2. Restriction Enzyme Digestion, Gel Extraction and Primer Design

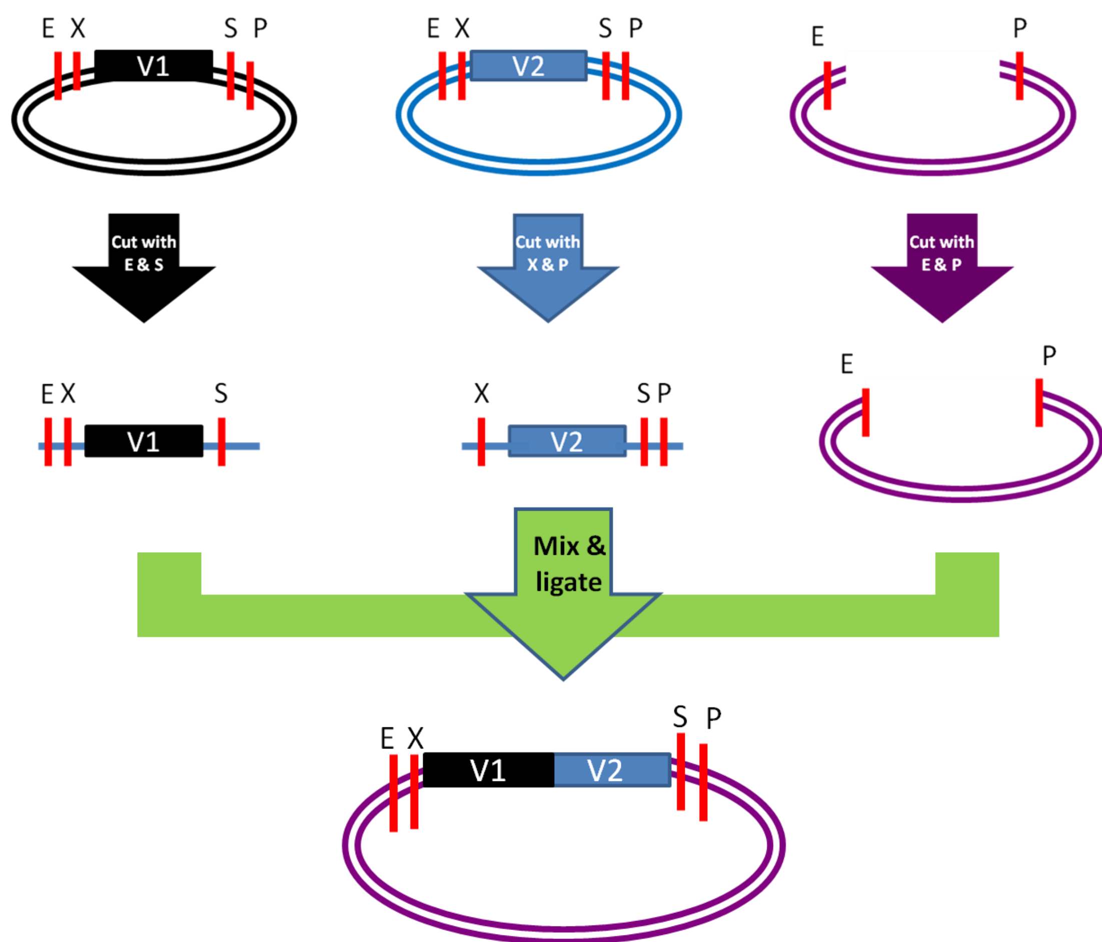
Molecular cloning requires manipulation of nucleic acid, including copying (synthesize), cutting (digest) and pasting (ligate). In bacteria, it is usually done in plasmid form. To insert your target gene into a vector, you need to digest the gene and the vector to produce joining sites. Restriction enzymes are needed because it recognizes and digests DNA at specific site. According to the cleavage end, it can be divided into two groups, blunt end and staggered end (or sticky end); According to recognition mechanism and cleavage sites, it can be classified into 5 types. The common restriction endonucleases we use belong to type II. It requires no ATP, but Mg^{2+} as co-factor. It recognizes and cleaves at the same palindromic sites, i.e. same sequence from either strand of DNA from 5’ to 3’ (e.g. GAATTC).

Depending on the digestion template and enzymes used, following step may be different. If only 1 restriction enzyme is used to linearize a circular DNA, heat inactivation of enzymes is enough. If 2 or more restriction enzymes are used at a distance from each other to cut out a DNA fragment, agarose gel electrophoresis is required to separate the DNA fragments and then recover the desired one from gel.

Blunt end	Staggered end
GAA/TTC	G/AATTC
CTT/AAG	CTTAA/G

A. *Restriction Enzyme Digestion*

This step is to generate sticky ends or blunt ends of the 5' end/ 3' end of the DNA, or to change a circular plasmid into a linear form. The figure below illustrates double enzyme digestion of DNA for biobrick construction.



Materials

Restriction enzyme(s)

100 × BSA (bovine serum albumin)

10 × NEB buffer

ddH_2O (deionized, sterile, DNase-free)

DNA template

37 ºC incubator or dry bath

Procedure

1. Place the required reagents on ice. Label them.
2. Follow the table below to prepare restriction digestion reaction mixture:

Reaction Volume	**10 µl**	**25 µl**	**50 µl**
DNA template	0.1 µg	0.5 µg	1 µg
10X Buffer (1-4, according to enzymes)	1	2.5	5
100X BSA	0.1	0.25	0.5
Enzyme	1 U	5 U	10 U

* *Enzymatic units differ among enzymes and companies. Refer to the documents of the supplier to calculate the needed amount.*

3. Pipette the solution up and down to ensure all reagents are mixed well.
4. Place the reaction mixture at 37 ^{o}C incubator or dry bath for 2-4 hours.
5. Purify the DNA by PCR purification kit/gel extraction kit for downstream process.

Notes

- Water is always added first.
- Buffer is always added before that of enzyme.
- Make sure that you use the relevant buffer.
- Beware of the temperature for optimal enzyme digestion and DO NOT over-digest your DNA.
- Enzyme dissolved in glycerol sticks to the sides of the pipette tip. Therefore, just touch the pipette tip to the surface of the enzyme solution to ensure correct volume of the enzyme being pipetted.
- Total enzyme volume should be lower than 10 % of the reaction volume to avoid star activity

(This video shows the procedures of restriction enzyme digestion)

B. DNA Purification from Gel by spin column

This step is to purify the DNA (70 bp – 10 kb) by removing the nucleotides, enzymes and ions from solution, and separate different pieces of DNA for target DNA selection. The purified DNA is suitable for downstream process.

Materials

Gel clean kit

ddH_2O (deionized, sterile, DNase-free)

60 ^{o}C dry bath

Procedures

(Modified from QIAGEN Gel clean kit protocol)

All centrifugation steps are carried out at 10,000 – 13,000 rpm (17,900 x g) in a conventional tabletop micro centrifuge at room temperature.

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by trimming off extra agarose gel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (300 µl QG to 100 mg gel). For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIA quick column is 400 mg.

3. Incubate the tube at 60℃ for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by inverting the tube every 2 min during the incubation. Solubilize agarose completely. For >2% gels, increase incubation time.

4. If the color of the mixture is orange or violet, add 10 μl of 3 M sodium acetate, pH 5.0, and mix the reagents. The color of the mixture will turn to yellow. The adsorption of DNA to the QIA quick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator, which is yellow at pH ≤7.5 and orange or violet at higher pH.

5. Add 1 gel volume of isopropanol to the sample and mix the reagents well. For example, if the agarose gel slice is 100 mg, add 100 μl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. Place a QIA quick spin column in a provided 2 ml collection tube.

7. To bind DNA, apply the sample to the QIA quick column, and centrifuge for 1 min. The maximum volume of the column reservoir is 800 μl. For sample volumes of more than 800 μl, simply load and spin again.

8. Discard flow-through and place QIA quick column back in the same collection tube. It can be reused to reduce plastic waste.

9. Add 0.75 ml of Buffer PE to wash the QIA quick column, and centrifuge for 1 min. If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifugation.

10. Discard the flow-through and centrifuge the QIA quick column for one more min. Residual ethanol can inhibit downstream process. Ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

11. Place QIA quick column into a clean 1.5 ml micro centrifuge tube.

12. To elute DNA, add 30-60 μl of DNA extraction buffer (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) (pre-warmed at 60 ºC) to the center of the QIA quick membrane, let the column stand for 1 min, and then centrifuge the column for 1 min.

Notes

One advantage using Gel purification kit over PCR purification kit is that it can purify single type of DNA when there is any other suspected DNA contamination (such as primer-dimer, non-specific amplification, etc).
You don't have to use QIA quick from Qiagen, other brand names will do just fine but you need to follow their protocols carefully.

C. *Primer design*

To initiate nucleotide synthesis, primers are needed to specify the location. Especially in PCR, there is no topoisomerase to stabilize unwinding double helix, the specificity is defined by the complementarity of primer sequence and the annealing temperature of the primer:template duplex. Following requirements are suggested for good primer design:

- **GC content**: 40 – 60%. As GC pair has 3 hydrogen bonds, while AT pair has only 2, higher GC content means higher binding strength of primer to the template. However, the primer may lose its specificity if its GC content is too high.
- **Length**: A primer usually has around 15 bp, but it can be as long as 30 or even more in some cloning method, e.g. overlapping PCR. The longer the length, the higher the binding strength and melting temperature.
- **Melting temperature (Tm)**: The temperature at which 50% of the oligonucleotide and its perfect complement are in duplex. Wallace rule gives

the most basic calculation for short oligonucleotides at 0.9 M NaCl, Td = 2℃(A+T) + 4℃(G+C). More accurate thermodynamic calculation gives the nearest neighbour melting temperature. The Tm difference between primer pair should be within 5˚C.

- **Secondary structure**: Primers are single-stranded. It tends to for secondary structure, e.g. hairpin, to stabilize itself. PCR will fail if formation of primer hairpin is more preferable than primer:template duplex. Some online tool can do prediction, e.g. sequence manipulation suite.
- **Self-annealing**: Primers have chance to form dimer, or even multi-mer in some cases. It may not be "lethal", but better avoid it.
- **Runs of A/T**: It may cause frameshift.
- **GC clamp**: 2 G/C at the 3'-end is essential to ensure the primer the tightly bound to the template for elongation. More than 3 G/C at the 3'-end may cause non-specific binding.