Five Points in this training program

1. Plasmid handling
Plasmid vector, principle of miniprep, restriction enzyme
Agarose gel electrophoresis

2. Protein handling
Crude extract from cell culture, SDS-PAGE, CBB staining

3. Cell culture
How to start cell culture from frozen stock,
Aseptic technique, cell count

4. Subcloning
Principle of subcloning, gel extraction, ligation, transformation

5. RNA handling
Total RNA extraction from cell culture,
Electrophoresis in a denaturing agarose gel

Plasmid Vector

Bacterial plasmids are double-stranded closed circular DNA that can replicate and inherited independently of the bacterial chromosome. Plasmid vectors are most frequently used as a tool in the field of molecular biology. They have multiple cloning sites to insert exogenous clones and drug-resistant genes to selectively grow in the presence of antibiotics. One of major purpose of plasmid vectors is to produce exogenous proteins.

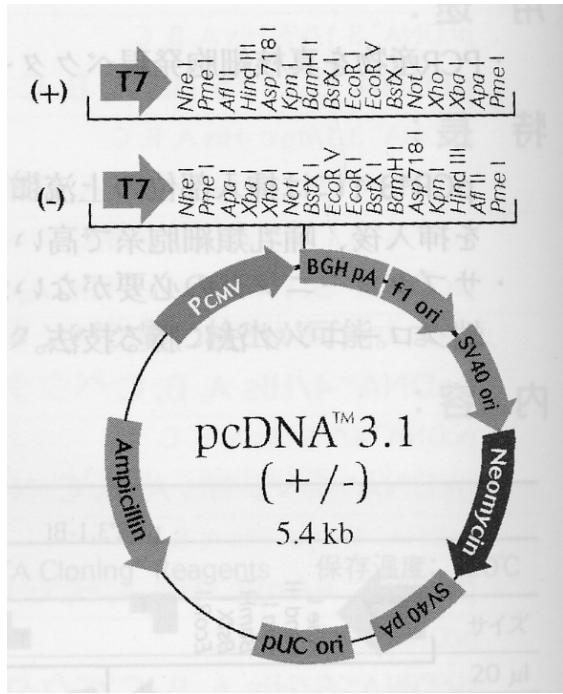
For example, pcDNA3 series ---

Use

Expression vector in various mammalian hosts.

Characteristics

Human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells.
Multiple cloning sites to facilitate cloning.
Ampicilin resistant gene for selection of transformed bacterial cells.
Neomycin resistant gene for selection of stable cell lines.
BGH (bovine growth hormone) polyadenylation signal stabilize messenger RNA.
Replicate in cell lines expressing SV40 large T antigen (e.g. COS7) because it possesses SV40 origin.



Principle of small scale DNA preparation

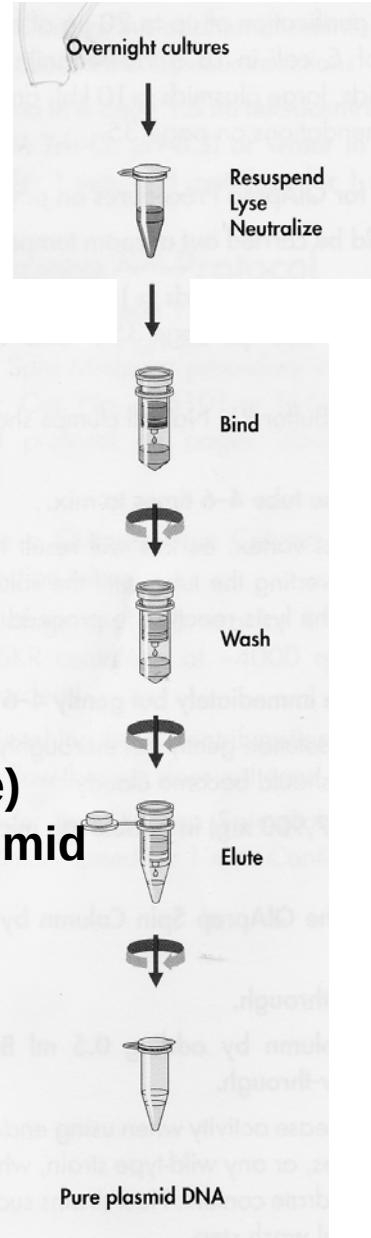
Using QIAprep (Qiagen)

Three basic steps

1. **Preparation of bacterial lysate by alkaline lysis**
2. **Adsorption of plasmid DNA onto silica**
3. **Washing and elution of plasmid DNA**

Protocol

- **Culture bacterial cells containing plasmid DNA over night**
- **Centrifuge the cultured medium to harvest bacteria**
- **Resuspend the bacterial pellet in Buffer P1**
- **Lyse the bacterial cells by adding Beffer P2 (NaOH + SDS)**
- **Neutralize the lysate by adding Buffer N3 (Potassium acetate)**
- **Centrifuge the lysate to take the supernatant containing plasmid**
- **Adsorb the plasmid of the supernatant onto anion-exchange resin column**
- **Wash the column to eliminate contaminant**
- **Elute the plasmid DNA with H2O or TE**



Restriction enzymes

They bind specifically bind to and cleave double-stranded DNA at specific sites known as recognition sequence (palindrome). They were originally identified as bacterial enzymes which restrict growth of bacteriophage. Therefore, they are termed restriction enzymes.

Ex). EcoRI 5'-GAATTC-3' BamHI 5'-GGATCC-3
3'-CTTAAG-5' 3'-CCTAGG-5'

Agarose gel

Agar with high purity, extracted from seaweed.

It has even mesh structure through which nucleotides (DNA, RNA) are size-fractionated under electrophoresis. Negatively charged nucleotides migrate toward the anode.

Ethidium Bromide

It binds to Nucleotides (DNA and RNA).

It absorbs energy of ultraviolet radiation and re-emits light in the red-orange region of the visible spectrum.

Extraction of Proteins

Various methods

We will extract them by simply adding lysis buffer.

SDS-PAGE (SDS-polyacrylamide gel electrophoresis)

Polyacrylamide is polymer of acrylamide

Even mesh structure through which proteins and DNA are separated under electrophoresis.

SDS is detergent which disrupts 3D-structure of proteins and linearize them.

Therefore, proteins can be size-fractionated by SDS-PAGE.

Coomassie brilliant blue (CBB)

Dye which can stain proteins with high sensitivity.

Cell culture

Please study basic technique to keep aseptic condition.

Hemocytemeter

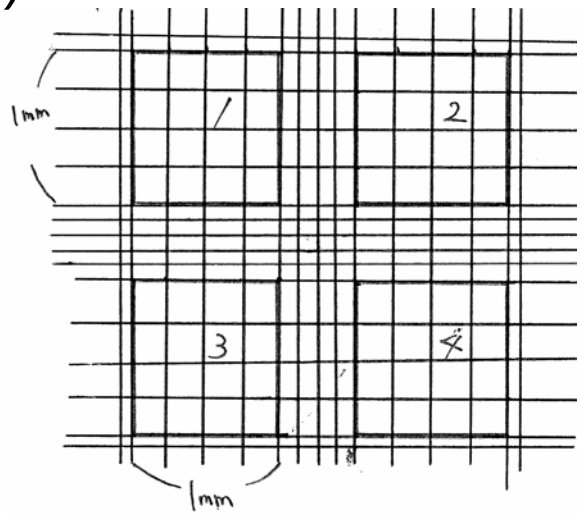
A glass plate with ruler to caluculate cell number.
Press cover glass until Newton ring can be seen.

Volume

1mm x 1mm x 0.1mm = 0.1 mm^3
length width height

Cell number/ml = (area1 + area2 + area3 + area4)/4 x 10^4

Transfer 2 x 10^5 of cells to a 60 mm dish.



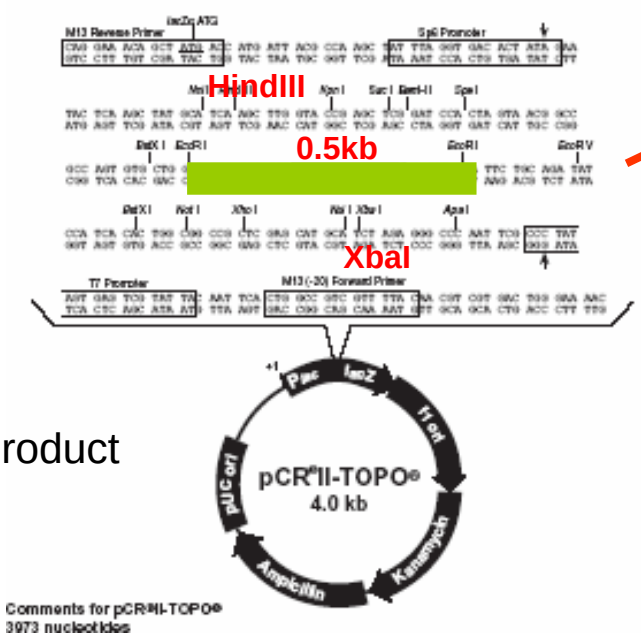
Subcloning

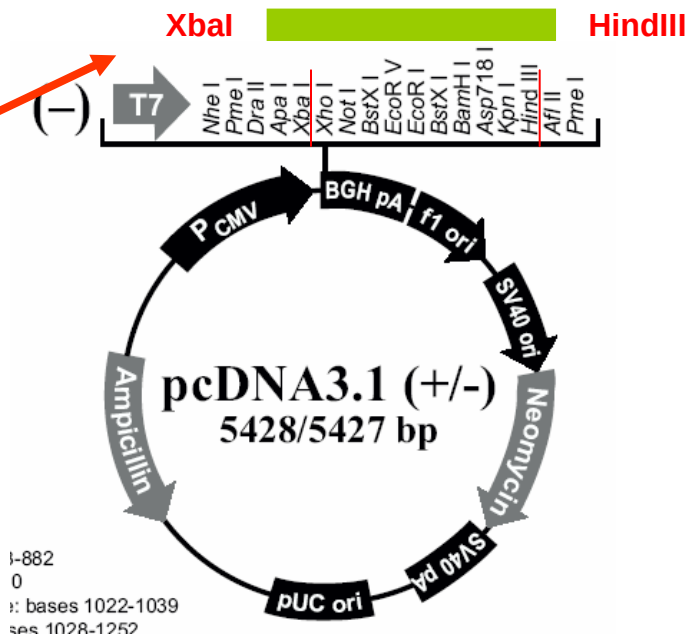
To transfer DNA fragment from one type of vector to another.
For example, from TA cloning vector to pcDNA3-expression vector.
When the restriction sites at the termini of the target fragment are identical to those of the new vector, subcloning is simple.

XbaI XX T CTAGAXX—— XXA AGCTTXX HindIII
XXAGATC TXX—— XXTTCGA AXX

vector side insert side insert side vector side

Insert:
PCR product
0.5kb





Main steps of subcloning

1. Digestion of DNAs with restriction enzymes
2. DNA extraction
3. Ligation
4. Transformation

Ligation

T4 ligase is the enzyme which joins a plasmid vector and foreign DNA. It is easy to ligate those DNAs when two ends of the DNAs have identical restriction sites.

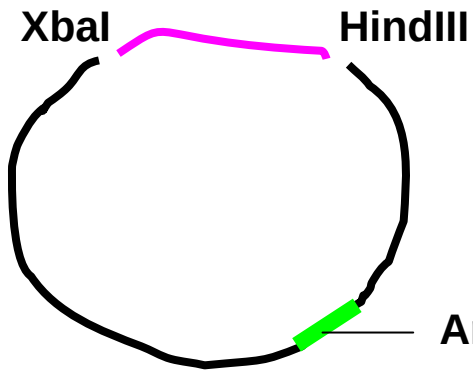
Transformation

Bacterial cells that can uptake exogenous DNA in a certain condition are called competent cells. When the competent cells uptake exogenous genes, they acquire additional characteristics. This process of the competent cells to change its phenotype is called transformation. In this training, we will transform E.coli competent cells with ligated DNA by heat shock method. After transformation, the bacterial cells will be selected on an agar plate with ampicillin because plasmid DNA encodes ampicillin-resistant gene.

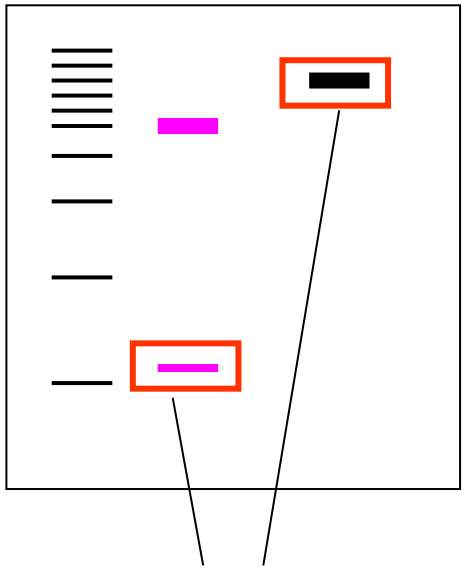
Main steps of subcloning

1. Digestion of DNAs with restriction enzymes
2. DNA extraction
3. Ligation
4. Transformation

3. Ligation with T4 ligase

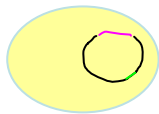


1. Digestion and electrophoresis
2. DNA extraction



Cut agarose gel with razor blade
Extract plasmid DNA using
gel extraction kit (Qiagen)

4. Transformation by heat shock method



Can grow on Amp plate
Plasmid can replicate in E. coli.

Cannot grow on Amp plate

E. coli (DH5α)

Messenger RNA (mRNA)

Cellular RNA is comprised of ribosomal-, transfer- and messenger-RNA (mRNA). mRNA makes up between 1 and 5 % of total RNA. mRNAs are transcribed from genomic DNA, and proteins are translated from mRNAs. To study expression level of mRNA, which reflects its function of cells/tissues/organs, is one of the most common technologies in biological science.

Principle of RNA extraction

Trizol reagent (Invitrogen) is a ready-to-use reagent for the isolation of total RNA. The reagent contains a mono-phasic solution of phenol and guanidine isothiocyanate. The reagent maintains the integrity of RNA while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains in the aqueous phase. After transfer of the aqueous phase to a new tube, the RNA is recovered by precipitation with isopropyl alcohol.
The point of RNA extraction is to minimize RNAse activity because RNA is easily degraded by RNAse. For the purpose, please wear latex gloves, use RNAse-free reagents and apparatus.

Electrophoresis of total RNA in a denaturing agarose gel

Because single-strand RNA forms 3D-structure, they cannot be size-fractionated in a usual agarose gel. In the presence of denaturing reagents such as formaldehyde, they are linearized and therefore size-fractionated. After electrophoresis, ribosomal RNAs (5S, 18S and 28S RNA) are visualized under UV. (loading dye contains ethidium bromide)

Denaturing agarose gel
1.2% agarose
1 x MOPS
18% formaldehyde

Loading sample

Total RNA	**3 μl (20 μg)**
10 x MOPS buffer	**2 μl**
Formaldehyde	**3.5 μl**
Formamide	**10 μl**
H2O	**1 μl**
Dye	**0.5 μl**
Total	**20 μl**