(Preparation of plasmid and subcloning)

Digestion:

Perform a restriction reaction. Mix the following solution:

Check the buffer conditions and reaction temperature. It is possible to perform a restrictive enzymatic reaction using more than 2 enzymes at the same time if the buffer conditions and reaction temperature of the restriction enzymes are the same.

Prokaryotic Methylation:

DNA methyltransferases (MTases) that transfer a methyl group from S-adenosylmethionine to either adenine or cytosine residues, are found in a wide variety of prokaryotes and eukaryotes. Methylation should be considered when digesting DNA with restriction endonucleases because cleavage can be blocked or impaired when a particular base in the recognition site is methylated. Popular strains of E. coli contain three site-specific DNA methylases. Plasmid DNA isolated from dam+ E. coli is completely resistant to cleavage by MboI, which cleaves at GATC sites.

1. Dam methylase–methylation at the N6 position of the adenine in the sequence GATC (1,2).
2. Dcm methyltransferases–methylation at the C5 position of the second cytosine in the sequences CCAGG and CCTGG (1,3).
3. EcoKI methylase–methylation of adenine in the sequences AAC(N6)GTGC and GCAC(N6)GTT.

Eukaryotic Methylation:

CpG MTases, found in higher eukaryotes (e.g., Dnmt1, Dnmt3a, Dmnt3b), transfer a methyl group to the C5 position of cytosine residues. Patterns of CpG methylation are heritable, tissue specific and correlate with gene expression. Consequently, CpG methylation has been postulated to play a role in differentiation and gene expression. The effects of CpG methylation are mainly a concern when digesting eukaryotic genomic DNA. CpG methylation patterns are not retained once the DNA is cloned into a bacterial host.

DNA(BSSK PEBPβ2(3.8kb) 　 10μl (adjust ≦2.5μg)

10×H　 　 5μl

10×BSA 　 5μl

EcoRⅠ(Takara) 　 5μl

NotⅠ (Takara) 　 5μl

DDW 　　20μl

Total 　　50μl

Incubate the solution at 37℃for 2 ～ 3 hours.

Cut check:

Check the band sides by electrophoresis on agarose gel.

Check the complete cut(2 bands).

Wizard® SV Gel and PCR Clean-Up System(Promega)

Electrophoresis:

1. Extract insert DNA after cutting out a band from the agarose gel. Load and run the gel. DNA can be extracted from agarose gels run with either TAE or TBE buffer. Cut out the band from the agarose gel on a transilluminator.
2. Weigh a 1.5ml micro centrifuge tube for each DNA fragment to be isolated and record the weight.

Ex)

BSKS　PEBPβ１ 449mg

BSKS PEBPβ 2 303mg

MSCV EN GFP 479mg

MSCV EN hCD4 369mg

1. Visualize and photograph the DNA using a long-wavelength UV lamp and an intercalating dye such as ethidium bromide. To reduce nicking, irradiate the gel for the absolute minimum time possible. Excise the DNA fragment of interest in a minimal volume of agarose using a clean razor blade. Transfer the gel slice to the weighed micro centrifuge tube and record the weight. Subtract the weight of the empty tube from the total weight to obtain the weight of the gel slice.

Dissolving the Gel Slice:

1. Add Membrane Binding Solution at a ratio of 10μl of solution per 10mg of agarose gel slice.
2. Vortex and mixture and incubate at 50-65℃ for 10min or until the gel slice is completely dissolved. Vortex the tube every few minutes to increase the rate of agarose gel melting. Centrifuge the tube briefly at RT to ensure the contents are at the bottom of the tube. Once the agarose gel is melted, the gel will not resolidify at RT.

Binding of DNA:

1. To purify the DNA using a microcentrifuge, Place one SV Minicolumn in a Collection Tube for each dissolved gel slice.
2. Transfer the dissolved gel mixture to the SV Minicolumn assembly and incubate for 1min at RT.
3. Centrifuge the SV Minicolumn assembly in a microcentrifuge at 16,000 ×g(14,000rpm) for 1 min. Remove the SV MInicoumn from the Spin Column assembly and discard the liquid in the Collection Tube. Return the SV Minicolumn to the Collection Tube.

Washing:

1. Wash the column by adding 700μl of Membrane Wash Solution, previously diluted with 95% ethanol, to the SV Minicolumn. Centrifuge the SV Minicolumn assembly for 1 min at 16,000×g(14,000rpm).Empty the Collection Tube as before and place the S Minicolumn back in the Coellecition Tube. Repeart the wash with 500μl of Membrane Wash Solution and centrifuge the SV Minicolumn assembly for 5min at 16,000×g.
2. Remove the SV Minicolumn assembly from the centrifuge, being carefule not to wet the bottom of the column with the flowthrouhg. Empty the Collection Tube and recentrifuge the column assembly for 1min with the microcentrifuge lid open(or off) to allow evaporation of any residual ethanol.
3. Carefully transfer the SV Minicolumn to a clean 1.5ml micro centrifuge tube.Apply 30μl of Nuclease-Free Water directly to the center of the column without touching the membrane with the pipette tip. Incubate at RT for 1min. Centrifuge for 1min at 16,000×g(14,000rpm).
4. Discard the SV Minicoumn and store the microcentrifuge tube containing the eluted DNA at 4℃or －20℃.

DNA estimation:Gel electrophoresis

Load the 1μl of the sample and each 10μl of λ/HindⅢand BSM-13/HinfⅠ and Run the gel for 20 min.

Compare the sample bands with the λ/HindⅢor BSM-13/HinfⅠ.

Dephosphorylation:

It is needed to prevent self-ligation when one cut or homologous end cut are performed.

It lowers efficiency of ligation, so increase molar ratio : insert : vector = 1:10→1:30.



Ligation:

molar ratio: Vector:Insert=1:10(1st) (cf. 2nd 1:100) ※1base=330, 1base pair=660

Want 5ng of vector, so Insert=5×800/7000×10=6ng

BSKS PEBPβ1 　 2.93ng/μl

BSKS PEBPβ2 　 2.93ng/μl

MSCV EN hCD4(10/2) 　16.9ng/μl

MSCV EN hCD4(9/25) 　16.9ng/μl

TaKaRa DNA Ligation Kit <Mighty Mix>

Insert DNA(approximately 800bp)(PEBPβ1 or 2) 2μl(5.86ng)

10-fold dilution Vector DNA(7kb)(MSCV-GFP-4EN, MSCV-CD4-4NE) 3μl(5.07ng)

Ligation Mix 5μl

No insert:

10-fold dilution Vector DNA 3μl

Ligation Mix 3μl

 Vector Insert

MSCV-PEBPβ1-hCD4(10/2) MSCV-hCD4(10/2) 3μl PEBPβ1 2μl

MSCV-PEBPβ1-hCD4(9/25) MSCV-hCD4(9/25) 3μl PEBPβ1 2μl

MSCV-PEBPβ2-hCD4(10/2) MSCV-hCD4(10/2) 3μl PEBPβ2 2μl

MSCV-PEBPβ2-hCD4(9/25) MSCV-hCD4(9/25) 3μl PEBPβ2 2μl

MSCV-hCD4(10/2) MSCV-hCD4(10/2) 3μl －

MSCV-hCD4(9/25) MSCV-hCD4(9/25) 3μl －

Incubate it at RT for 10min.

Transformation:

Perform transformation, amplification in liquid media ,and purification of DNA.

1. Prepare for the vector(plasmid DNA).
2. Transfer E.coli(DH5α1.6×108CFU/μg pBSKS) from the deep freezer. Place the tubes containing E.coli on ice.
3. Transfer Amp Agarose gels from low-temperature chamber. ※Add 10～15ml of Amp(100mg/ml) into the old gels.
4. Add E.coli.

Plasmid DNA 10μl

E.coli 100μl(or 50μl)

1. Place the tubes on ice for 10 min.
2. Incubate the tubes in a water bath at 42℃ for 90sec.
3. Place the amp agarose gel(4℃) upside down in clean bench to avoid dew condensation.
4. Add 900μl of SOC(15ml tube with yellow seal)..
5. Centrifuge them at 15000rpm, for 1 min.
6. Immerse glass rod in alcohol and roast in burner in clean bench.
7. Place dish containing Amp agarose gel on a rotating wheel.
8. Discard 900μl of the supernatant, pipette it up and down using 100μl tip.
9. Spread the inoculum evenly over the surface of Amp agarose gel.Slowly shake the plate back and forth, turning the plate roughly 90°every few seconds. Dry them opening the cover for 5min.
10. Transfer the plates to an incubator and incubate them upside down for 16 hours～overnight at 37℃.
11. Perform small scale amplification and purification of plasmid DNA

Cut check:

1. One cut. Cut the juncture (5’ or 3’).
2. Two cut. Cute the junctures (5’ and 3’).
3. Cut the vector ligation site and inside the insert (3 cut).

→Select the colonies for sequencing.

1. (Option) Check the direction. Cut the ligation site and the outside the insert (2 cut).
2. (Option) Cut the site outside the insert to avoid triple insert (2 cut).

Digest the purified DNA after ligation, and electrophorese the digested DNA and the DNA after ligation.