Laboratory Molecular Biology

Recombinant DNA (rDNA) Techniques

(PCR, Geneclean, Preparation of Inserts and Vectors, Ligation, Preparation of competent cells, Transformation)

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1、Introduction

In the early 1970s, biochemists at Stanford University showed that genetic traits could indeed be transferred from one organism to another. In this experiment, the DNA of one microorganism recombined with the inserted DNA sequence of another, and thus had been edited to exhibit a very specific modification. The process can be explained in terms of editing a written text: scissors and "glue" are used to "cut" and "paste."

The methods used in **rDNA technology** are fairly simple. We take, for example, the sentence (gene) for insulin production in humans and paste it into the DNA of E.coli. The bacterial cells divide very rapidly, and each bacterium carries in its DNA a faithful replica of the gene for insulin production. Each new E. coli cell has inherited the human insulin gene sentence. And we cut the appropriate gene from human DNA and paste, or splice, it into plasmid DNA, a special kind of DNA that takes a circular form and can be used as a vehicle for this editing job. Our "scissors" are the class of enzymes called **restriction enzymes**. There are well over a hundred restriction enzymes, each cutting in a very precise way a specific base sequence of the DNA molecule. With these scissors used singly or in various combinations, the segment of the human DNA molecule that specifies insulin production can be isolated.

This segment is "glued" into place using an enzyme called **DNA ligase**. The result is an edited, or recombinant, DNA molecule. When this recombinant plasmid DNA is inserted into E. coli, the cell will be able to process the instructions to assemble the amino acids for insulin production. More importantly, the new instructions are passed along to the next generation of E. coli cells in the process known as gene cloning.

By using rDNA techniques, it is possible to produce substances of medical and economic value. And we could obtain desired recombinant of all kinds. (1)

2、Abstract

In order to express our target gene and then the protein in bacteria, we used the traditional recombinant DNA (rDNA) techniques. First, we amplified our target gene DNA sequence (hsp90) by the well-known PCR techniques. And we ligated the insert with our vector (PET23a) in order to transform it into the vector and thus we can do further exploration of protein expression or other functions or effects of the target genes. (In our experiment, the target protein is hspt, a heat-shock protein)

To prevent re-ligation of the vector and to confirm the correct orientation of our insert introduced into the vector, we apply directional cloning with two different restriction endonuclease (NdeI and XhoI) to “cut” our target insert and vector. And to confirm our successful transformation into the bacterial *E.coli* , we spread the germs onto LB agar plates with antibiotics(Ampicillin) in it. After antibiotics screening, we select positive clones into LB medium and culture overnight to make a single stable expression clone. Then we can obtain plasmid with target genes by miniprep technique. And after enzyme digestion, we run the product on agarose gel to confirm whether successful ligation occur or not. If so, we culture the single cell clone on LB agar plate to produce large quantity of cells with our target protein.

3、Materials And Methods

1. Polymerase Chain Reaction (PCR)

Chamicals: *Taq* DNA polymerase, 10X reaction buffer, dNTPs, DNA, mineral oil.

Equipment: Pipetman, microcentrifuge, vortex mixer, thermocycler.

Enzyme	DNA polymerase	
	Taq DNA polymerase	*Pfu* DNA polymerase
Source	*Thermus aquaticus*	*Pyrococcus furiosus*
Thermostability	Yes	Yes
Proofreading	No	3'→5' exonuclease act.
10x Rxn Buffer	100 mM Tris (pH 8.8) 500 mM KCl 15 mM $MgCl_2$ 0.1% gelatin	200 mM Tris (pH7.5) 80 mM $MgCl_2$ 400μg/mL BSA

Template: pET 23a-hspt

Primer:

Primer 1: $^{5'}$TAATACGACTCACTATAGGG$^{3'}$

Primer 2: $^{5'}$GCTAGTTATTGCTCAGCGG$^{3'}$

Polymerase:

Tag DNA polymerase 5U/μl.

Reaction condition:

Add each component in the following sequence to a 0.5 mL Eppendorf tube. Mix with a pipette tip (no air bubbles allowed!) and cover the surface with 50μl of mineral oil.

	[stock]	Volume	working concentration
QH_2O	-	37.5μl	-
DMSO	100%	2.5μl	5%
Rxn. Buffer	10×	5μl	1×
dNTP	20 mM	1μl	200μM
Tag	10/λ	1μl	0.04U/μl
$T_{7\text{-}P}$ primer	10μM	1μl	200μM
$T_{7\text{-}T}$ primer	10μM	1μl	200μM
Template(PET23a-hspt)	1-100 ng/μl	1μl	＜2 ng/μl
Final		50μl	

Reaction Program

Stage	Function	Temperature	Time	Cycle
1	Denaturation	95℃	5 min	1
2	Denaturation	95℃	30 sec	35
	Annealing	42℃	30 sec	
	Extension	72℃	30 sec	
3	Extension	72℃	7 min	1

Store the PCR product at 4℃

2.Isolation and Purification of PCR Products

Chemicals: Low melting agarose, restriction enzymes, TBE buffer, DNA loading dye, DNA molecular weight marker, ethidium bromide, NaCl, EtOH.

Equipment: Pipetman, microcentrifuge, vortex mixer, DNA eletrophoresis apparatus, fluorescence light box, camera, -20℃ freezer.

1. Use electrophoresis to isolate vector and insert (PCR product) from protein (polymerase) or other fragments.
2. Use a clean, sharp scalpel or razor blade to excise the gel slice containing the DNA fragment of interest.
3. Measure the weight of the gel slice (about 50~200 mg) and place it into a sterile 1.5 ml or 2 ml centrifuge tube. Add 0.5 ml GEX Buffer to it.
4. Place a Gel-M™ Column onto a Collection Tube. Load no more than 0.7 ml dissolved gel mixture into the column.
5. Centrifuge at full speed for 45 seconds. Discard the flow-through. Repeat step 4 for the rest of the mixture.
6. Wash the column with 0.5 ml of WF Buffer by centrifuging for **45** seconds. Discard the flow-through.
7. Wash the column once with 0.7 ml WS Buffer by centrifuging for 45 seconds Discard the flow-through.
8. Centrifuge the column at full speed for another 3 minutes to remove residual ethanol.
9. Place the column onto a new 1.5 ml centrifuge tube. Add **30**μl of Elution Buffer (provided) onto the center of the membrane.
10. Stand the column for 1~2 minutes, and centrifuge for 1~2 minutes to elute DNA.
11. Store DNA at -20℃.

3.Preparation of cloning vector and insert

Chemicals: DNA, restriction enzymes

Equipment: Pipetman, microcentrifuge, vortex mixer, dry bath

	Vector	Insert
DNA	40μl	30μl
*Nde*I (20U/λ)	1μl	1μl
*Xho*I (20U/λ)	1μl	1μl
#4	5μl	4μl
RNase	1μl	1μl
QH_2O	2μl	3μl
Final	50μl (×2)	40μl

4.Ligation:

Chemicals: DNA of unknown concentration, T4 DNA ligase (NEB), 10X T4 DNA ligase buffer (NEB), ddH_2O.

Equipment: Pipetman, spectophotometer, UV-cuvette, microcentrifuge, vortex mixer, dry bath, water bath, handy cooler.

Spectrophotometric Determination of the Amount of DNA and RNA

1. Samples are diluted to 200-fold with ddH_2O (or TE buffer).
2. Turn on the spectrophotometer to warm up.
3. Add ddH_2O (or TE buffer) to the quartz cuvette and take readings as background valus.
4. Remove ddH_2O (or TE buffer) and insert sample.
5. Take readings at wavelengths of 260 nm and 280 nm.
6. OD260nm allows calculation fo the concentration of nucleic acid in the sample.

1 OD260nm corresponds to 50μg/mL for double stranded DNA.

40μg/mL for single stranded DNA and RNA.

33μg/mL for single stranded oligonucleotides.

7. The ration between the readings at 260 nm and 280 nm represents the purity of the nucleic acid. Pure DNA and RNA have OD260/280 values of 1.8, and 2.0, respectively.

Ligation:

1. Calculate the concentration of the unknown DNA solution; put appropriated volume (Less than 0.1μg.) of insert (hspt fragment) and Vector (pET-23a) in eppendorf. * For blunt end ligations it best to use 10x molar excess of insert to vector (e.g. for a 1kb insert that would be 30 ng of insert to 1 ng of vector.) For ligation with overhangs, use a 4 to 1 molar insert to vector ratio.
2. Add 1μl of 10X T4 DNA ligase buffer (working concentration: 50mM Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25μg/ml bovine serum albumin).
3. Add 0.5μl T4 DNA ligase (NEB, 400U /μl) and ddH_2O to final volume =10μl * One NEB unit is defined as the amount of enzyme required to give 50% ligation of *Hind* III fragments of lambda DNA in 30 minutes at 16℃ in 20μl of the above assay mixture and a 5' DNA termini concentration of 0.12μM(300μg/ml)
4. Incubate the reaction mixture at 16℃ for 16 hr.

Vector	3.5μl
Insert	5μl
Buffer	1μl
Ligase	0.5μl
Total	10μl

5. Competent cells and transformation

Chemicals: LB medium, LB agar plate (Amp + Tet), 1 M $CaCl_2$, tetracycline, ampicillin.

Bacterial strain: E.coli Top10F' (Tet^r), BL21

Equipment: Pipetman, microcentrifuge, spectrophotometer, orbital shaker incubator, water bath, rubber floater, incubator, L-shape glass rod.

Preparation of Competent Cells

1. Inoculate a single colony of bacteria to 5 mL of LB medium comtaining 12.5g/mL tetracycline and incubate at 37℃ with shaking for 16~20 hr.
2. Dilute the overnight culture 100 fold into 20 mL of LB medium containing 12.5μg/mL tetracycline and incubate at 37℃ with shaking at 250 rpm for 3 to 4 hr until OD_{600} reaches 0.4~0.6.
3. Collect the cells by centrifugation at 6000 rpm for 5 min at r.t.
4. Decant the supernatant and place the tube on ice for 10 min.
5. Resuspend the cells in 10 mL of ice-cold 100 mM $CaCl_2$ and store on ice for 30 min.
6. Collect the cells by centrifugation at 4000 rpm for 5 min at 4℃.
7. Remove the supernatant and drain away any fluid left by inverting the tube.
8. Resuspend the cells in 0.5 mL of ice-cold 100 mM $CaCl_2$ and place the tube on ice for 1 hr.

Transformation

1. Preheat the water bath to 42℃
2. Transfer 5μl of DNA sample into 100μl of competent cells, swirl the tubes gently several times to mix.

   *Approximately 40 ng of supercoiled DNA is generally preferred in the process.

*The volume of the DNA solution should not exceed 5% of the volume of the competent cells for optimal results.

3. Add 5µl of ddH_2O or TE to 100µl of cells as the negative control.
4. Add 10 ng of supercoiled plasmid vector to 100µl of cells as the positive control.
5. Chill the mixture on ice for 5 min.
6. Transfer tubes to a floater in a 42℃ circulating water bath. Leave the tubes in the water bath for exactly 90 sec. Do not shake the tube.
7. Rapidly transfer the tubes to an ice bath. Allow the cells to chill for 1~2 min.
8. Add 500µl of LB medium (no antibiotic) to the cells and mix gently.
9. Incubate the tubes at 37℃ with shaking at 220 rpm for 45 min to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
10. Gently spread the transformed cells over the surface of the agar plate with a sterile L-shape glass rod. ** Rinse the glass rod with absolute EtOH and sterile it by flame. * For cells transformed with circular plasmid DNA, spread 30µl of culture onto an LB agar plate containing 50µg/mL ampicillin and 12.5µl/mL tetracycline. * For cells transformed with ligation mixture, spread 100µl of culture onto an LB agar plate containing 50µg/mL ampicillin and 12.5µg/mL tetracycline.
11. Leave the plates at r.t. until the liquid has been absorbed.
12. Invert the plates and incubate at 37℃ for 12~16 hr.
13. Count the numbers of single colonies obtained from the plate.

Transformation Efficiency = No. of colonies x dilution factor /µg DNA used

4、Results

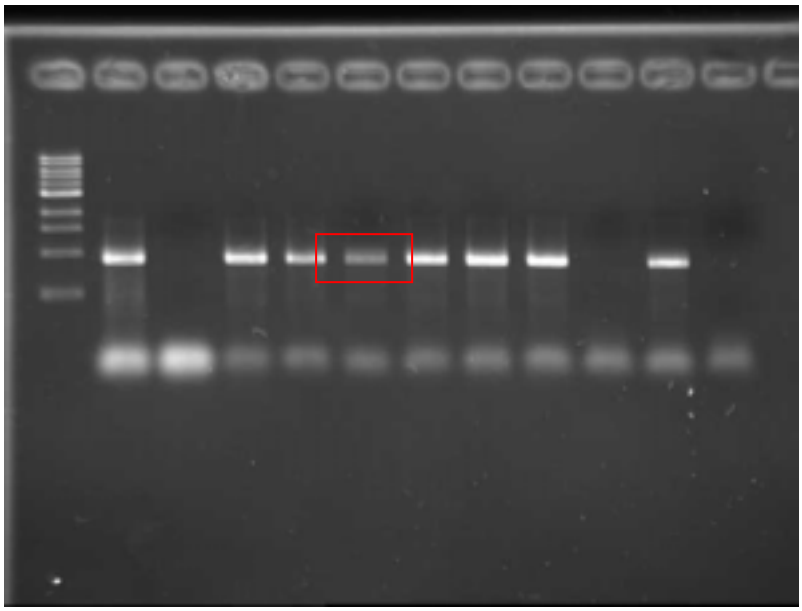

Fig 1. gel electrophoresis and ethidium bromide staining of target gene PCR products.

(1^{st} lane: marker ; 6^{th} lane: our PCR product,hsp90 from rats)

1 2 3 4 5

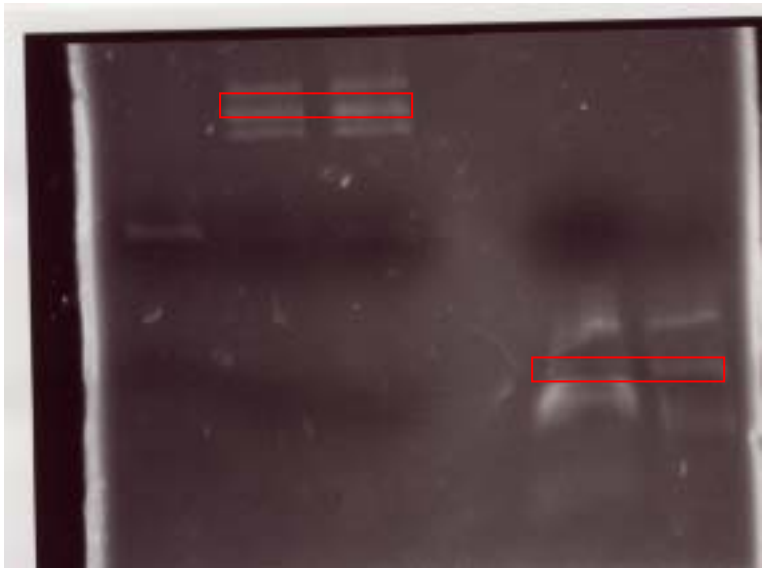

Fig 2. enzyme-digested vector(lane 2,3) and insert(lane 4,5) which will be cut and purified later.

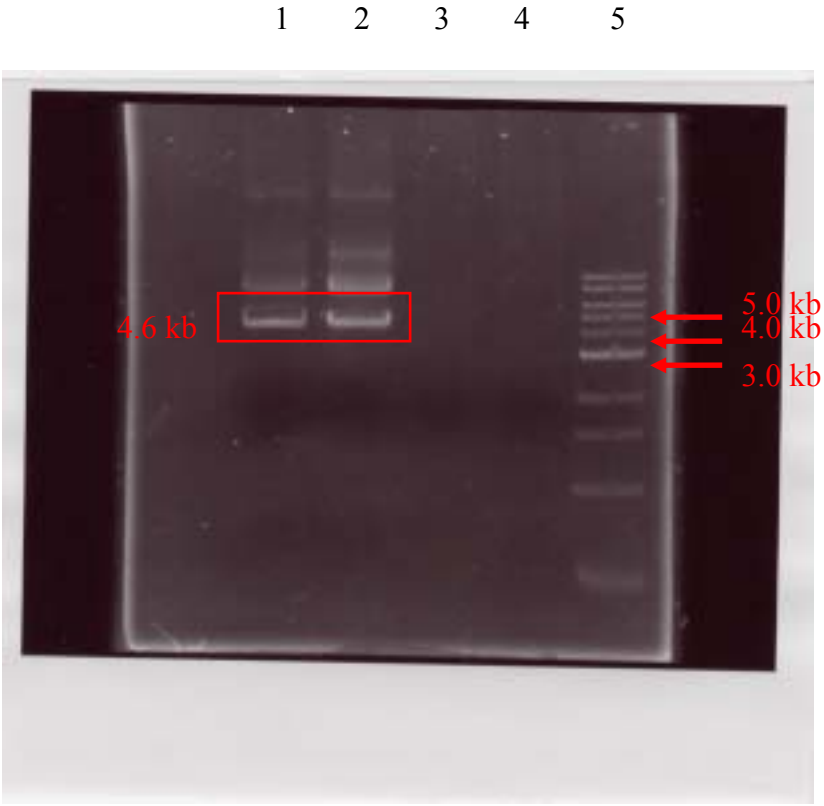

Fig 3. successful ligation products purified by miniprep technique(lane 1,2: successful ligation insert-vector products ; lane 3,4: unsuccessful ligation ; lane 5: marker)

5、Discussion

PCR (Polymerase Chain Reaction) has revolutionized molecular genetics and continues to be applied to many fields of biology. The PCR technique is basically a primer extension reaction for amplifying specific nucleic acids in vitro. The use of a thermostable polymerase (ex:*Taq,Pfu*) allows the dissociation of newly formed complimentary DNA and subsequent annealling or hybridization of primers to the target sequence with minimal loss of enzymatic activity. We use *Taq* DNA polymerase to amplify our target sequence and run it with agarose gel electrophoresis (**Fig 1.**) A very important property of *Taq* DNA polymerase is its error rate, which was initially estimated at $2* 10^{-4}$ nt/cycle (Saiki et al., 1988). (2)

Then as in the Gel-M^{TM} Gel Extration System , we should minimize the size of the

gel sliced by removing extra agarose and cutting the gel slice into small pieces can facilitate dissolution. After enzyme digestion, we run insert and vector on the gel again(**Fig 2.**) and purified them with the same kit. The mess in Fig 2. results from the preparation of gel. (3)

In the process of ligation , we should carefully control the condition of the reaction. If it's sticky end, we control the temperature at about 16℃, wherease in the blunt end ligation, one often controls the temperature at about 12℃.In order to prevent self-ligation, we take advantage of the directional cloning using two different restriction enzymes. In case of only one enzyme reaction, we also can use the chemical Alkaline phosphatase to remove the phosphates at 5' ends of the vector, preventing it from relegation with itself. In spectrophotometry, we measure OD260nm to calculate the amount of DNA, by the " Beers Law ":

$A = \varepsilon bc$

A: Absorpance

ε: Extinction coefficient

b: path length

c: concentration

In preparation of competent cells , *E.coli* cells are grown to log phase . Cells are centrifugation and resuspended in a solution containing calcium chloride.Exposure to calcium ions renders the cells able to take up DNA, or *competent*. Plasmid DNA is mixed with the cells and are together heat shocked, which allows the DNA to efficiently enter the cells . The cells are grown in nonselective medium to allow synthesis of plasmid-encoded antibiotic resistence proteins, then plated on antibiotic-containing medium to allow identification of plasmid-containing colonies, which can be done directly in the air.(4)

We again used the miniprep technique to purify our ligation product. After enzyme digestion, we run them on the agarose gel (**Fig 3.**) , And we can obtain the successful products from the 4.6-kb bands.(**Fig 3.** lane 1,2). The upstream several bands are also present on all the other groups in the laboratory and the reasons remain unknown so far.

6、Questions

1. Polymerase Chain Reaction (PCR)

1. Please calculate the T_m value of each primer. [$T_m = 2\times(A+T)+4\times(G+C)$].

Ans: Primer 1—TAATACGACTCACTATAGGG

$2\times(7+5)+4\times(4+4)=58℃$

Primer 2—GCTAGTTATTGCTCAGCGG

$2\times(3+6)+4\times(5+3)=50℃$

2. How do you determine what temperature for anneling to apply in a PCR?

Ans: Annealing temperature depends on the primer GC content. Primers with high GC content, higher annealing temperatures may be necessary, and vice versa. We can experiment to find the optimal temperature.

3. Is mineral oil absolutely necessary for the reaction? How can we avoid using it?

Ans: (1) mineral oil keeps the water from vaporization to sustain the buffer concentration

: (2) close system to reduce vaporization or reduce the reaction cycles.

4. What is the function of DMSO?

Ans: DMSO is solubility-enhancing solvent. DMSO inbhbits antibody binding and

should not be used with TaqStart.

2. Isolation and Purification of PCR Products

˙Why can DNA be released from agarose by the operation?

Ans: Low melting agarose would be melted at moderate temperature, which DNA wouldn't denature at same temperature. As a result, we can isolate intact double-strand DNA from agarose.

3.Preparation of cloning vector and insert

Can you digest PCR product directly in the PCR buffer prior to DNA isolation and purification?

Ans: Theoretically yes, however, the restriction enzyme activity may be less efficient without isolation and purification.

4.Ligation:

1. Based on the OD260nm/OD280nm ratio, examine the purity of the DNA solution

Ans: Normally:

With DNA: OD260nm/OD280nm = 1.8~2.0

With RNA: OD260nm/OD280nm = 1.6~1.8

Which result from the extinction coefficient.

And our result: OD260nm/OD280nm 2.3

2. For DNA ligation reactions, how do you design "positive" and "negative" control for the experiments?

Ans: Cells without ligased plasmid vector are called negative control.

Cells with ligased supercoiled palsmid vector are called positive control.

5. Competent cells and transformation

1. Why do we have to keep the competent cells on ice during the experiment?

Ans: For higher transformation efficiency.

2. What is the transformation efficiency of your competent cells?

Ans: Normally, we have transformation efficiency of 10^7 colonies /μg DNA.

In our result, we have only two colonies on our plate, so we have:

(dilution factor= 500μl+ 100μl / 200μl = 3)

Transformation Efficiency = No. of colonies x dilution factor /μg DNA used

= 2x 3/ 0.01=600.

3. List three factors that affect the efficiency of transformation.

Ans: (1) plasmid size, (2) calcium ion concentration, (3) temperature (ice)

(4) supercoil form has higher efficiency.

7、Reference

1. MIT Biology Hypertextbook (A website which contains the basic molecular biology that is the basis of MIT's core Biology course, "Introductory Biology")
2. Current Protocols in Molecular Biology(2001), contributed by Martha F. Kramer and Donald M. Coen.
3. Protocol for Spin Method, Gel-M™ Gel Extration System.
4. Current Protocols in Molecular Biology(1997), contributed by Christine E.Seidman, Kevin Struhl, Jen Sheen, and Timm Jessen.