Restriction Digest, Dephosphorylation, Gel Purification, Ligation

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Materials:
Sterile dH_2O
Restriction Enzyme Buffer (comes with restriction enzyme)
BSA (comes with restriction enzyme)
Restriction enzyme (From NEB)
DNA
Sterile 0.5ml Microfuge tubes

10 U/µl CIP, cow intestine alkaline phosphatase (NEB M0290S)
1X NEB Buffer #3, (50 µl 10X #3 in 450 µl H_2O)
Promega PCR Purification Kit (Promega, A2981)

Sterile 1.5 ml microfuge tubes
Gel electrophoresis apparatus etc
Qiagen MinElute Gel Extraction Kit (Qiagen, 28604)
Isopropanol

Procedure:

Note the order that seems to work best is
1. **Restriction digest, do not heat inactivate,**
2. **Gel Purification, elute vector in 1X NEB buffer #3**
3. **Dephosphorylation, make sure to dilute phosphatase in 1X NEB buffer #3**
4. **PCR cleanup**
5. **Ligation, use Promega ligase**

If possible make a positive and negative controls
1. **Positive control = destination vector that is not cut but goes into ligation reaction**
2. **Negative control = destination vector that is cut and dephorphorylated (if doing non directional cloning that goes into ligation reaction**

The positive control will tell you if your transformation is working i.e. you should see lots of colonies
The negative control will tell you if your dephorphorylation is working i.e. you should see few colonies

Restriction Digest
One Unit digests 1 µg DNA in 1 hour
1. Using the concentration of restriction enzymes given on the tube (or the table below) calculate how many units and then how many µl you need to digest your given amount of DNA (want at least 5 – 10 µg DNA) in 1 hour. Keep in mind the 8 well comb holds 45µl in each well and a double well made by taping 2 wells together will hold 125 µl. So ideally keep the volume to less than 45 µl

** If doing a double digest remember if one enzyme only has partial activity in the buffer you are using to increase the amount of that enzyme porportionatly ex. if it only has 50% activity in the buffer you are using and you calculate you need 5 µl then in reality you should add 10 µl

** Many of the NEB restriction enzymes work in multiple buffers, however if you are doing a dephosphorylation after the restriction digest try to use an NEB buffer with $MgCl_2$, this would be buffer 2 or 3. NEB Alkaline phosphatase does not work in NEB buffer 1 or 4.

**We have found that ClaI does not cut well if at all in pUC57 in DH5α, this may be due to ClaI being *dam* methylation sensitive

2. In a sterile 0.5 ml microfuge mix the following
+ DNA
+ 10% of total volume 10X restriction enzyme buffer (ex. 5.5µl buffer in 55µl total volume)
+ 10% of total volume BSA (if needed, check your restriction enzyme)
+ Restriction enzyme (volume determined in step 1)
+ Sterile dH_2O to bring total volume to desired amount (usually between 20 and 70 µl)
3. Incubate at 37°C for 3 – 4 hours, 4 hours is definitely better
4. Do not heat inactivate restriction enzyme. Run digested DNA on agarose gel, even if you your just opening a vector and no insert is being removed. The gel can help separate uncut from cut vector. The cut vector will move slower through the gel as the supercoiling has been relaxed. Gel purification and clean up should also remove the restriction enzyme leaving the sites open for dephosphorylation. Elute DNA from gel clean up with water

NEB Restriction Enzymes			
Restriction Enzyme	**NEB Buffer**	**BSA required**	**Concentration**
AsiSI	3, 2	Yes	10,000 U/ml (10 U/µl)

AvaI	4, 2	No	
BamHI	3, 2, 4	Yes	
BbvCI	4, 2	No	2,000 U/ml (2 U/µl)
ClaI (R0197S)	4	Yes	
EcoRI	U[dd], 1, 2, 3, 4	No	
EcoRV	3, 2	Yes	
HindIII (R0197S)	2	No	
KpnI	1, 2	Yes	
NcoI (R0193S)	3, 1, 2, 4	No	10,000 U/ml (10 U/µl)
NotI	3	Yes	10,000 U/ml (10 U/µl)
SbfI	4, 1	No	10,000 U/ml (10 U/µl)
SmaI 25°C	4	No	
SpeI	2, 1 , **4**	Yes	10,000 U/ml (10 U/µl)
SphI	2, 1, 4	No	

Gel Purification
If using gel purification kit for the first time add ethanol to Buffer PE according to dirctions on bottle. Date bottle

Note: The 8 well comb holds 45µl in each well and a double well made by taping 2 wells together will hold 125 µl.

1. Run as much DNA as you can on agarose gel as you would normally (ex 1% Agarose in 1X TAE)
2. Pre weigh 2 ml microfuge tubes, you will need to determine to volume of the band you cut out later
3. Excise the DNA fragment of interest from the agarose gel with a clean sharp razor blade, minimize the amount of excess agarose
4. From the weight of the gel piece determine the volume of the gel piece (0.1g = 100 µl) add 3 volumes Buffer QG
5. Incubate at 50°C in a dry bath until the gel slice has dissolved completely, make sure the color of the mixture is yellow.
6. Add 1 gel volume of isopropanol to the sample mix and mix by inverting several times
7. Place sample mix on a MinElute Column that is on top of a 2 ml collection tub
8. Centrifuge at max speed for 1 minute
9. Discard flow through and place the MinElute column back in the same collection tube
10. Add 750 µl of PE buffer to column, centrifuge for 1 minute
11. Discard flow through and centrifuge for an additional minute to get the remainder
12. Discard flow though with a 10 µl pipette carefully remove remaining PE buffer around the edge of the column filter
13. Place column in a sterile 1.5 ml microfuge tube
14. Add 15 µl dH_2O (if using water make sure pH is between 7.0 and 8.5) and allow to stand for 1 minute. If you are going to dephophorylate the DNA elute with NEB 1X buffer #3
15. Centrifuge for 1 minute

Dephosphorylation

Note: Total volume for dephosphorylation reaction can be from 10 - 30 µl, NEB recommends 10 µl but this is often impossible and we have had success with 20 and 30 µl rxns.

1. Add 0.5 Units of phosphatase (0.05 µl) per µg of DNA. If necessary dilute the phosphatase down to the appropriate concentration using 1X buffer #
3. Too much phosphatase enzyme will cling tightly to the DNA ends and prevent the ligase from working.
2. DNA should be in 1X NEB buffer 3 (requires $MgCl_2$)
3. Incubate at 37°C for 1 hour
4. Once the dephosphorylation is complete purify the DNA with a PCR clean up kit, elute with water

DNA Cleanup Kit (PCR Purification kit)
If using Promega Wizard PCR clean up kit for the first time add 95% ethanol to the membrane wash bottle according to directions on bottle. Date bottle

1. Add an equal volume of Membrane Binding Solution to the DNA or PCR amplification. ex. if restriction digest + dephosphorylation had a total volume of 45 µl add 45 µl of Membrane Binding Solution.
2. Transfer DNA with membrane binding solution to spin column with collection tube
3. Centrifuge the spin column at 16,000g for 1 minute, discard liquid in the collection tube
4. Add 700 µl Membrane Wash Solution
5. Centrifuge for 1 minute at 16,000 g, discard liquid in collection tube
6. Add 500 µl Membrane Wash Solution
7. Centrifuge for 5 minutes at 16,000 g
8. Transfer spin column to a clean 1.5 ml microfuge tube, add 50 µl nuclease free H_2O. You can add as little as 15 µl water if you need more concentrated DNA
9. Incubate for 1 minute at room temperature
10. Centrifuge for 1 minute at 16,000g save DNA in 1.5 ml tube

Ligation
Materials:
T4 DNA Ligase 3U/µl (Promega M1801)
10X DNA Ligase Buffer (comes with DNA Ligase)
0.5 ml Microfuge tubes
Sterile dH_2O

Procedure
Note: Because the ligase buffer contains ATP, if using Ligase for the first time aliquot out buffer into 10 - 20 µl aliquots, and freeze. Once you thaw a tube with buffer discard, do not reuse or refreeze.

Note: I have tried Promega T4 ligase and Invitrogen T4 ligase side by side with the same DNA and found the Promega ligase works better, that said I have had limited success with the Invitrogen ligase

Note: Promega recommends starting with 100 ng of vector DNA but if your having trouble with empty vector try using 10 ng of vector. You will get few colonies so pipette at least 200 µl from step 7 may want to plate more if you have it. You can also try a 1:5 ratio of vector to insert, i.e. multiply by 5 instead of 3 in the equation in step 2.

1. In a 0.5 ml microfuge tube place 100 ng destination vector DNA
2. Determine the amount of insert to add by using the following formula
   [100 ng * Size of insert (Kb)] ÷ Size of vector (Kb) * 3 = DNA (ng) of insert needed
   If you want to be precise about this you want 3 - 30 fmol (10^{-15}) of vector DNA and 9 - 90 fmol insert DNA
3. Add 1 µl 10X DNA Ligase Buffer
4. Add 1 µl ligase
5. Add enough sterile dH_2O to bring volume to 10 µl
6. Make sure to make both a negative and positive control using only digested and undigested vector
7. Incubate at room temp for 1 hour followed by 4°C overnight
8. Transform 50 µl of DH5α with 4 µl of sample from step 5 and plate 200 µl
9. Plate a second plate with remainder of transformed DH5α (304 µl)
10. If you did not plate remainder of DH5α cells, keep transformed cells in -4°C
11. Choose 5 - 16 colonies for sub culturing and PCR checks

** We have found that BbvCI does not ligate well