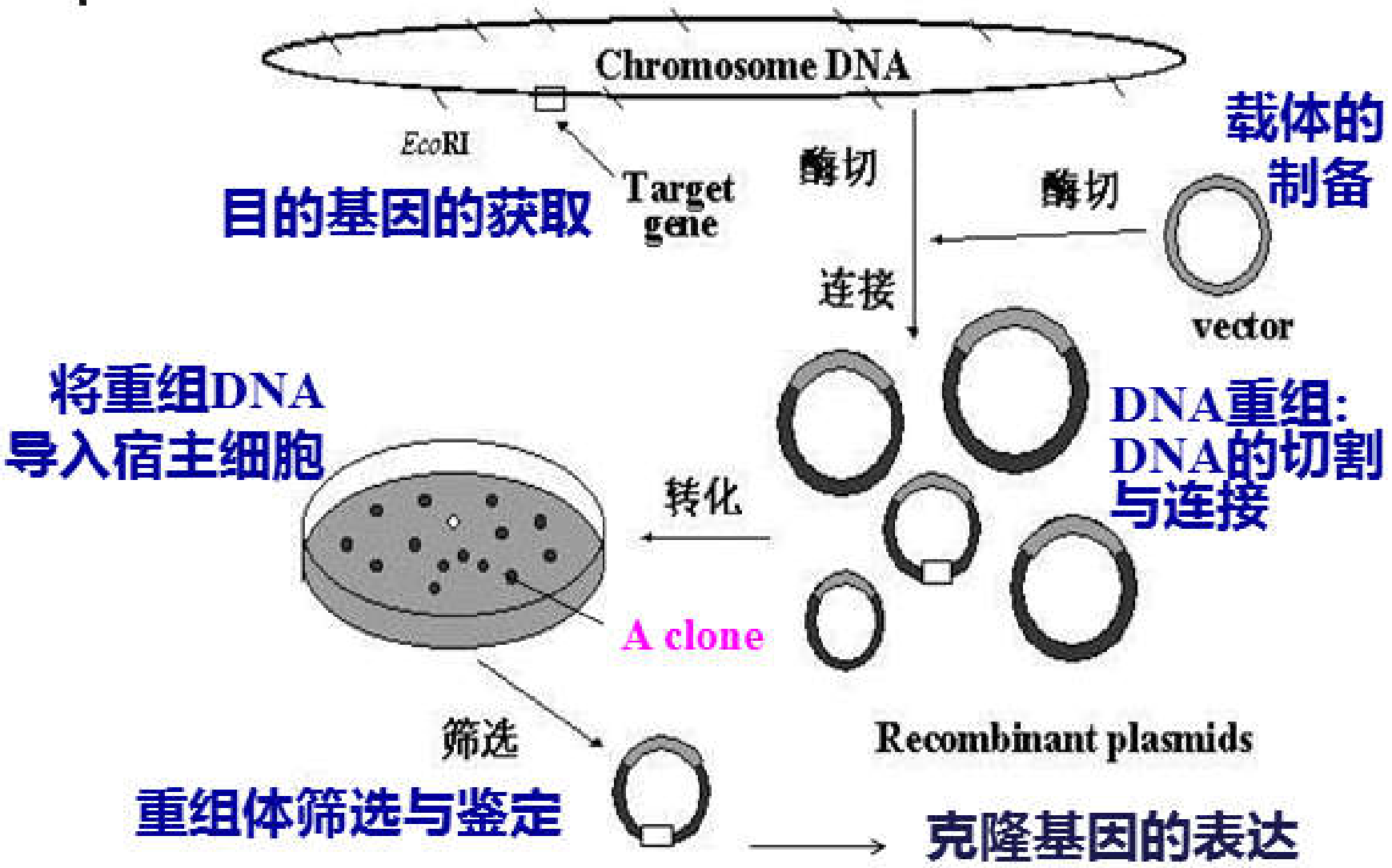
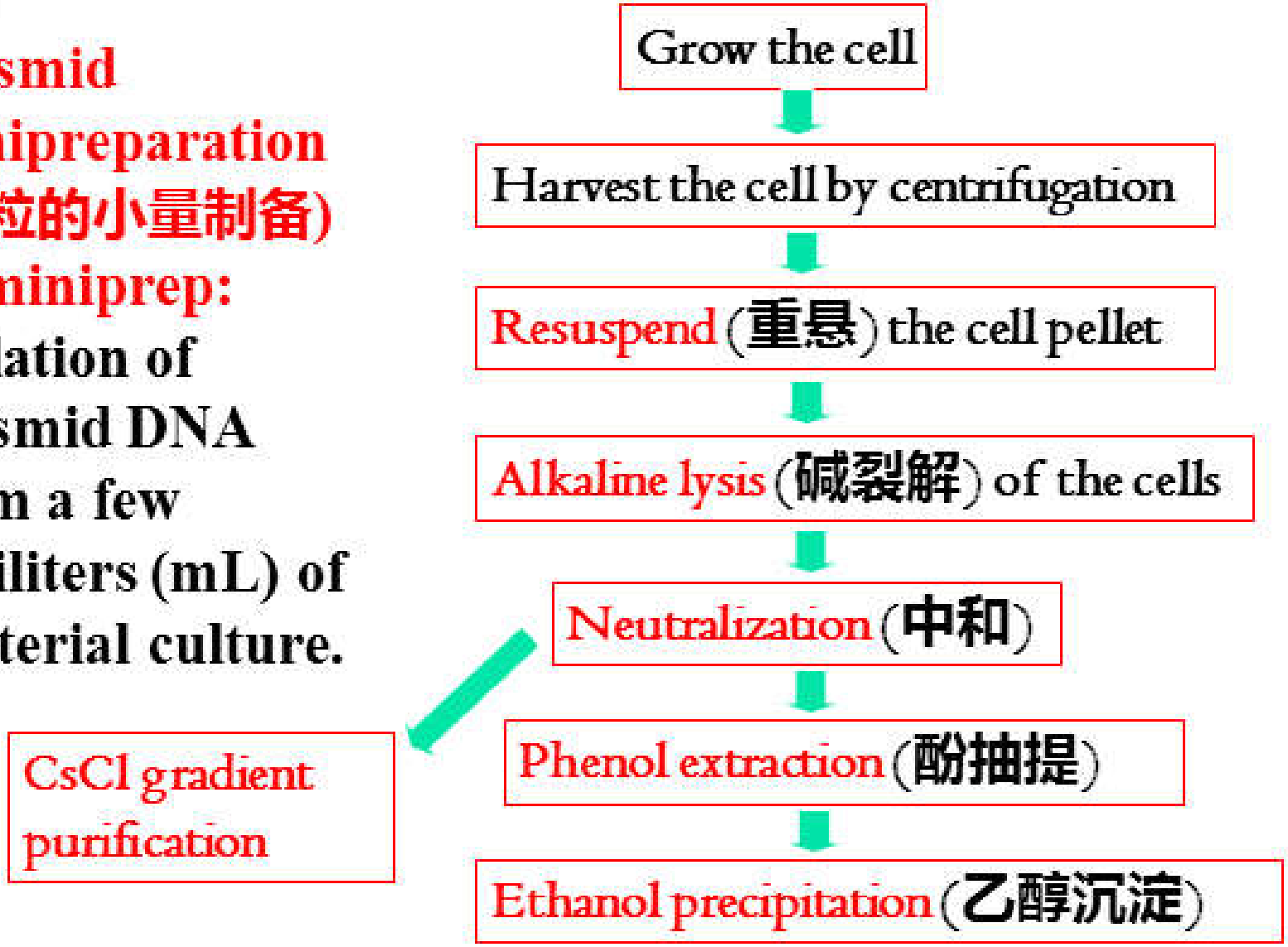
2. Steps of DNA cloning



2.1 Preparation of the plasmid

Plasmid minipreparation (质粒的小量制备) or miniprep: Isolation of plasmid DNA from a few mililiters (mL) of bacterial culture.



2.1.1 Resuspension

- **Solution I - 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0)**
- **Glucose prevents *E. coli* from quickly falling to the bottom of the tube, and the Tris-HCl buffers the cell at pH=8.0.**
- **After cell lysis (next steps), EDTA limits DNA degradation by binding (chelating, 螯合) Mg^{2+} ions that are a needed cofactor for bacterial nucleases**

2.1.2 Alkaline lysis

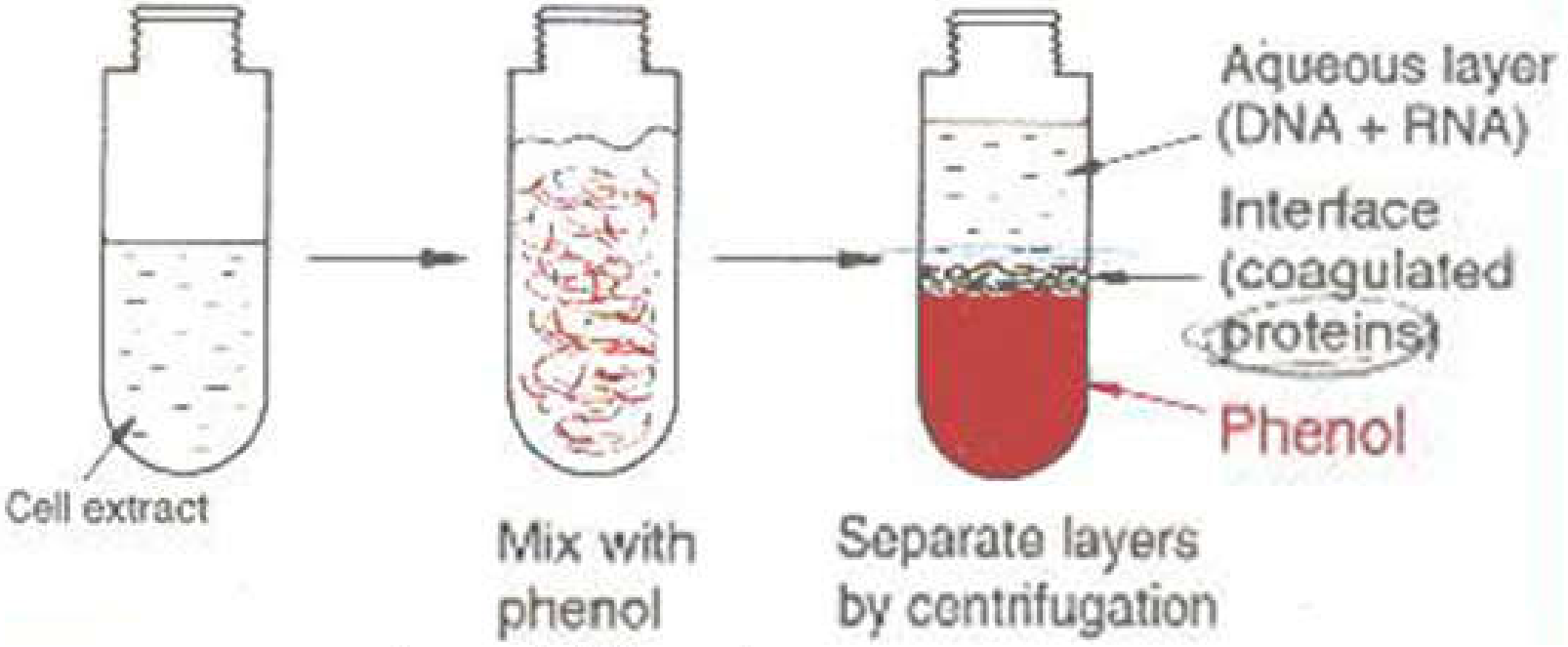
- Solution II - 0.2 M NaOH, 1% SDS
- NaOH denatures DNA and hydrolyzes RNA.
- SDS disrupts cell membrane and denatures proteins. (碱性条件下裂解效率高)

2.1.3 Neutralization

- Solution III - KAc-HAc buffer (pH 5), 5 M KAc
- KAc renatures plasmid DNA (supercoiled) and precipitates denatured proteins, along with the chromosomal DNA and most of the detergent (potassium dodecyl sulfate is insoluble in water).

2.1.4 Phenol extraction

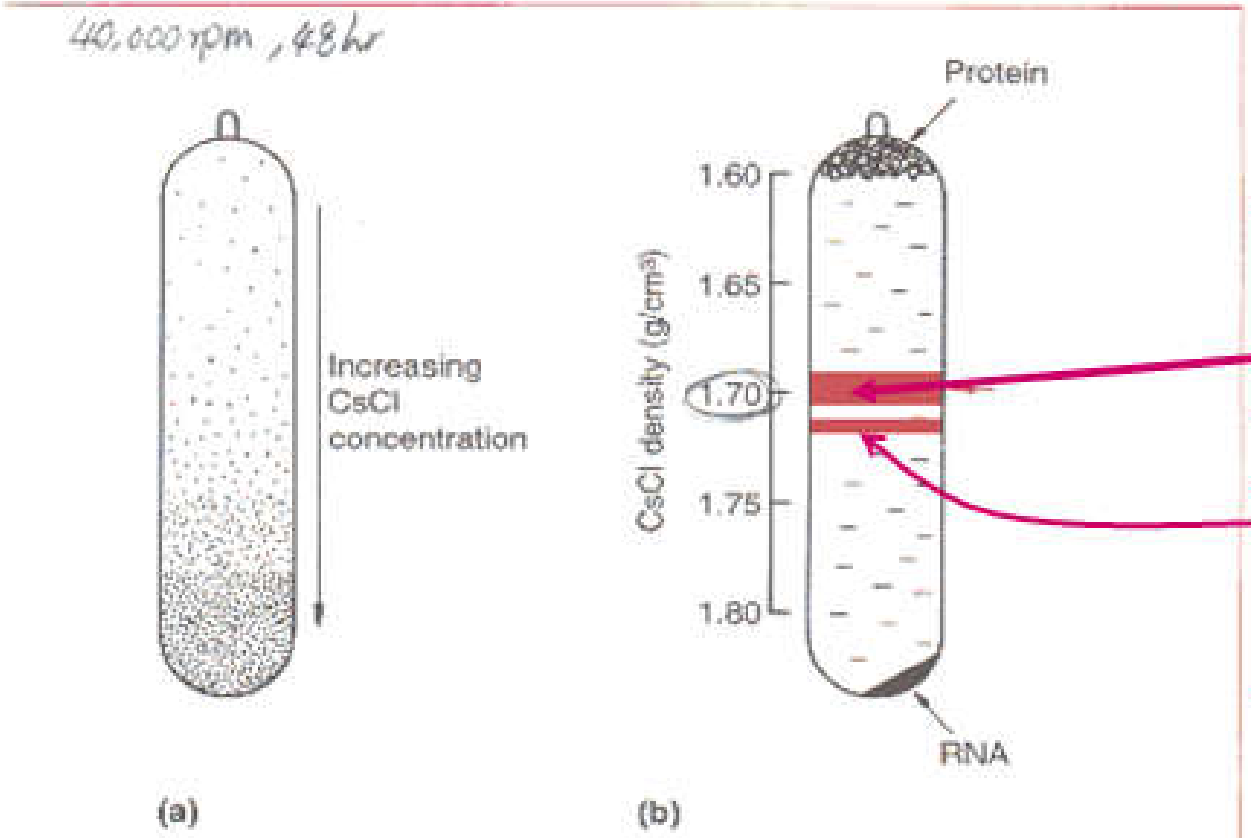
Phenol extraction to get rid of the protein contaminants



or phenol-chloroform

酚-氯仿

2.1.5 Cesium chloride gradient purification



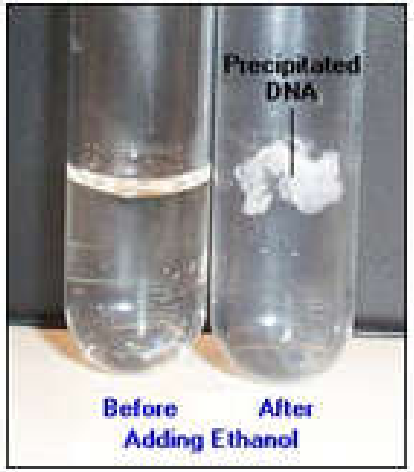
Chromosomal /linear DNA

Supercoiled plasmid

Supercoiled DNA may be purified from protein, RNA, chromosomal DNA and nicked plasmid DNA in one step!

2.1.6 Ethanol precipitation

- 0.3 M Na^+ (NaAc, pH 5.2), 3 volumes of ethanol
  - 乙醇消除核酸的水化层，使带负电荷的磷酸基团暴露出来。
  - Na^+与磷酸基团结合，在沉淀形成部位降低多核苷酸链之间的排斥力。



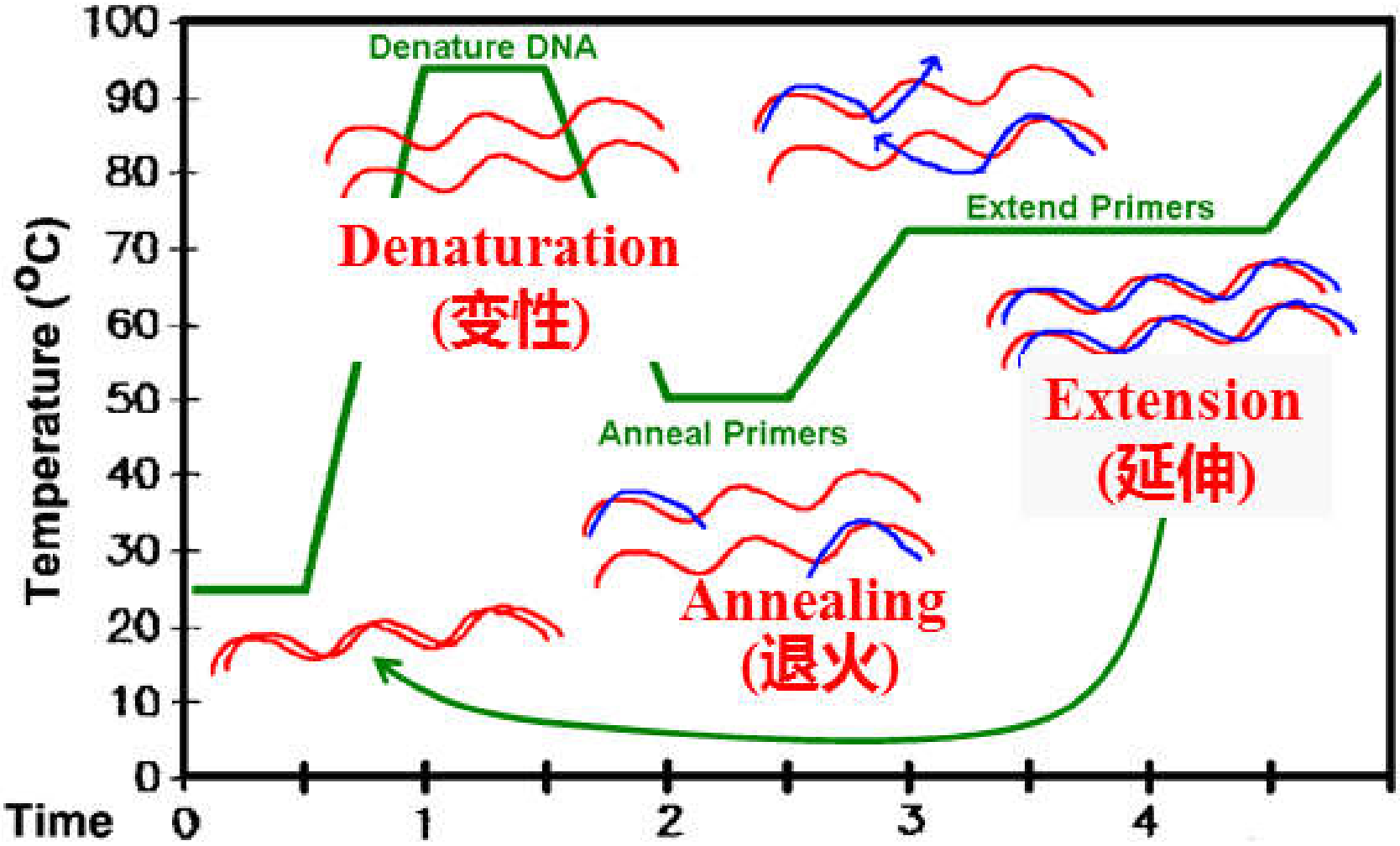
- Centrifuge 13 000g and air dried
- Tris-EDTA (TE) buffer (EDTA chelates Mg^{2+})
- RNase A digests remaining RNA contamination

2.2 Get the target gene

- (RT-)PCR amplification of the target gene
- Chemical synthesis of the target gene
- Restriction digestion from DNA library
  - Genomic library (基因组文库)
  - cDNA library

2.2.1 PCR amplification of the target gene

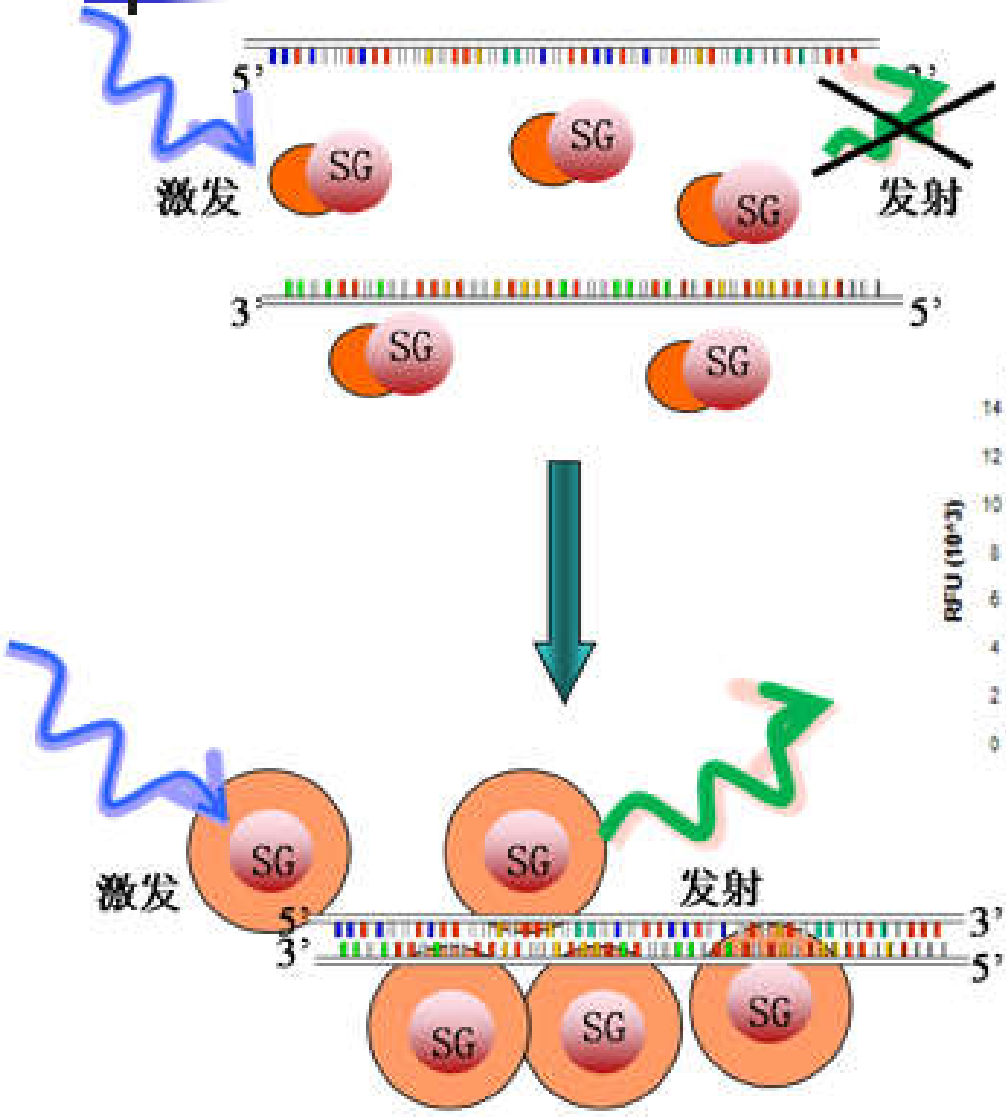
(1) The PCR cycle



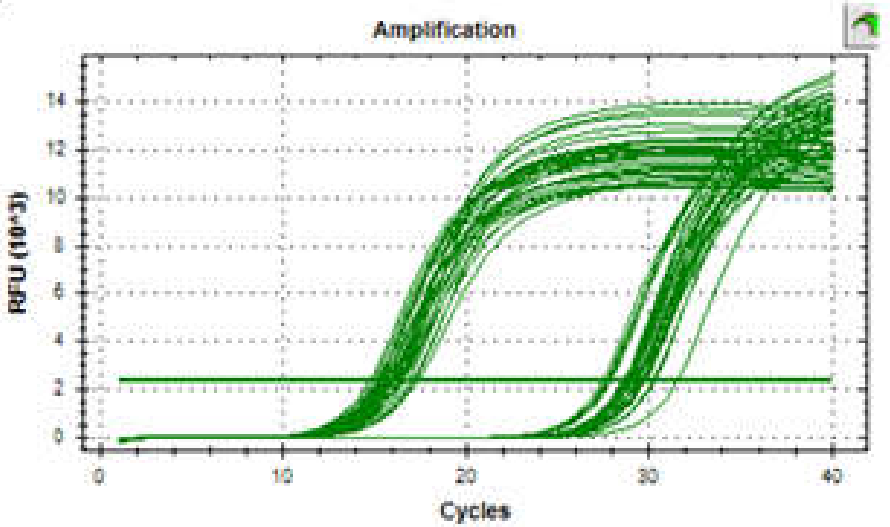
(2) Features of PCR

- **引物设计：** 引物的长度一般为18~25 bp，且引物自身及两个引物之间不能过多配对。3’端不得有任何修饰。**5’端加酶切位点。**
- **扩增最有效长度：**一般在200~800bp之间
- **扩增效率：**2^n，20次后不成此关系，因此n=25~30次（**荧光定量PCR** n=40）。
- **变性温度与时间：**94℃，第一次模板链较长，在5~10 min。每次循环中变性1~2 min。
- **退火温度：**50~60℃，引物G+C含量一般在40~60%之间，两条Tm值相差2~3 ℃以内。

SYBR-Green I qPCR原理示意图

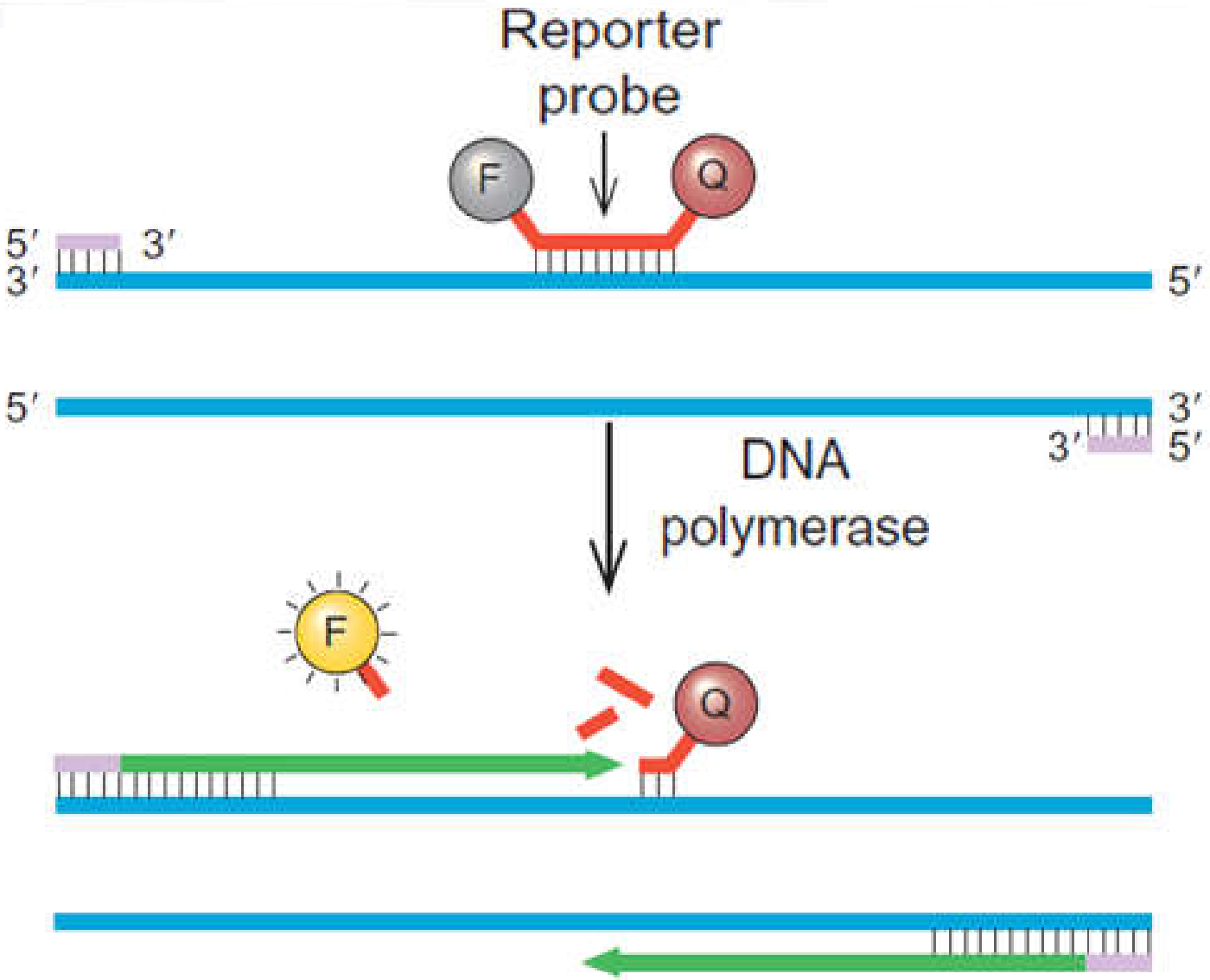


不掺入双链中的SYBR染料分子不会发出任何荧光信号

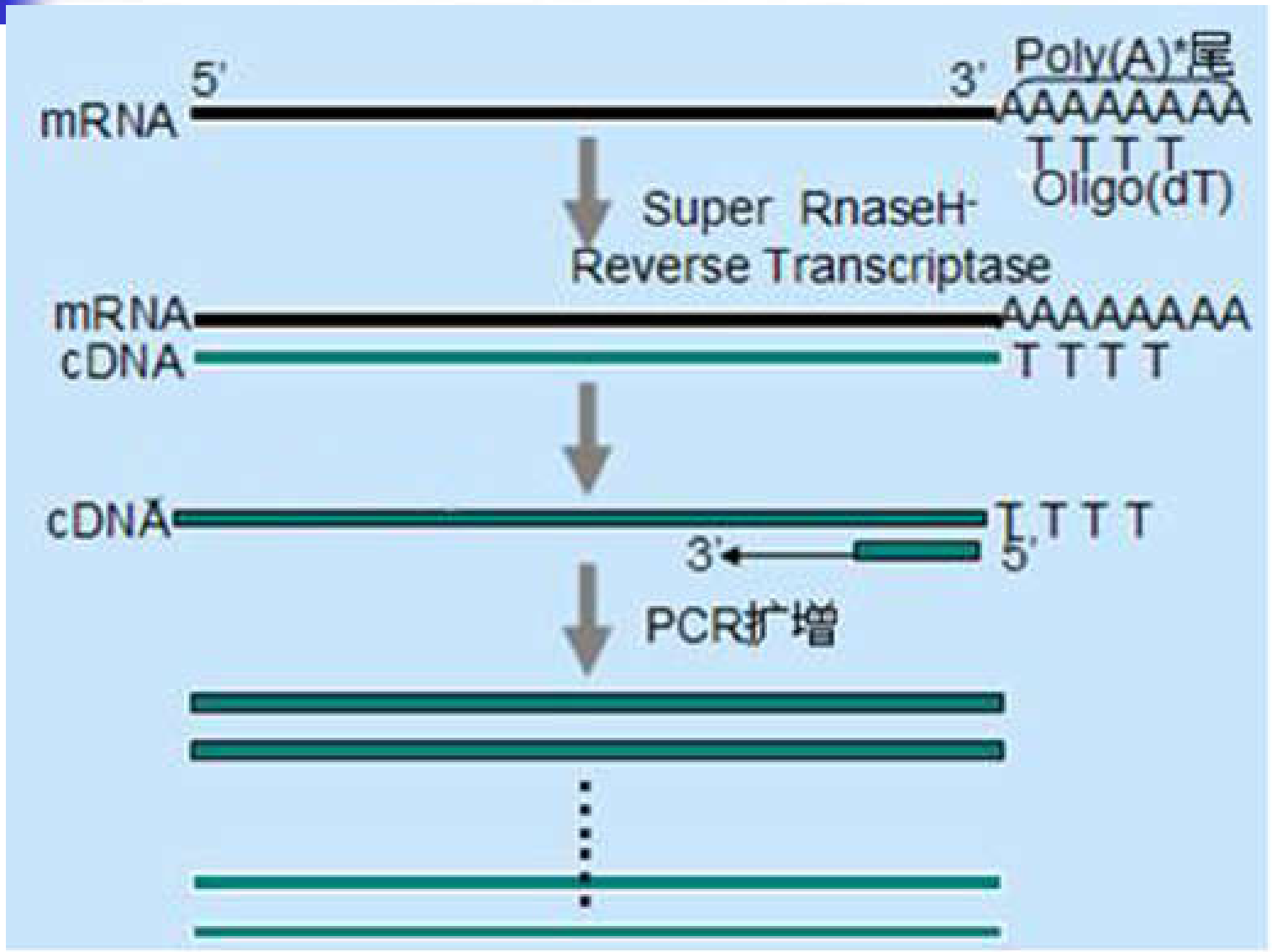


SYBR荧光染料特异性地掺入DNA双链,发出荧光信号

Probe qPCR原理示意图



(3) RT-PCR


mRNA
5'
3'
Poly(A)*尾
AAAAAAAA
TTTT
Oligo(dT)
Super RnaseH-
Reverse Transcriptase
mRNA
cDNA
AAAAAAAA
TTTT
cDNA
TTTT
3'
5'
PCR扩增

(4) Detection of PCR products

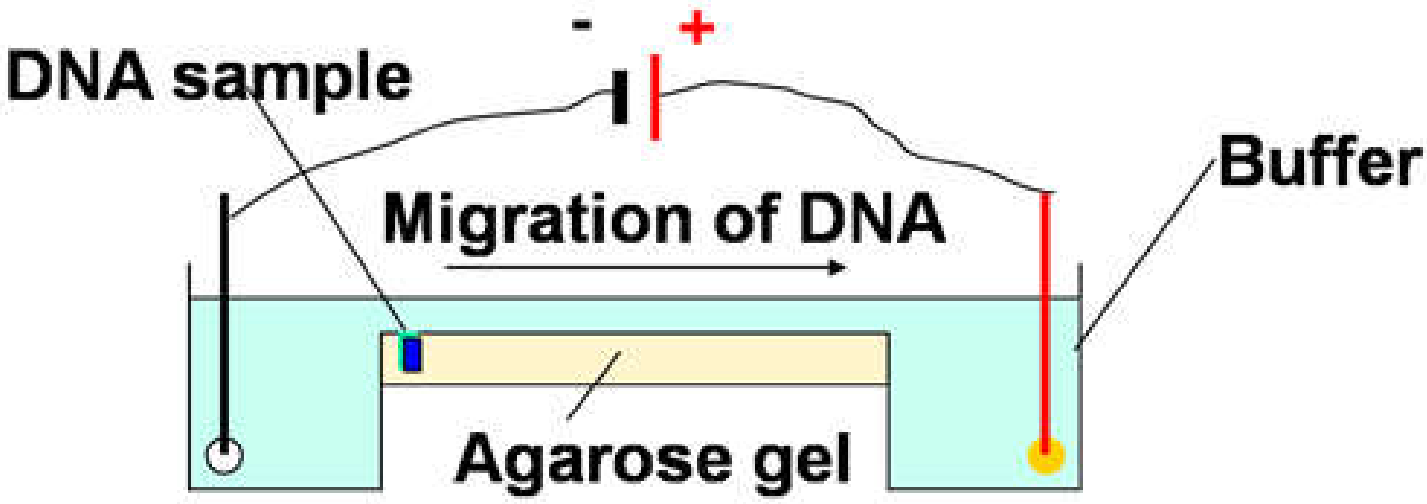
琼脂糖凝胶电泳 (agarose gel electrophoresis)

- **Agarose**
  - **Agarose is a polysaccharide derived from seaweed, which forms a solid gel when dissolved in aqueous solution at concentrations between 0.5% and 2% (w/v).**
  - **Agarose is a more purified form of the agar (琼脂) used to make bacterial culture plates.**

Principle

Migration

When an electric field is applied to an agarose gel in the presence of a buffer solution, DNA fragments move through the gel towards the + electrode, because DNA negatively charged.



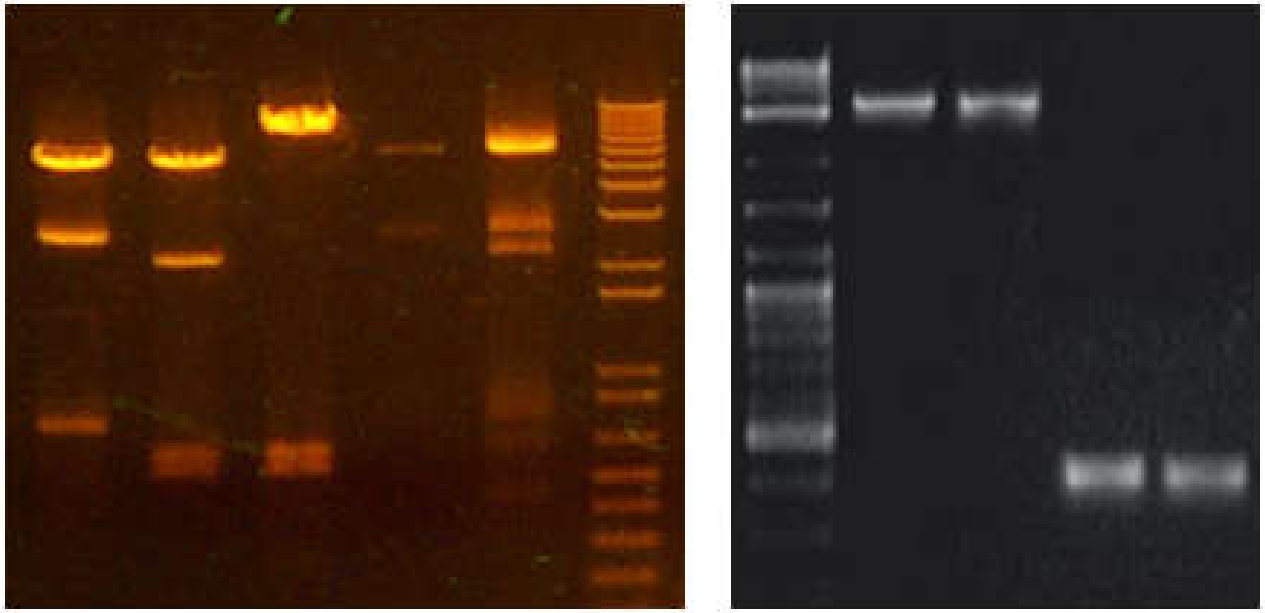
➢ Migration rate

The Migration rate is dependent on fragment sizes and shapes of the DNA and the electrophoresis may be used to separate mixtures of DNA fragments by the rate.

超螺旋>线型>开环

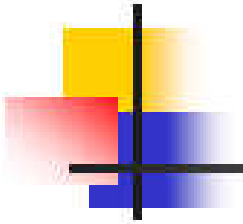
琼脂糖凝胶浓度	线性DNA的有效分离范围
0.3%	5-60 kb
0.6%	1-20 kb
0.7%	0.8-10 kb
0.9%	0.5-7 kb
1.2%	0.4-6 kb
1.5%	0.2-4 kb
2.0%	0.1-3 kb

➢ Staining for gel



The DNA is stained by the ethidium bromide (EB) in the gel, the DNA shows up as an orange band on illumination (光照) by UV light.

EB用量0.5 μg/mL，50 ng的微量DNA可成带



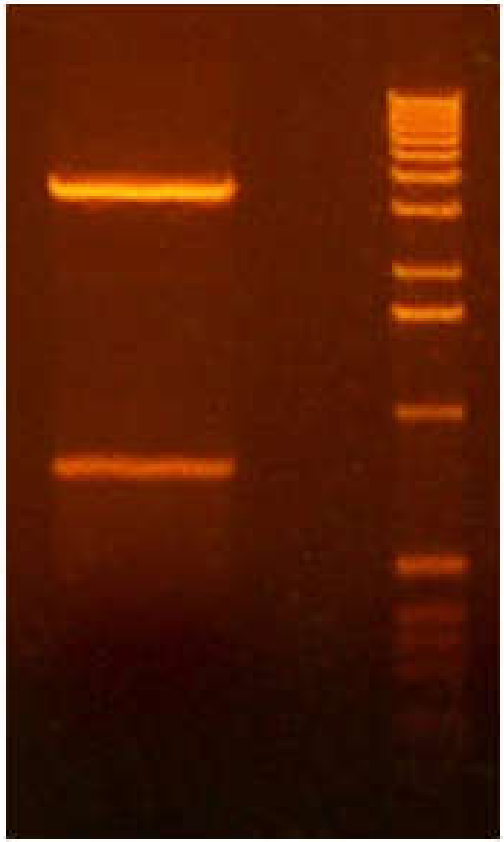
➢ Application

① Isolation of nucleic acids

② Recycling DNA fragment

③ Estimating the approximate molecular weight of nucleic acids

④ Blotting



Gel excision and purification

SanPrep柱式DNA胶回收试剂盒

磁珠法DNA胶回收试剂盒

2.2.2 DNA libraries

- **DNA libraries** are sets of DNA clones, each of which has been derived from the insert of a different **fragment** into a **vector** followed by propagation in the **host**.

  DNA文库是一套DNA克隆，每个克隆都是插入了不同片段的载体在宿主中扩增后的产物。

- **A clone** – Propagation of the host cell containing the recombinant DNA forms a set of **genetically identical** organism.

  单克隆：带有某一种重组DNA的宿主细胞，增殖构成的一群具有**遗传一致性**的个体。

(1) Genomic libraries (基因组文库)

- Genomic libraries are prepared from random fragments of genomic DNA.
- Physical shearing and restriction digestion;
- The approach may be an inefficient method of finding a gene, especially in eukaryotic genomes, where much of the DNA is noncoding.

(2) cDNA library

- mRNA→cDNA→dsDNA→Vector→Host
- cDNA library are derived from the mRNA by reverse transcription and are then inserted into a vector.
- cDNA libraries are efficient for finding and cloning a gene, but only the coding region, not the surrounding genomic sequences.

筛选文库 (screening libraries) :寻找目的基因

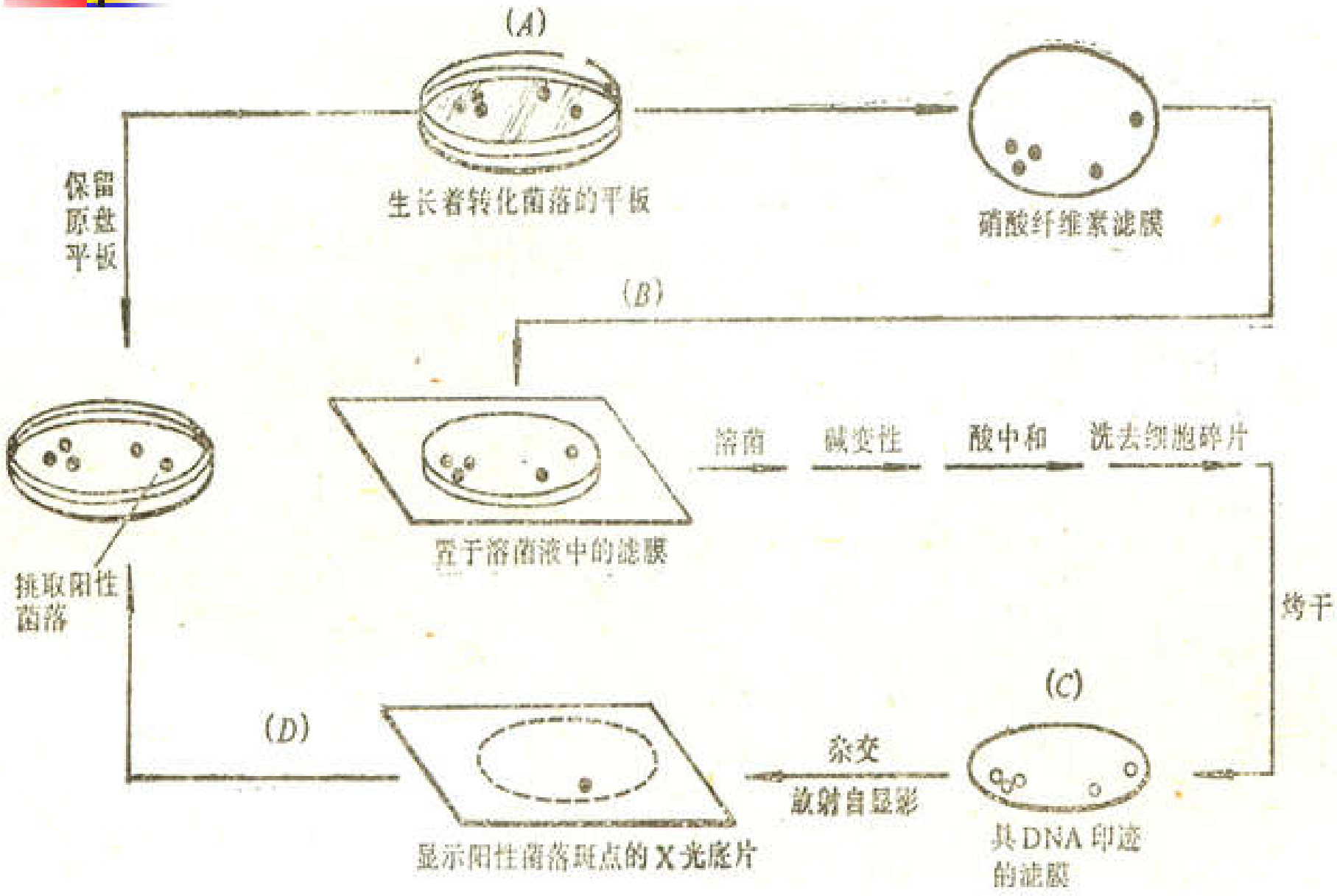
Nucleic acid hybridization - Southern blotting

A radioactively or fluorescently labeled DNA probe

Identification of the protein product

Antibody or enzyme activity measurement

菌落杂交或噬菌斑杂交有时也叫做原位杂交



2.3 DNA recombination

2.3.1 Restriction digestion (酶切)

将载体和扩增后的目的基因片段用相应的限制性内切酶消化→电泳，回收酶切产物

2.3.2 Ligation

(1) 具粘性末端的连接 √

(2) 平末端的连接

(3) TA克隆

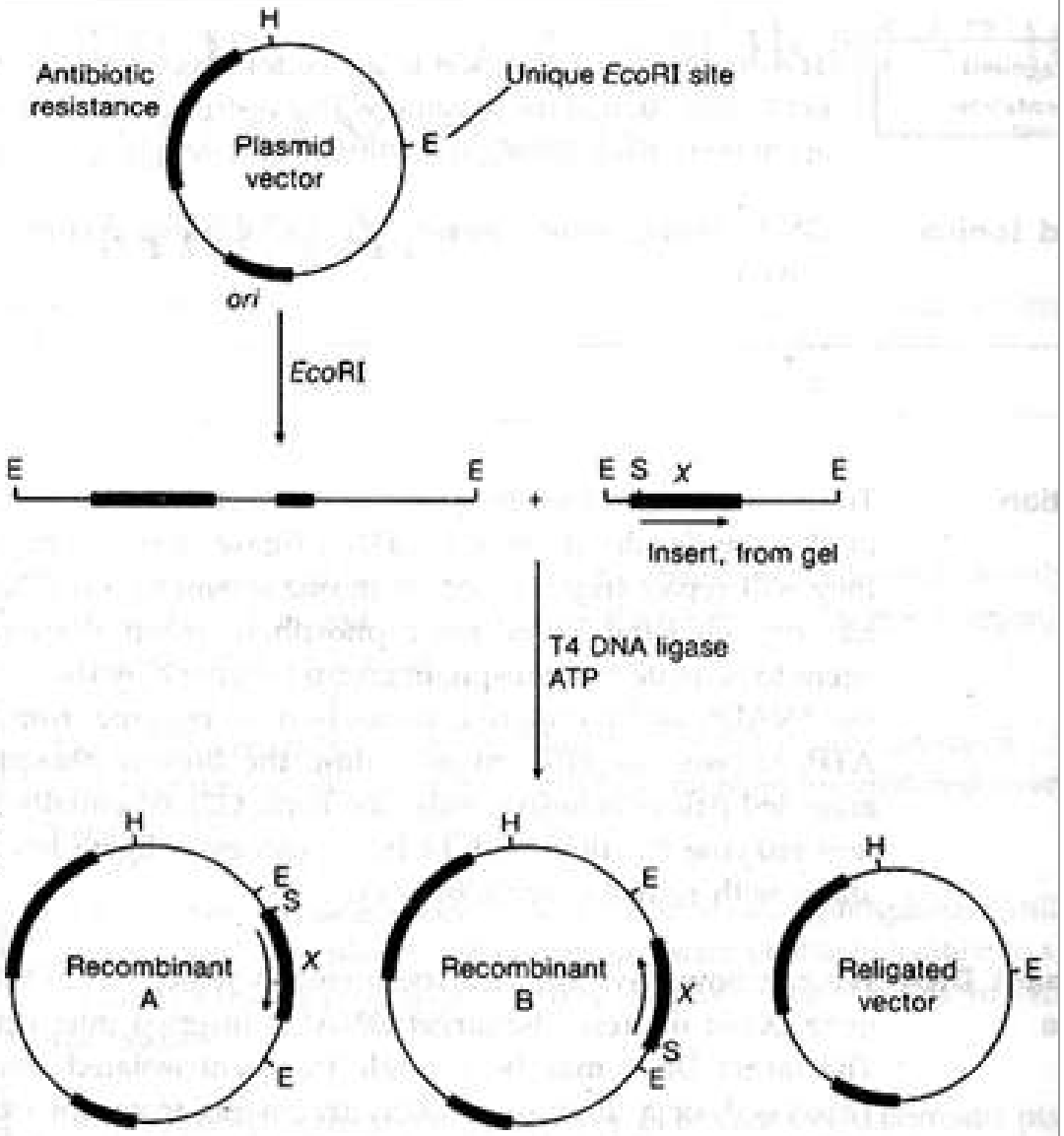
(4) 同聚物加尾后的连接

(5) 衔接物或人工接头的连接

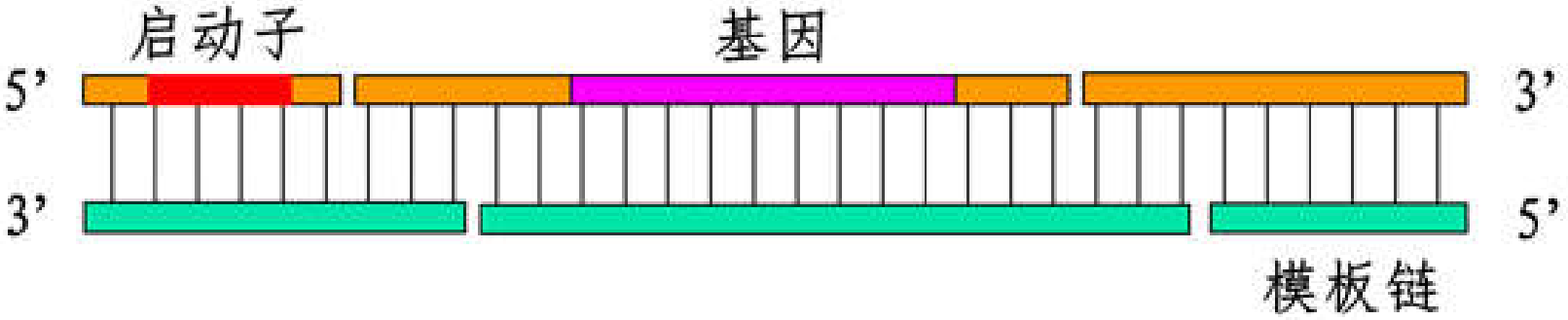
} (DNA文库)

单酶切的连接问题

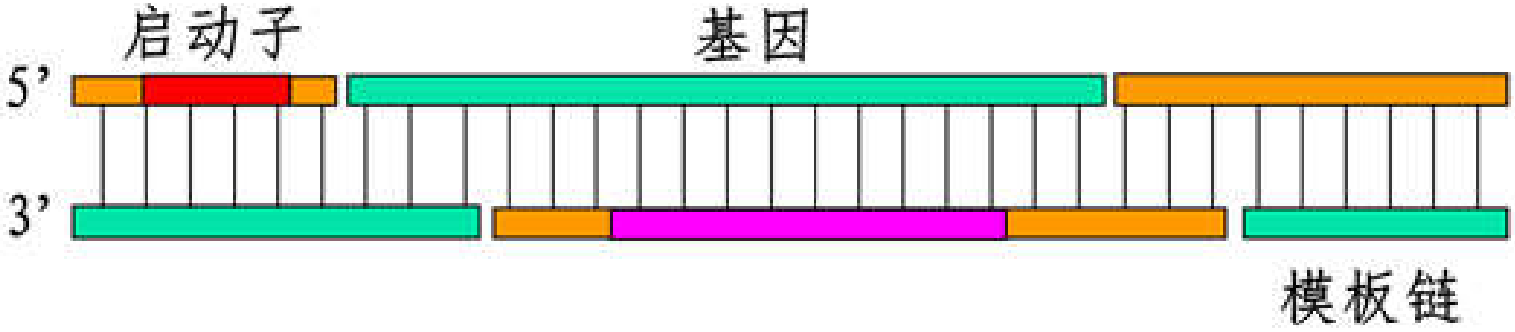
- **Religation** of the original vector plasmid.
  载体自身环化
- The target DNA fragment can be inserted into the vector with either **orientation** (**方向**) to form a recombinant molecule.

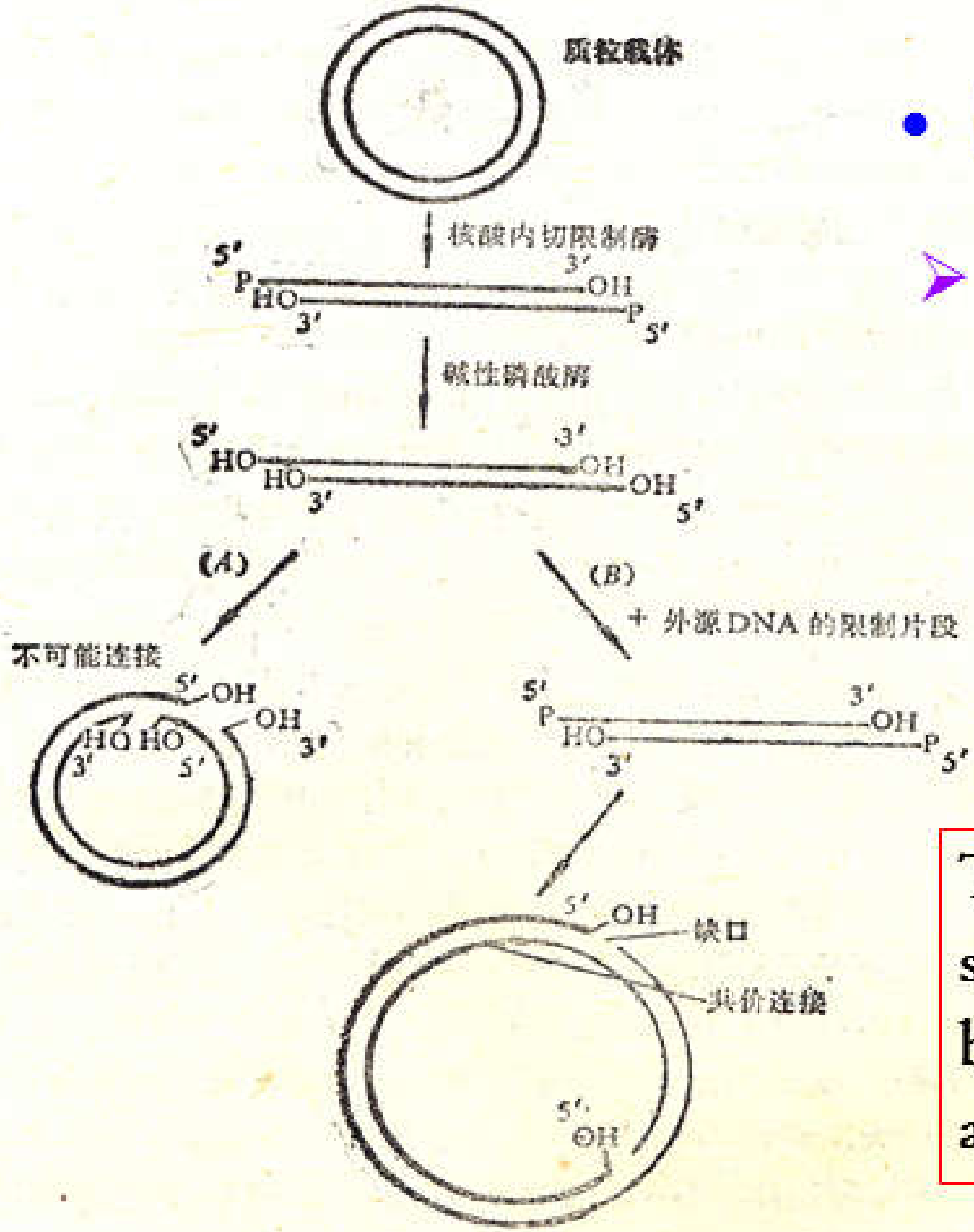


插入方向正确



插入方向错误





- **解决方法**

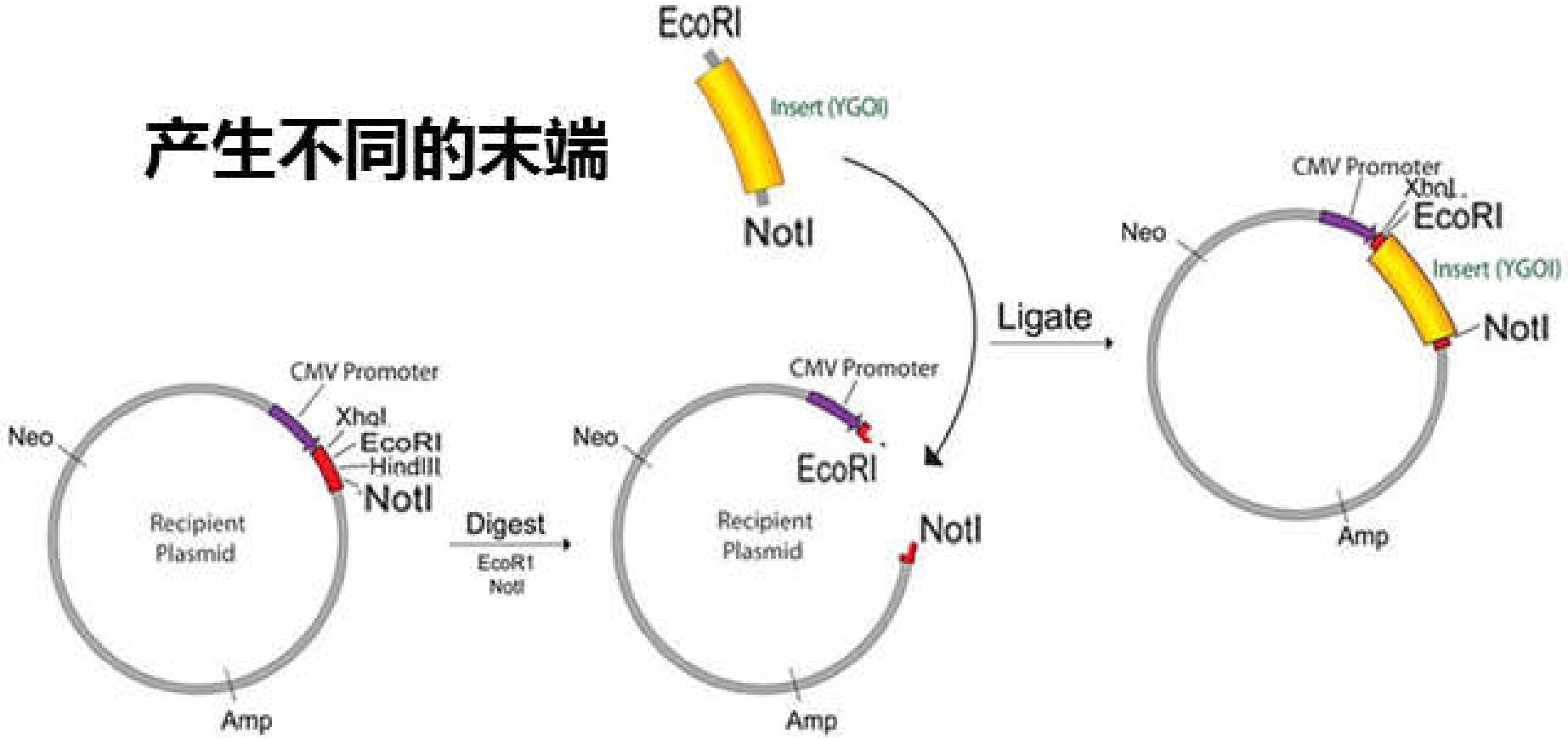
➢ **碱性磷酸酶(alkaline phosphatase)解决载体自连**

The nicks in the other strands will be repaired by cellular mechanisms after transformation.

双酶切定向连接

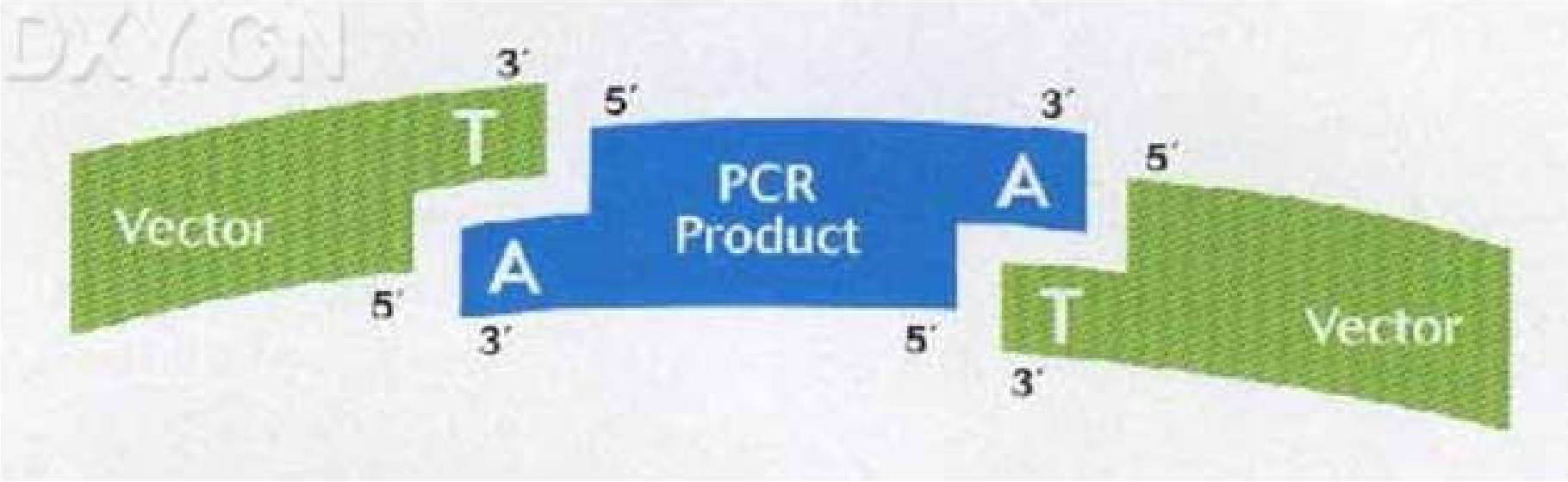
Double digestion　　同时解决载体自连

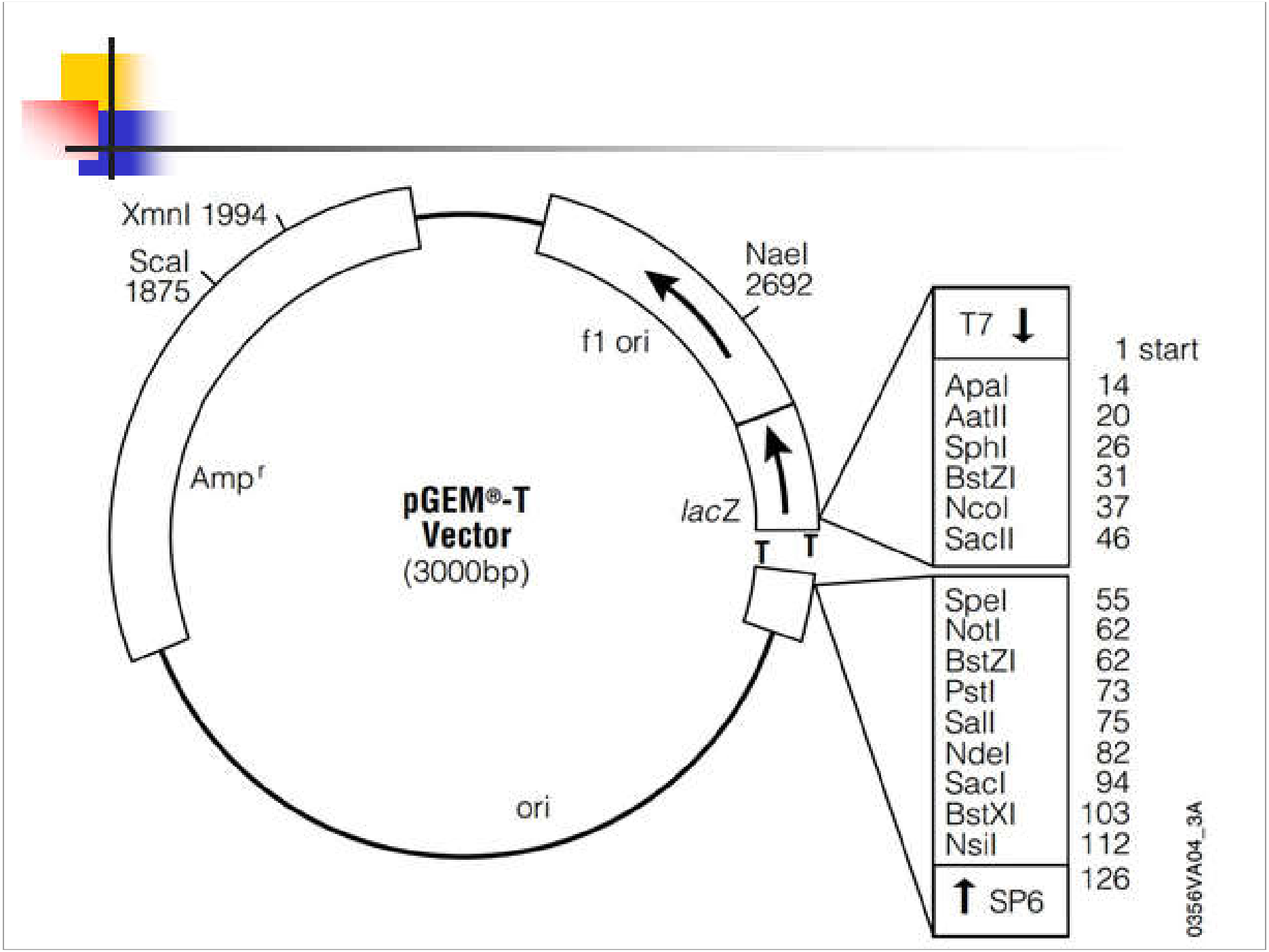
产生不同的末端



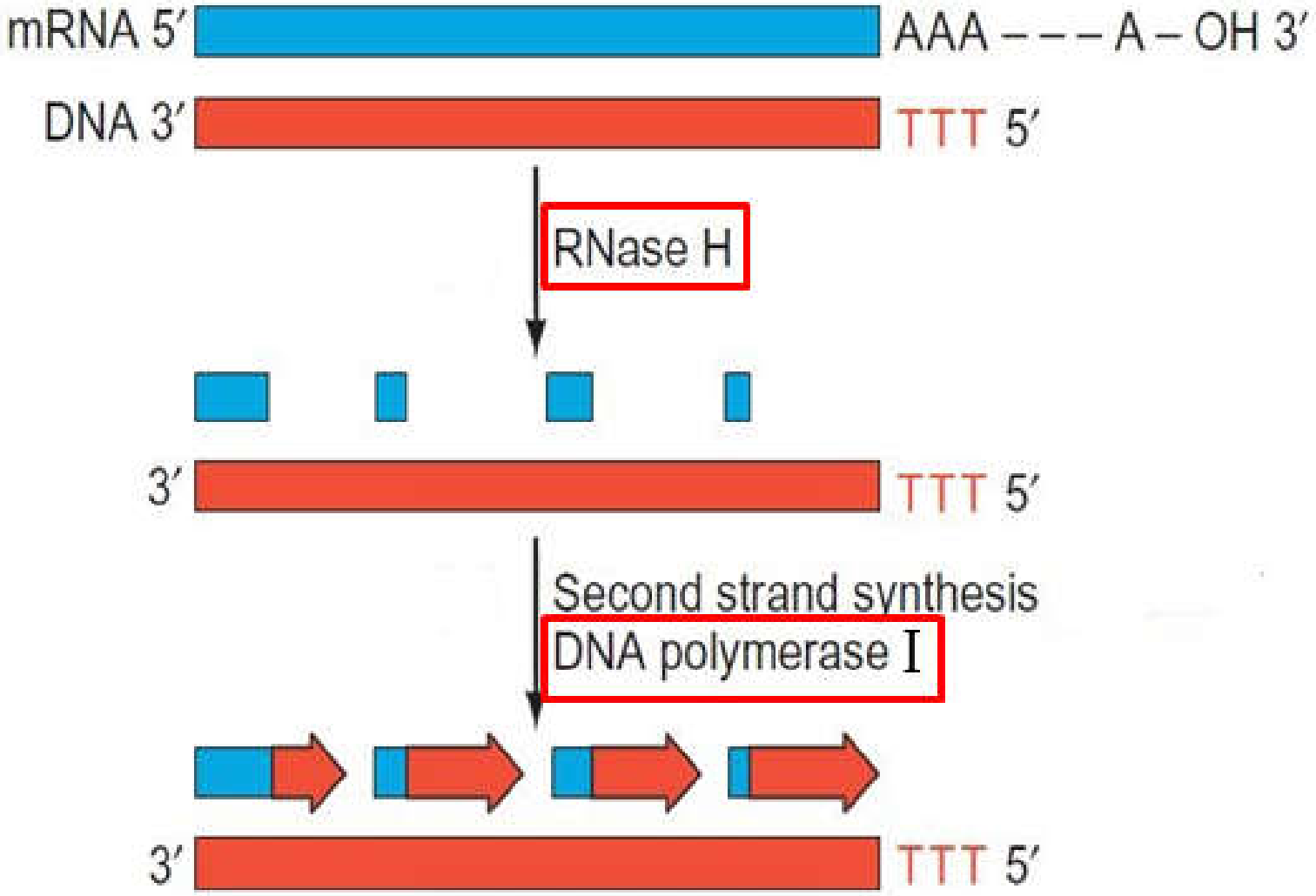
TA克隆

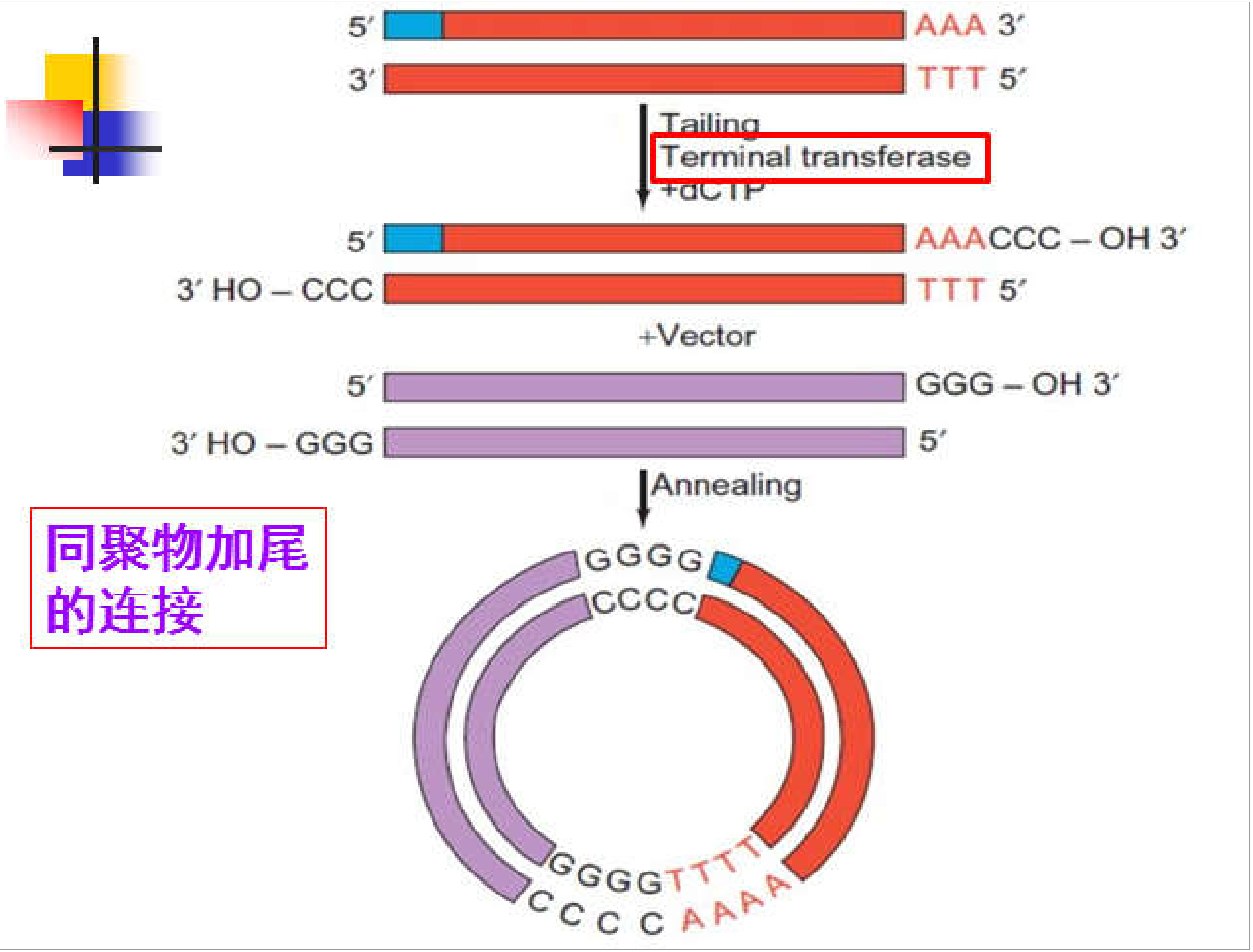
普通的Taq酶可在产物的3′ 端多加一个A，这是非模板依赖的。




XmnI 1994
ScaI
1875
NaeI
2692
f1 ori
Amp^r
pGEM®-T
Vector
(3000bp)
lacZ
T T
ori
T7
1 start
ApaI 14
AatII 20
SphI 26
BstZI 31
NcoI 37
SacII 46
SpeI 55
NotI 62
BstZI 62
PstI 73
SalI 75
NdeI 82
SacI 94
BstXI 103
NsiI 112
126
SP6
0356VA04_3A

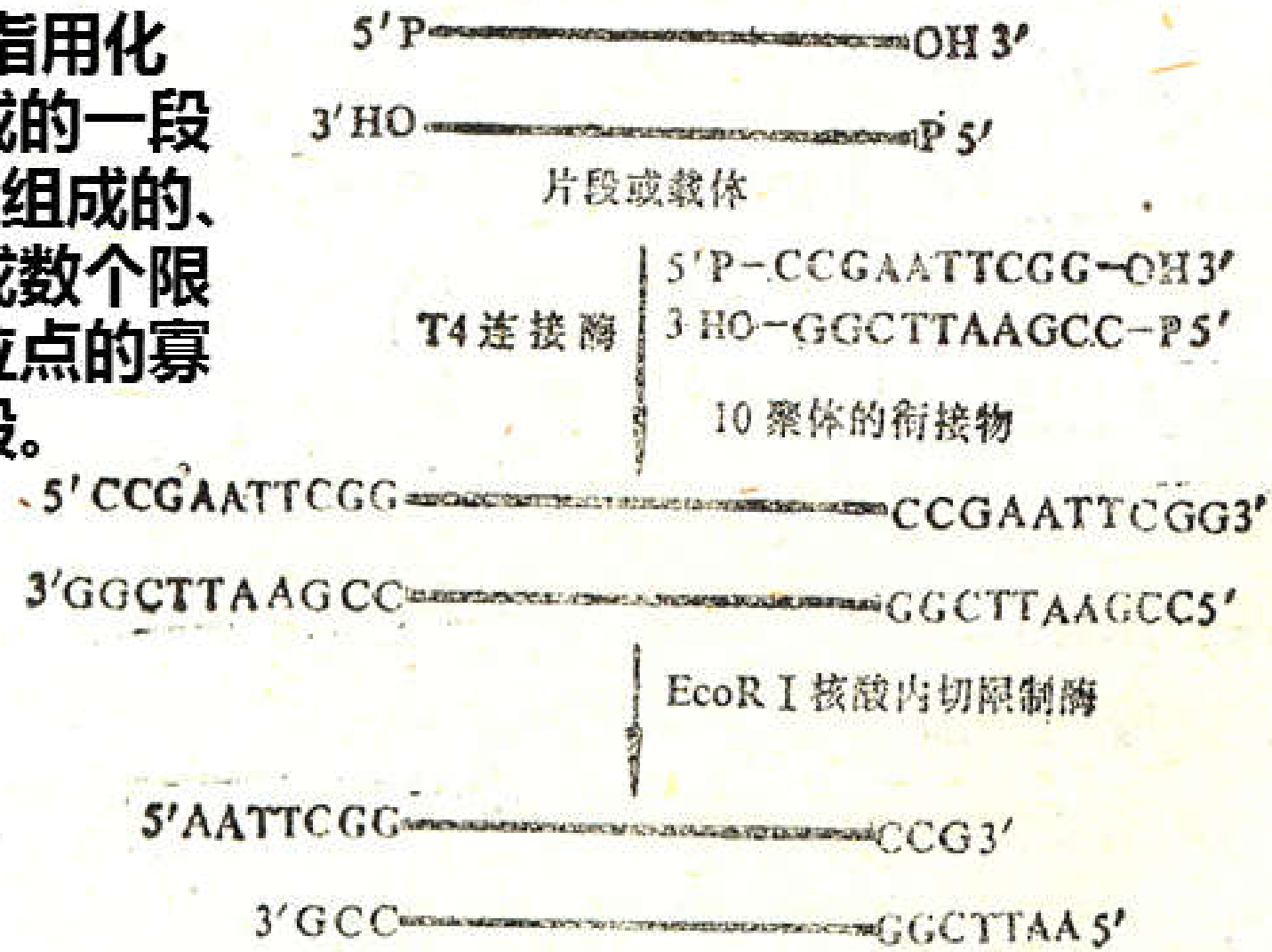
cDNA文库构建


mRNA 5′
AAA – – – A – OH 3′
DNA 3′
TTT 5′
RNase H
3′
TTT 5′
Second strand synthesis
DNA polymerase I
3′
TTT 5′


5′
AAA 3′
3′
TTT 5′
Tailing
Terminal transferase
+dCTP
5′
AAACCC – OH 3′
3′ HO – CCC
TTT 5′
+Vector
5′
GGG – OH 3′
3′ HO – GGG
5′
Annealing
同聚物加尾的连接
GGGG
CCCC
GGGGTTTT
CCCCAAAA

衔接物的连接

衔接物:是指用化学方法合成的一段由10-12 bp组成的、具有一个或数个限制酶识别位点的寡核苷酸片段。



人工接头的连接

^{OH}GATCCCCGGG
GGGCCC

DNA ligase

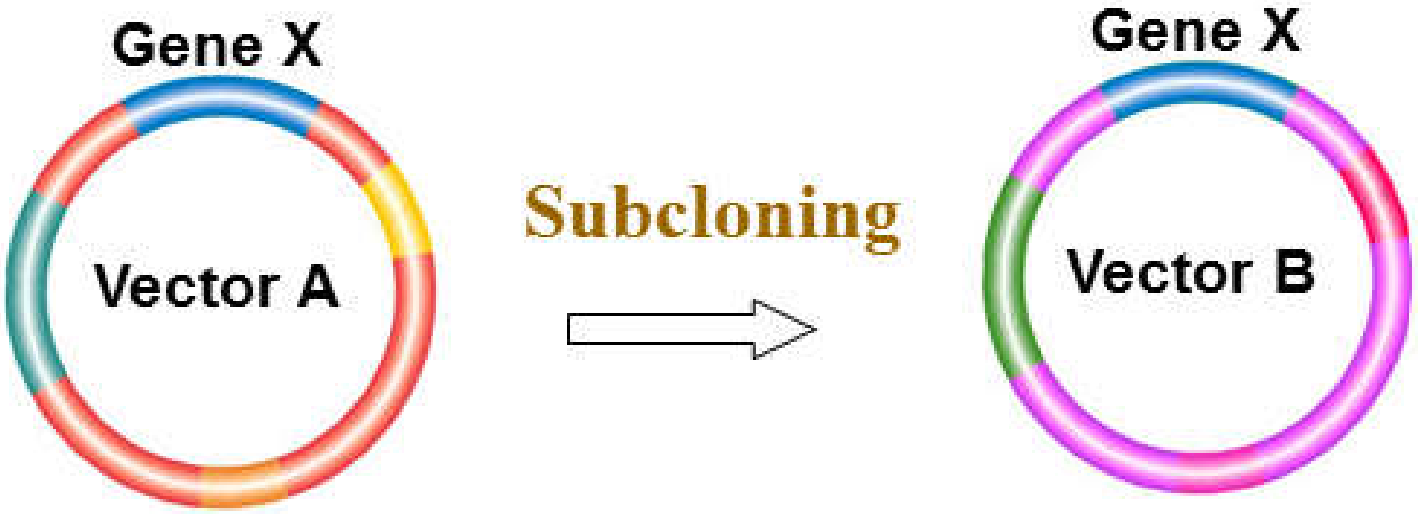
直接具备某一限制性内切酶黏性末端的寡核苷酸片段。

5'-^{OH}GATCCCCGGG
GGGCCC

CCCGGG
GGGCCCCTAG$_{OH}$-5'

亚克隆 (Subcloning)：从载体到载体

Subcloning is the process to transfer of a fragment of cloned DNA **from one vector to another**.



- **Express the gene** in a particular species
- Enables us to **investigate a short region** of a large cloned fragment in more detail.

2.4 Introduce recombinant DNA into host cells

2.4.1 Transformation (转化)

Transformation is a process of uptake of exogenous DNA (normally plasmids) by a host (bacteria or yeast) cell.

(1) Competent cells (感受态细胞)

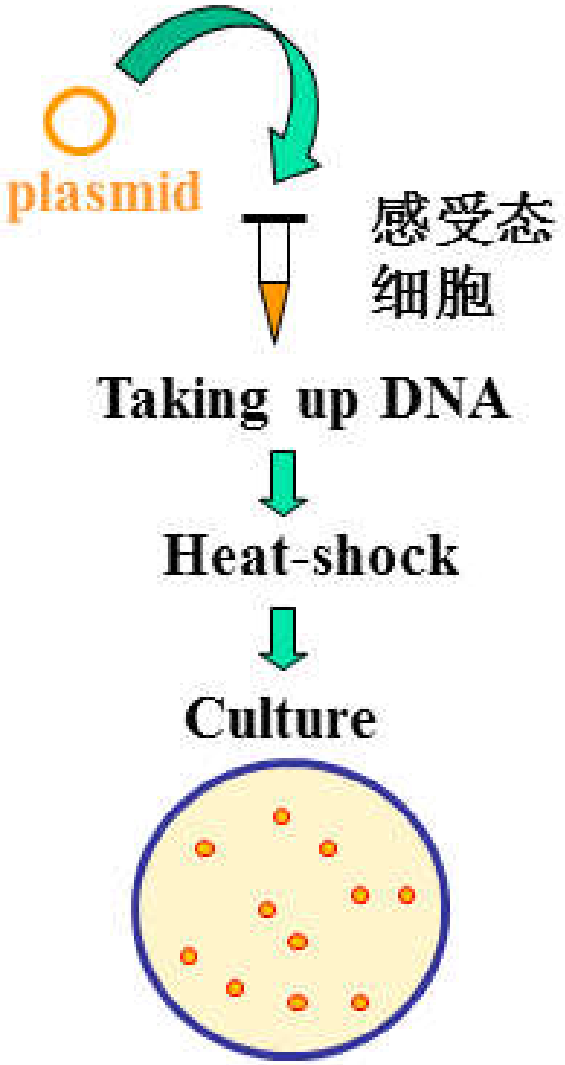
Competent cells are specially treated cells that foreign DNA can enter easily.

- Calcium ions (Ca^{2+}) treatment
- Electrical treatment

(2) Transformation process

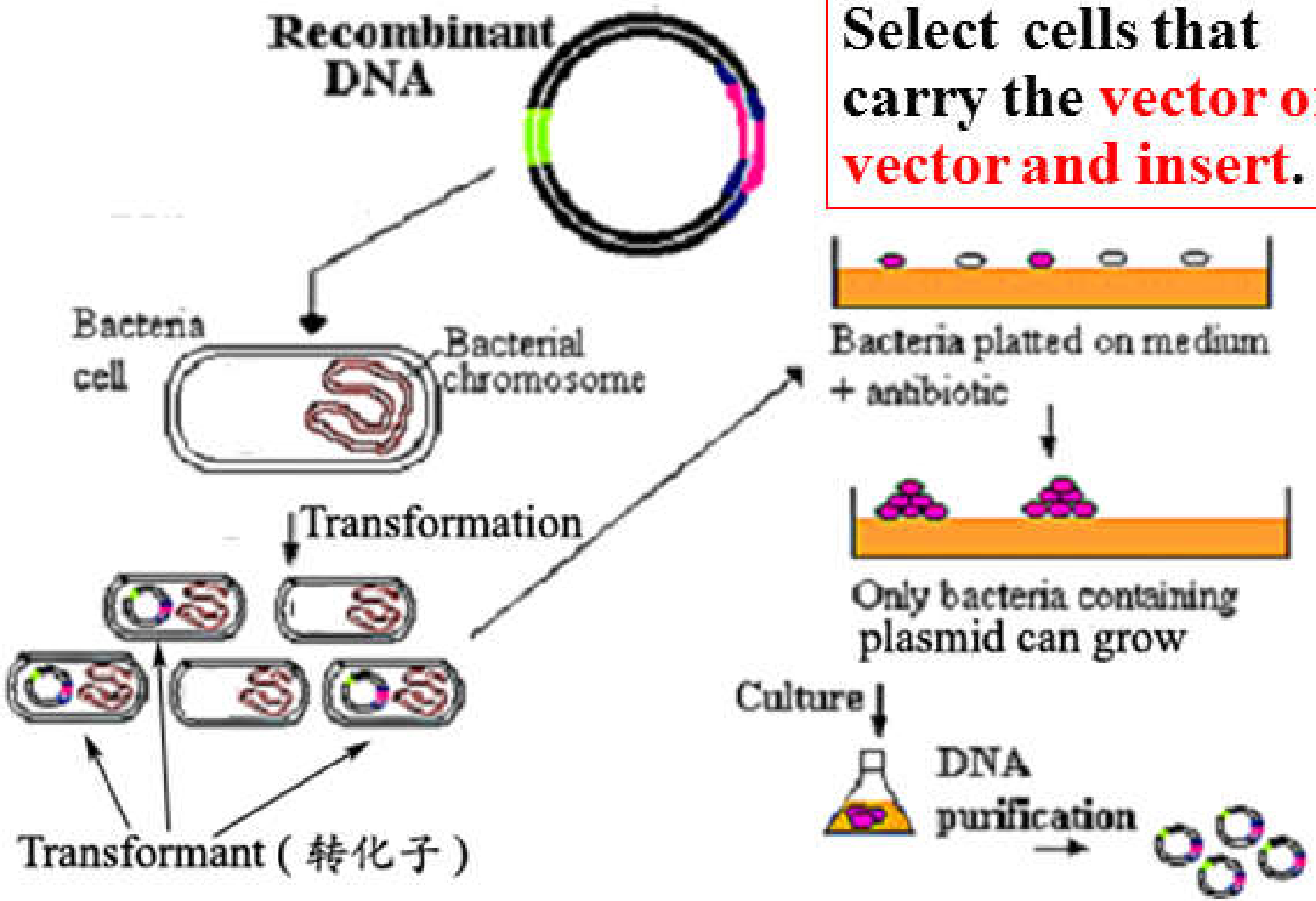
大肠杆菌(*E. coli*)转化过程：

重组DNA与competent cells在冰上混合 (Taking up DNA)→ 42 °C heat-shock (热激) 1~2 min →加适量液体培养基培养，平板（含抗生素）选择培养



Heat-shock induces enzymes involved in the repair of DNA, which allow the cells to recover from unusual conditions of the transformation process, and increases the efficiency.

Selection of plates (平板选择培养)



(3) Transformation efficiency (转化率)

- **Transformation efficiency: The number of colonies (菌落数) formed on a selective plate per μg transformed DNA (pure plasmid).**

- **10^5 colonies/μg plasmids: would be enough for a simple cloning experiment**
- **$>10^9$ colonies/μg plasmids: can be used for generation of libraries**

(4) Factors that influence transformation efficiency

- **Concentration, purity and conformation of transformed DNA**
  浓度越大、纯度越高，转化率越高；超螺旋>环状>线型，分子大的转化率比分子小的要低。
- **Physiological state of transformed cells**
  $CaCl_2$处理贮藏之后的成活率。对数生长期。
- **Environmental conditions of transformation**
  如温度，pH，离子浓度等。
- **Restriction modification system of receptor cells**
  一般用作转化受体的大肠杆菌菌株都是选用在宿主细胞控制的限制体系上有缺陷的突变体。

2.4.2 Transfection (转染)

- The introduction of DNA into mammalian cells is called transfection.
外源DNA导入到哺乳动物细胞的过程称为转染（相当于细菌的转化）。

- Bacteria can be transformed with DNA extracted from a bacterial virus, a process known as transfection.
外源DNA通过病毒载体的感染转移到宿主细胞的过程称为转染。

Transduction (转导): 通过病毒将一个宿主的DNA转移到另一个宿主的细胞中而引起的基因重组现象。

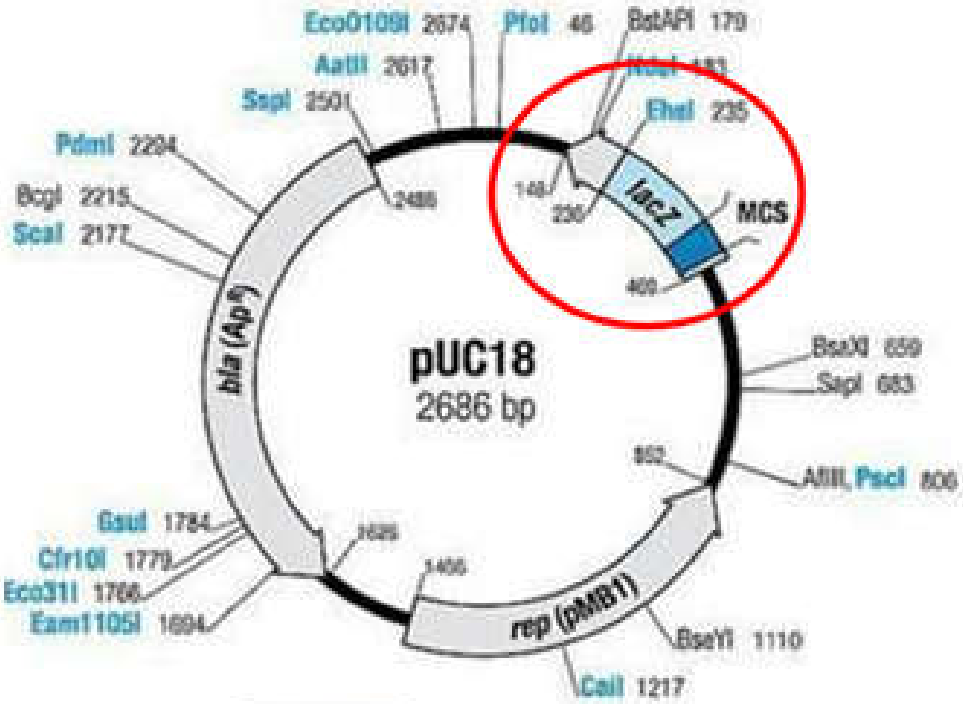
2.5 Screening and analysis of recombinants

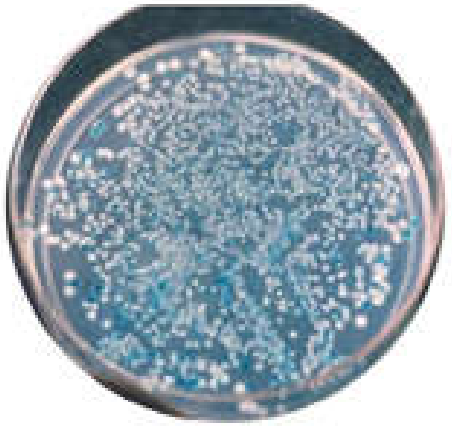
Screening transformants: Allows all cells to grow, but tests the resulting clones for the presence of the insert DNA in the vector.

- **Nucleic acid hybridization (screening libraries)**
- **Selectable markers**
- **PCR**
- **Ristriction digestion and electrophoresis**
- **Sequencing**

2.5.1 Blue-white screening (蓝白斑筛选)

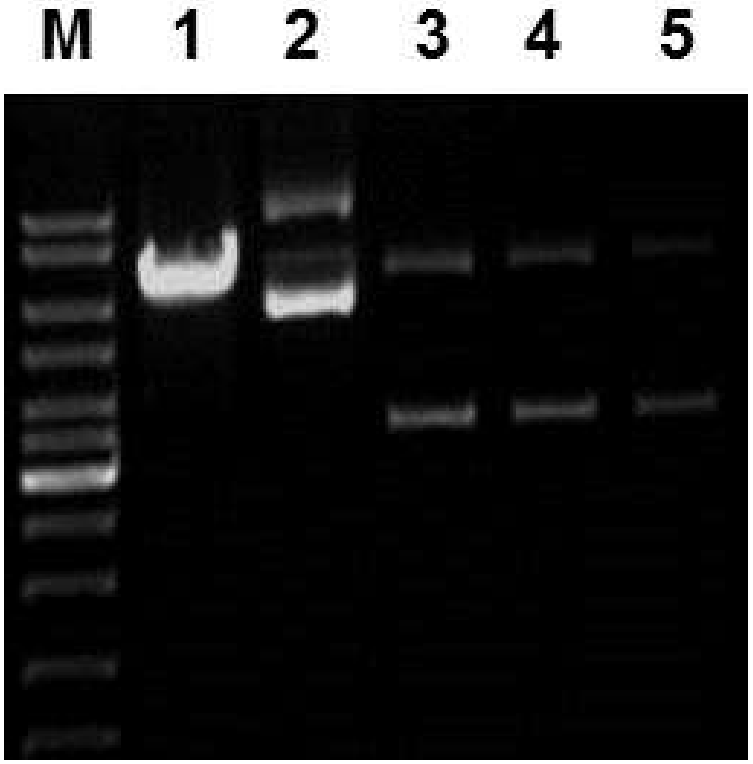
- Insertional inactivation of the *lacZ'*
- Plate containing IPTG and X-gal
- The X-gal is converted to a blue product if the *lacZ'* gene is intact (完整的) and induced by IPTG.
- Recombinants grow as white colonies.

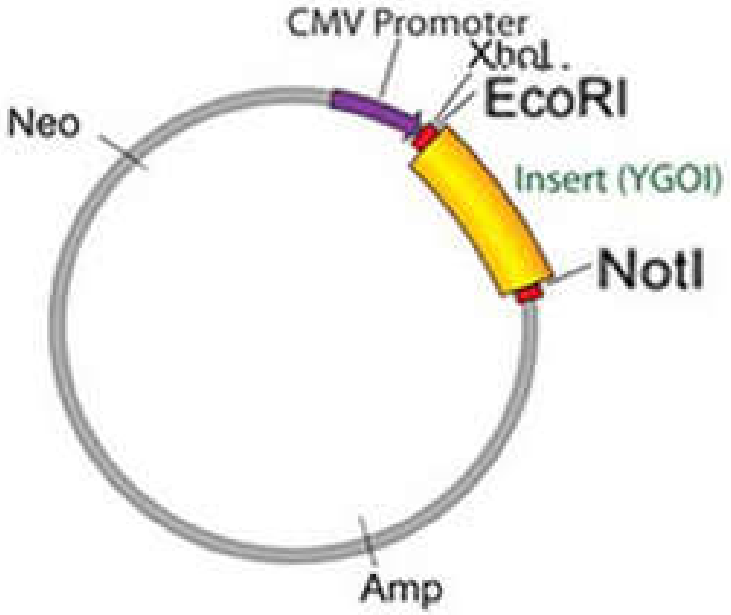




重组菌
白色菌落

2.5.2 Ristriction digestion and electrophoresis

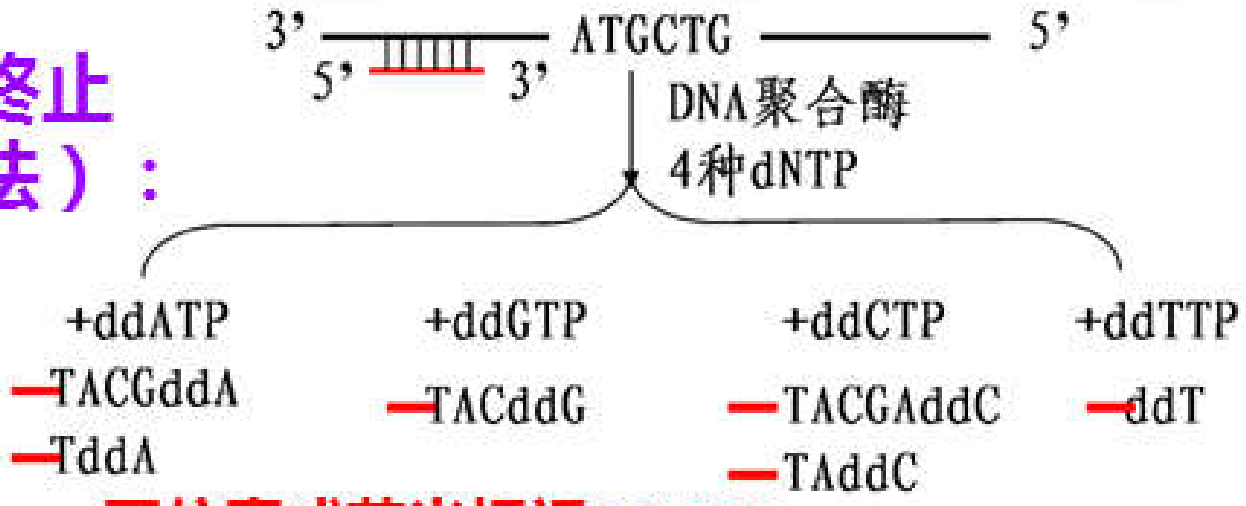




1. Plasmid digest by *Eco*RI and *Not*I
2. Plasmid
3~5. Plasmid with exogenous DNA digest by *Eco*RI and *Not*I

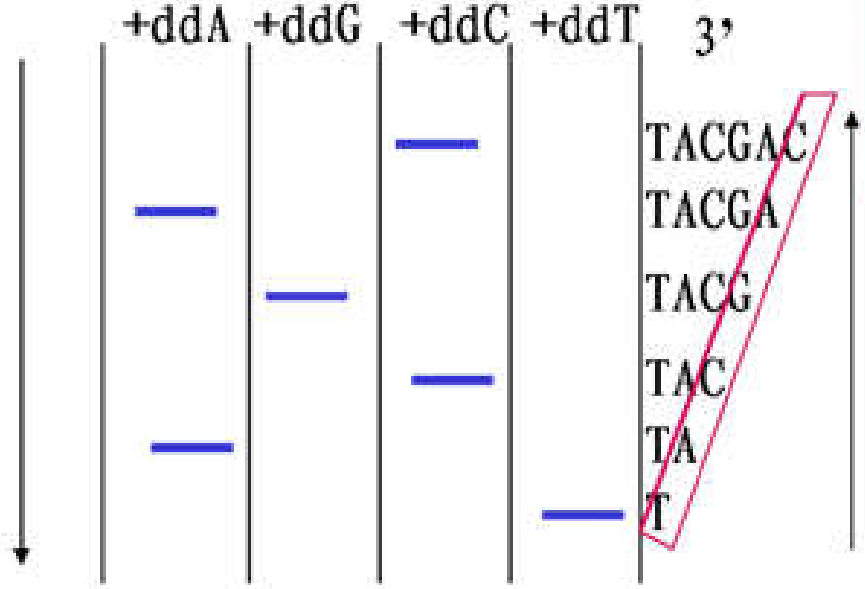
2.5.3 Sequencing

Sanger末端终止法（双脱氧法）：

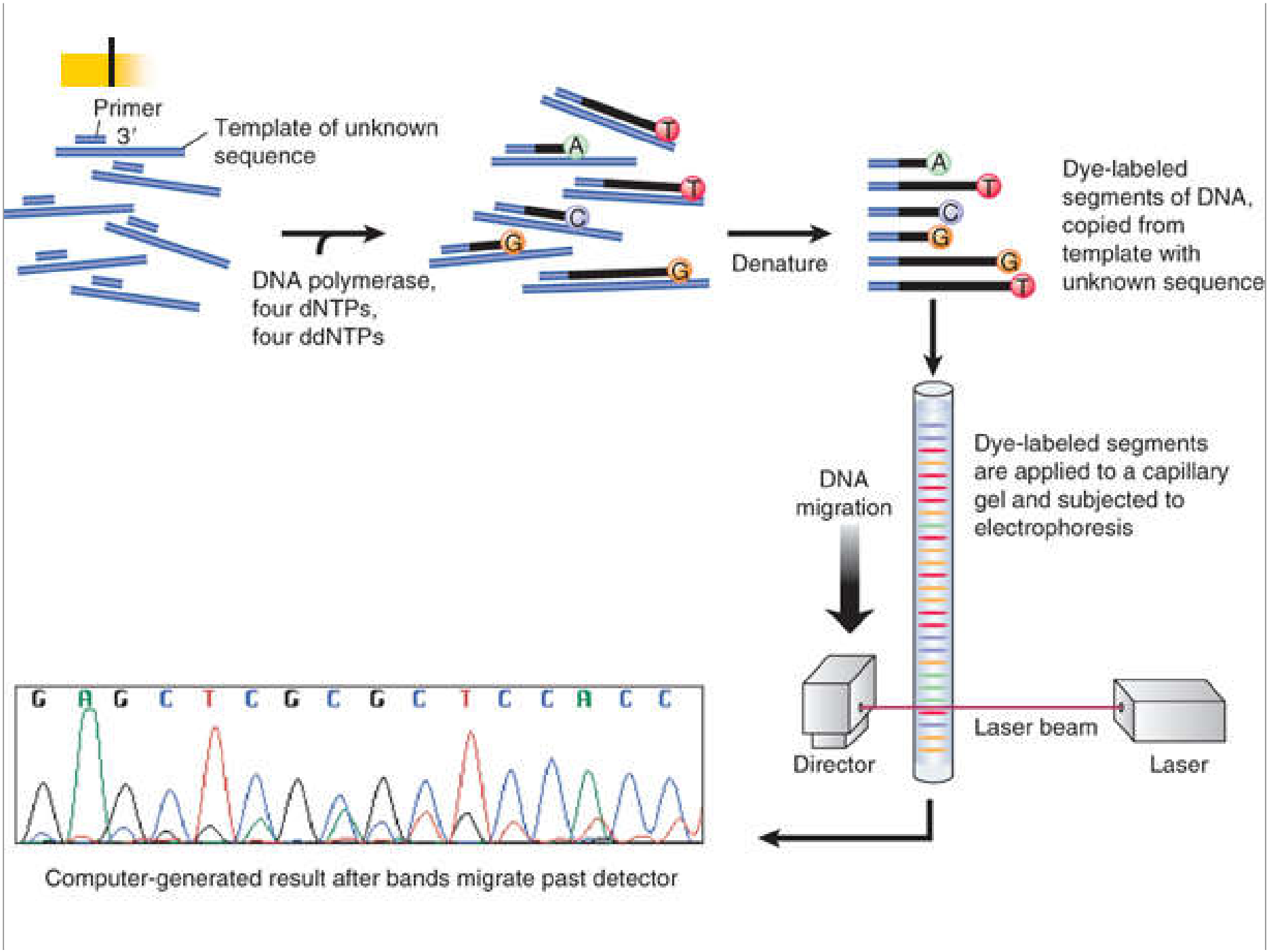


同位素或荧光标记ddNTP

利用 ddNTP 作为DNA合成链延伸的抑制剂，产生长短不一、并具有特定末端的DNA片段。



dNTP：ddNTP的比例最好为1：100


Primer
3′
Template of unknown sequence
DNA polymerase, four dNTPs, four ddNTPs
Denature
Dye-labeled segments of DNA, copied from template with unknown sequence
DNA migration
Dye-labeled segments are applied to a capillary gel and subjected to electrophoresis
Director
Laser beam
Laser
G A G C T C G C G C T C C A C C
Computer-generated result after bands migrate past detector

Proliferation and preservation of transformants

- Grow in liquid broth (液体培养基) with antibiotics.
- Store a portion of culture with 15-30% glycerol (甘油) at -20℃ or -70℃.

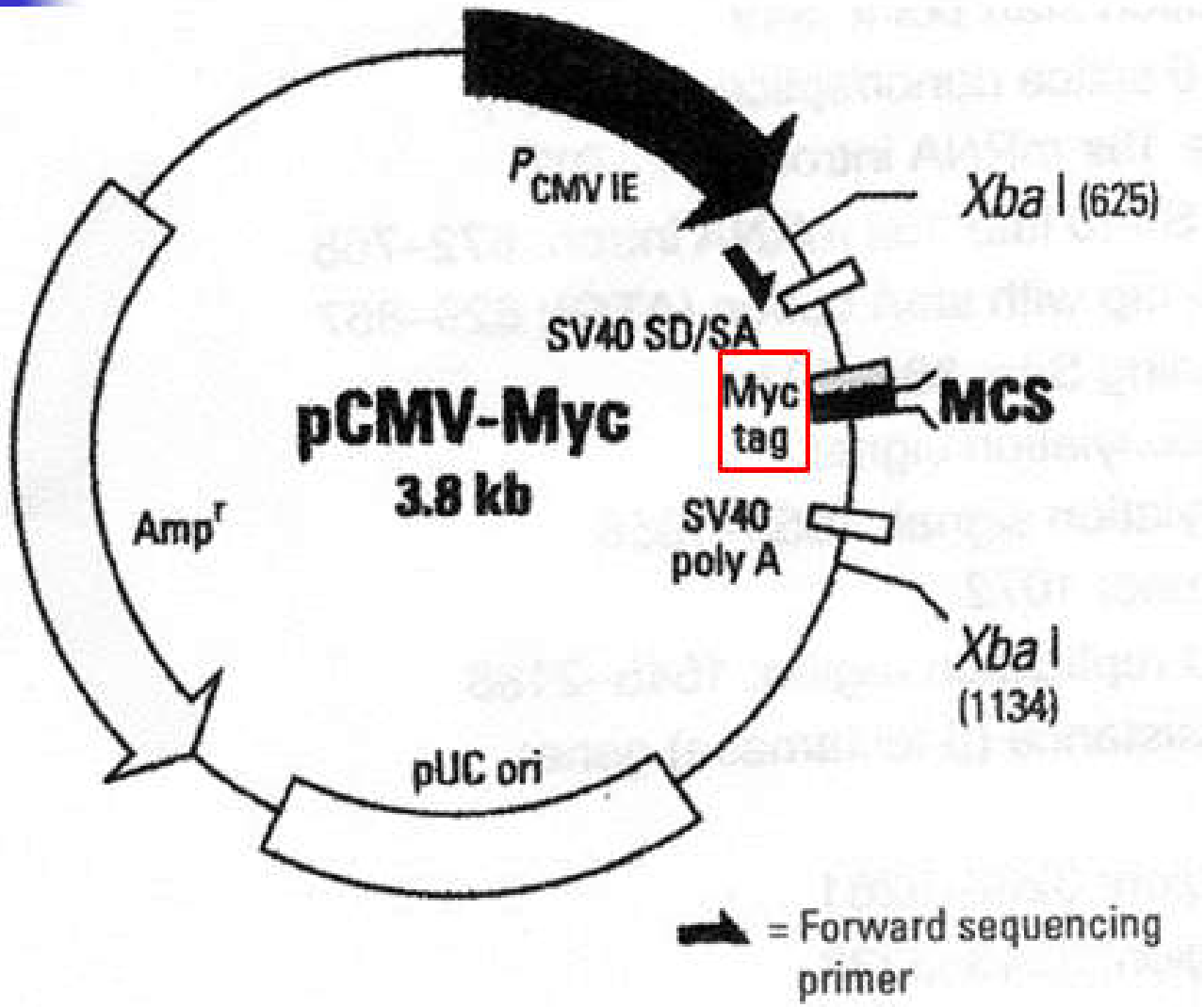
The effect of glycerol stock is to protect the cells from ice crystal formation.

2.6 Expression of cloned genes

- cDNA克隆
- 可诱导的启动子（IPTG诱导）
- SD/Kozak序列
- 报告基因 (reporter gene): 编码一种在目标细胞中不正常产生的但容易被检测和鉴定的蛋白质基因。

检测方法：

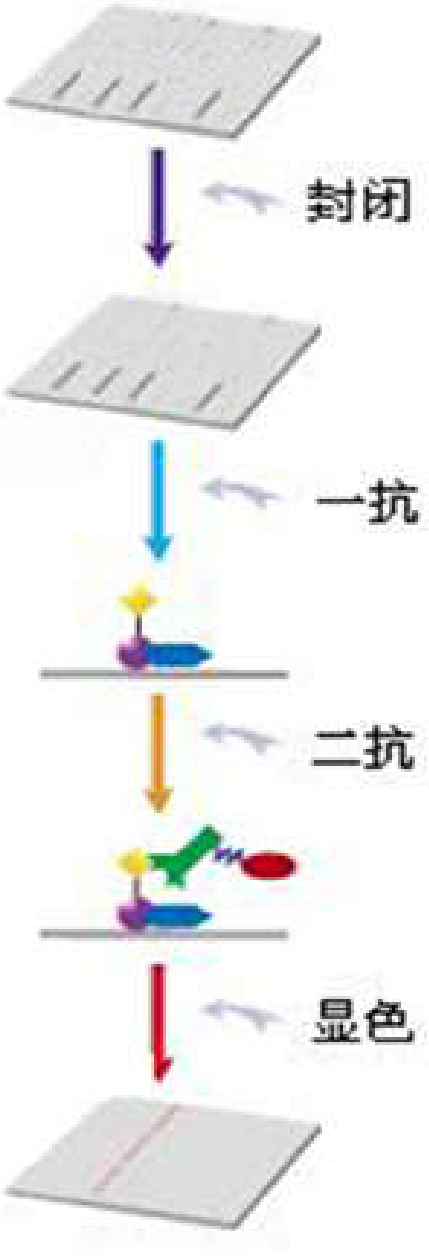
- 酶活检测（酶蛋白）
- 免疫化学检测法（Western blot）



HA
Flag
His
EGFP
…

SDS-PAGE

转膜



Western Blot 通过SDS-PAGE区分不同的蛋白组分，并转移至固相支持物 上，通过特异性抗体作为探针，标记的二抗显色（显影）对靶蛋白含量进行检测。

Summary

1. Definition and general protocol of DNA cloning
2. Types and properties of vectors
3. Types and properties of restriction endonucleases
4. Directional ligation of target DNA and vector
5. Transformation and transfection
6. Methods for screening recombinants

主观题 10分

怎样将一个平末端DNA片段插入到载体*Eco*RⅠ限制位点中去？