcloning

■ Primer design:

- ▶ Primer length: 20-30 nt
- ▶ Melting temperature (T_m) : 兩引子之差異小於 5°C
- ▶ G/C content: ~ 40%-60%
- ▶ PolyN stretches
- ▶ Complementary sequences
- ▶ Specificity
- ▶ 3'-end sequence

T_m & annealing temperature



$$T_m = \frac{\Delta H}{\Delta S + R \ln(c/4) - 273.15} + 16.6 \log_{10}[K^+]$$

H: the enthalpy for helix formation

S: the entropy for helix formation

R: the molar gas constant

c: the concentration of primer

$$T_m = 2(A+T) + 4(G+C) \text{ } ^\circ\text{C}$$

$$\text{Annealing temperature} = (T_m - 5) \text{ } ^\circ\text{C}$$

■ Primer design:

- ▶ Primer length: 20-30 nt
- ▶ Melting temperature (T_m) : 兩引子之差異小於 5°C
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■ Experimental approach: Standard PCR

Primer design, Preparation of template DNA



Determination of experimental parameters



PCR



Analysis of products



Purification of products



Subcloning

■ Determination of experimental parameters:



- ▶ Reaction buffer
- ▶ Enzyme
- ▶ Denaturation temperature and time
- ▶ Annealing temperature and time
- ▶ Elongation temperature and time
- ▶ Cycle number

Reaction buffer



General components:

- Tris-Cl, 10 – 50 mM, pH 8.3 ~8.8
- KCl, < 50 mM
- **MgCl₂**, 0.5 – 2.5 mM over total [dNTP]
- dNTPs, pH 7.0, 20 – 250 μM each
- Primers, 0.2 – 1 μM each
- Additives, e.g., BSA, gelatin, NP-40...etc.

■ Determination of experimental parameters:



- ▶ Reaction buffer
- ▶ **Enzyme**
- ▶ Denaturation temperature and time
- ▶ Annealing temperature and time
- ▶ Elongation temperature and time
- ▶ Cycle number

Enzyme

Taq polymerase (from *Thermus aquaticus*)

T. Thermophilus DNA polymerase

Vent polymerase (from *Thermococcus litoralis*)

.....

是否具 proofreading 功能？

目標基因的長度？

是否在產物末段多加上一個A？

■ Determination of experimental parameters:

- ▶ Reaction buffer
- ▶ Enzyme
- ▶ Denaturation temperature and time
- ▶ **Annealing temperature and time**
- ▶ Elongation temperature and time
- ▶ Cycle number

Experimental parameters



For *Taq* polymerase:

- Concentration: 0.5 – 5 units/100 μ l
- Denaturation: 94 – 95 °C, 30 sec - 1 min
- Elongation: 70 – 72 °C, 0.5 – 3 min
(2 – 4 kb/min)
- Cycle number: 25 - 35

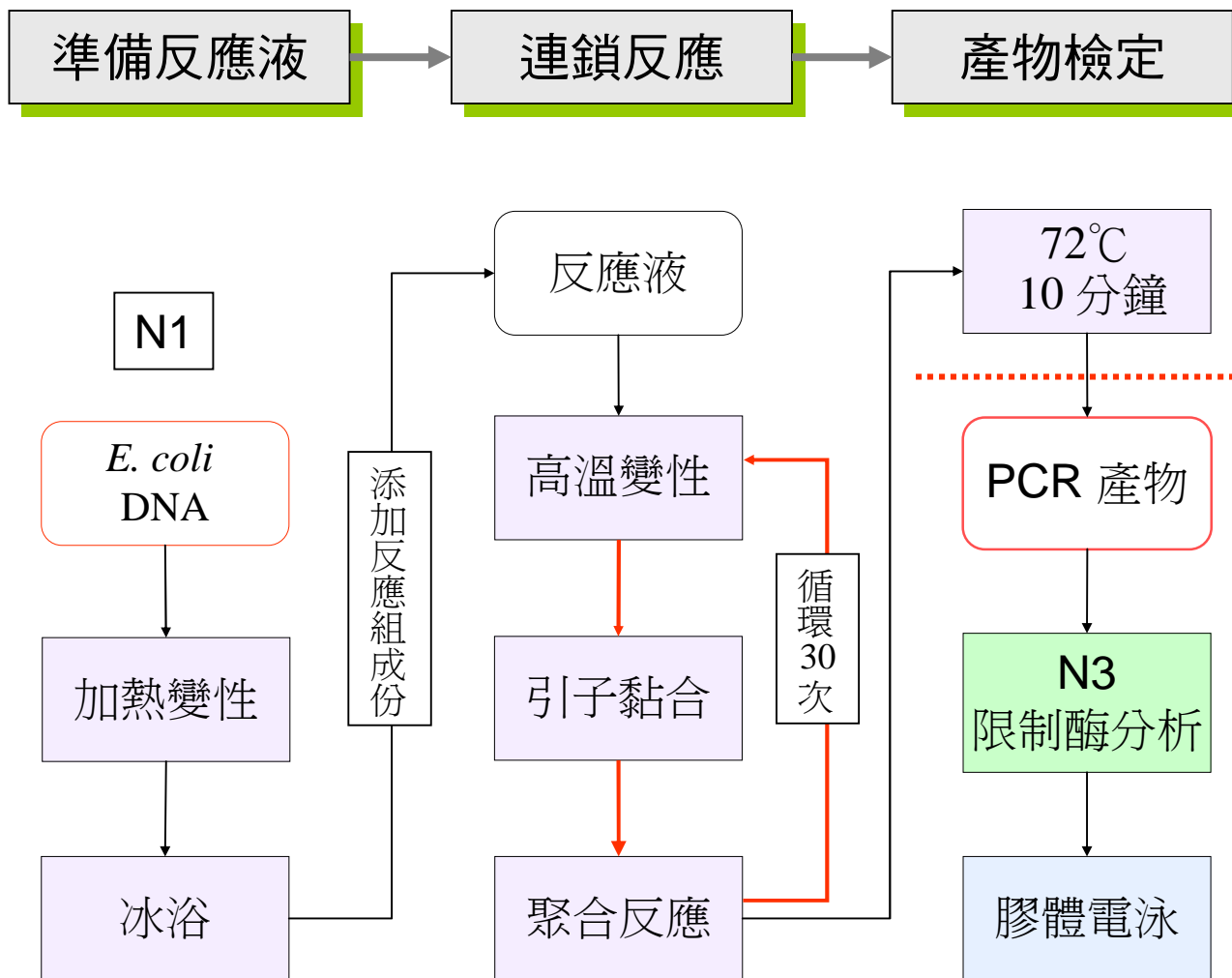
■ 為什麼你的 PCR 失敗？

- MgCl₂ 濃度不對：沒有試最適濃度？使用前沒有混合均勻？
- dNTP 濃度太高？已經降解了？
- 反應液中存在 inhibitors？
例如：EDTA, chloroform, phenol, ethanol, SDS, sarkosyl, tracking dyes,.....？
- Enzyme 太多？
- Template 過多或過少？
- Primers 濃度不對？ Primers 設計不良？
- Annealing temperature 不對？
- Reagents 有雜質？

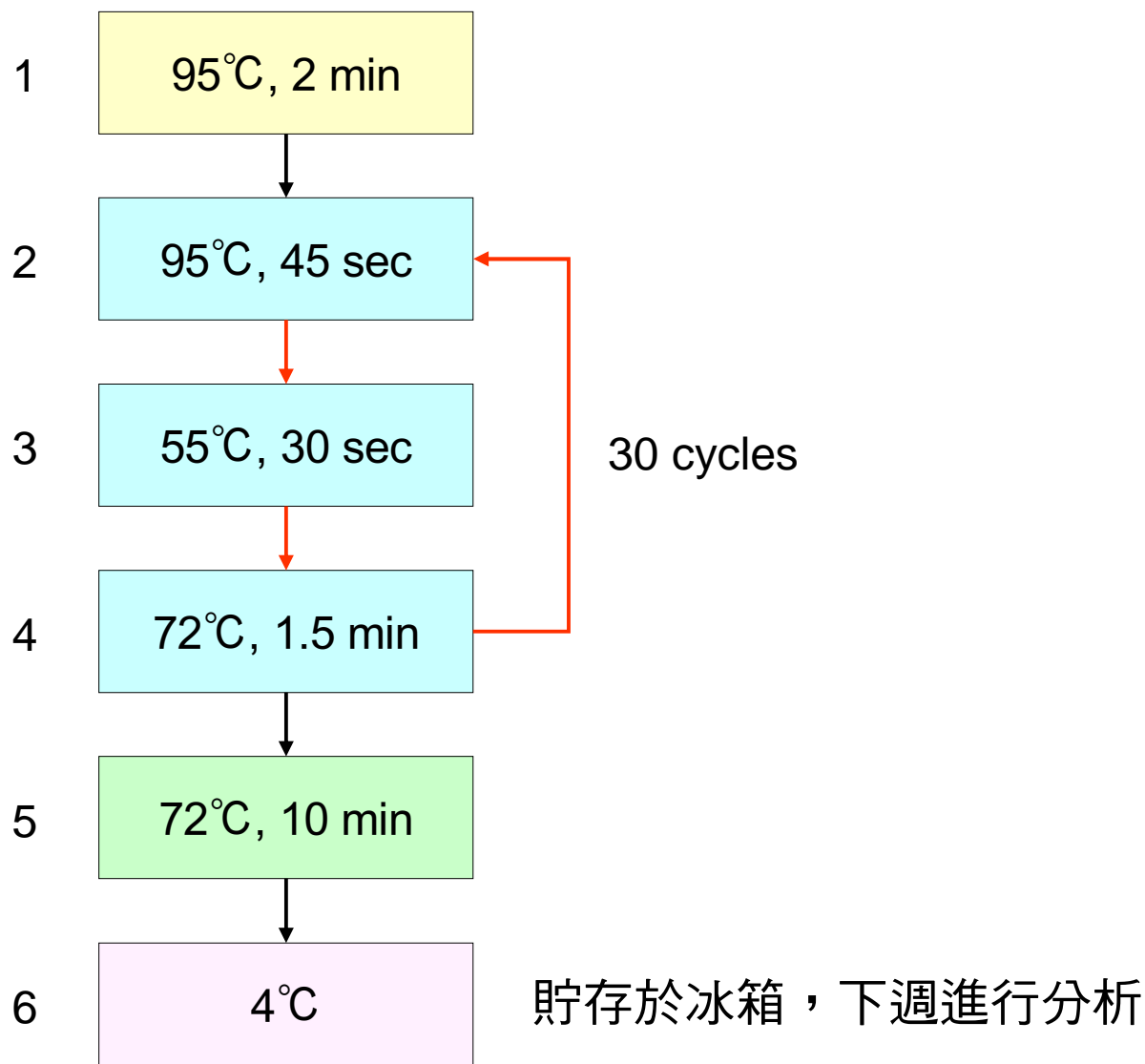
■ *Always remember –*

- Work clean.
- Titrate MgCl_2 .
- Do not use too much template DNA.
- Do not use PCR products in PCR preparation areas.
- Always include negative and positive control in every experiment.
- Wear gloves.

N2 以聚合酶連鎖反應增殖DNA



■ 本次實驗反應程式：



■ 本週實驗進行要點：

製備膠片

- TAs：示範鑄膠、說明儀器操作

定量

- 1,3 組：進行 DNA 稀釋 → 測定吸光值 → 準備電泳樣品
- 2,4 組：準備電泳樣品 → 進行 DNA 稀釋 → 測定吸光值

電 泳

- TAs：示範 sample loading

準備 PCR

- 1,3 組：進行 DNA 稀釋 → 準備 PCR 反應液 (DNA 及酵素除外)
- 2,4 組：準備 PCR 反應液 (DNA 及酵素除外) → 進行 DNA 稀釋
- TAs：說明 PCR 程式設定

膠片分析

- TAs: 協助判讀結果，決定 DNA 是否可用於 PCR

PCR

- 各組添加 PCR 反應之 DNA 及酵素