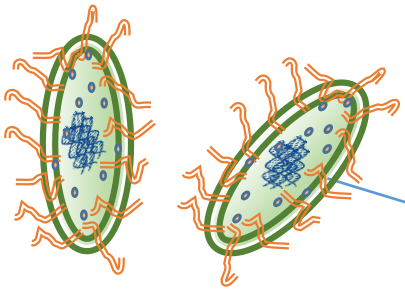
Cloning, expression, purification, and biophysical characterization of SSB from pathogenic bacteria

Week 2: Molecular Cloning of the *SSB* gene into the pCDF plasmid for protein expression.

Introduction – *SSB* gene was isolated from a specific species of pathogenic bacterium and cloned into the pUC57 plasmid.



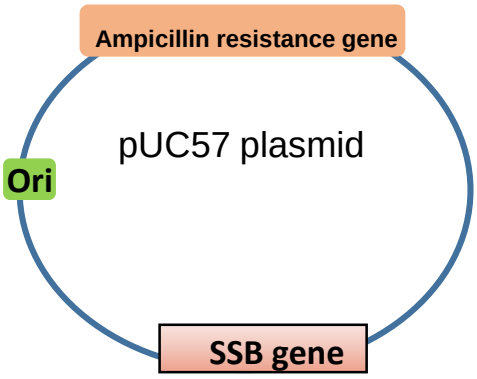
Pathogenic bacteria

Genomic DNA extracted from pathogenic bacteria

SSB gene

PCR using primers specific for the *SSB* gene was carried out to amplify SSB DNA

SSB gene was inserted into the pUC57 plasmid by molecular cloning



Week 2: Molecular Cloning of the *SSB* gene into the pCDF plasmid for protein expression.

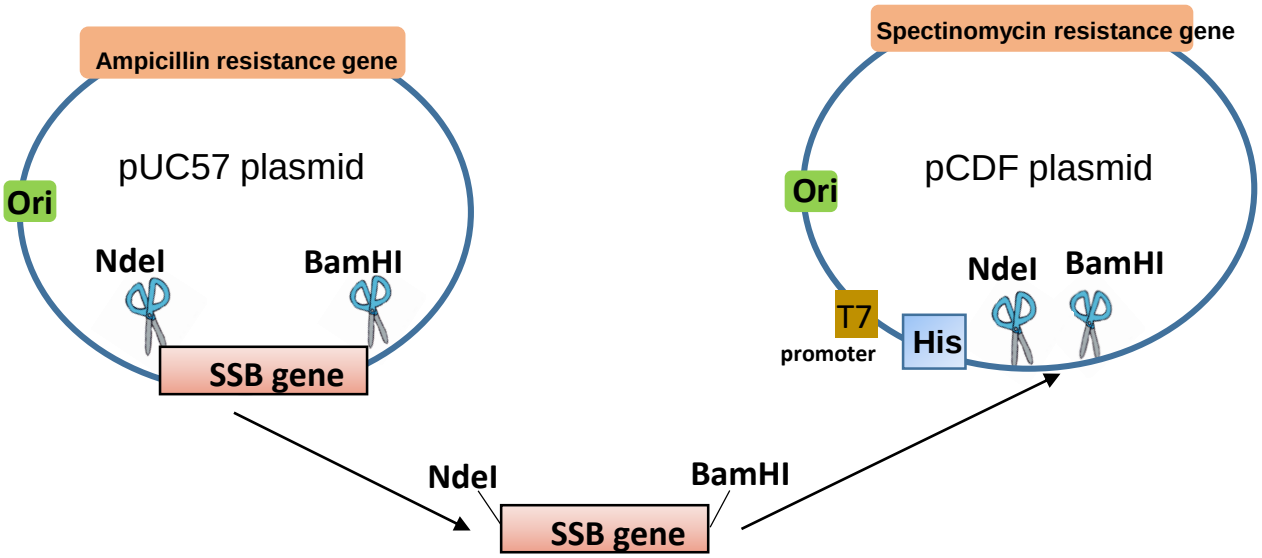
Introduction (cont'd.)

SSB gene is currently inserted in pUC57 plasmid. pUC57 plasmid lacks a T7 promoter, which is required for gene expression. Therefore, *SSB* gene has to be cut out from the pUC57 plasmid and inserted into the pCDF plasmid that carries the T7 promoter.

Step I - Restriction Digestion (Overview):

***SSB* gene- Insert** - The entire *SSB* gene has to be cut out from the pUC57 plasmid using restriction enzymes NdeI and BamHI that cut at specific restriction sites at the start and end of the SSB DNA respectively.

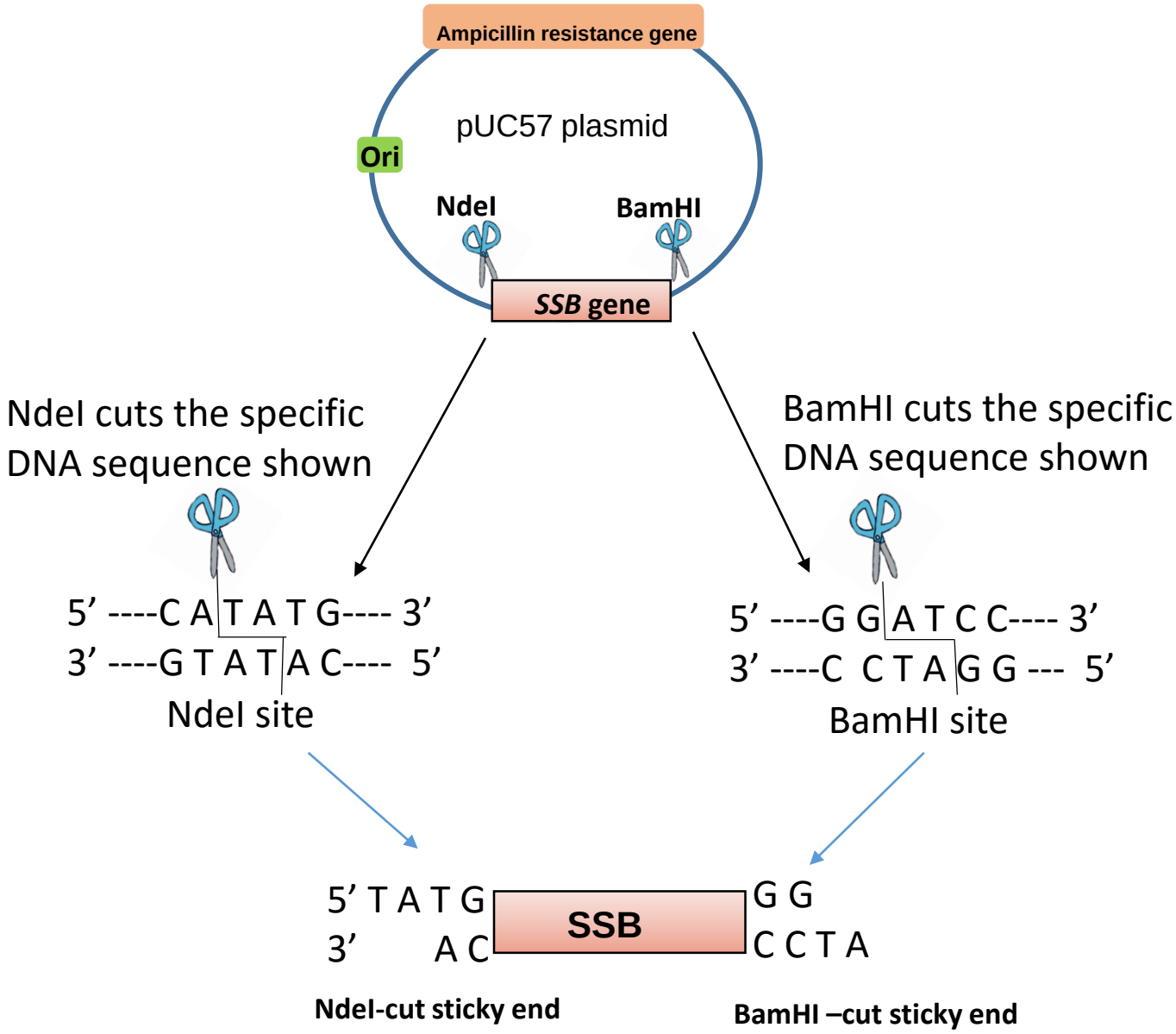
Vector – The vector-pCDF is also cut with the same enzymes - NdeI and BamHI to generate sticky ends that will be compatible with the restriction digested ends of SSB DNA. This will help in inserting SSB DNA into the pET28a vector.



Note- The pCDF plasmid also carries a His tag, which is a sequence of 6 histidine aminoacids. The SSB protein will be expressed with the His tag, which will be helpful for the protein purification protocol.

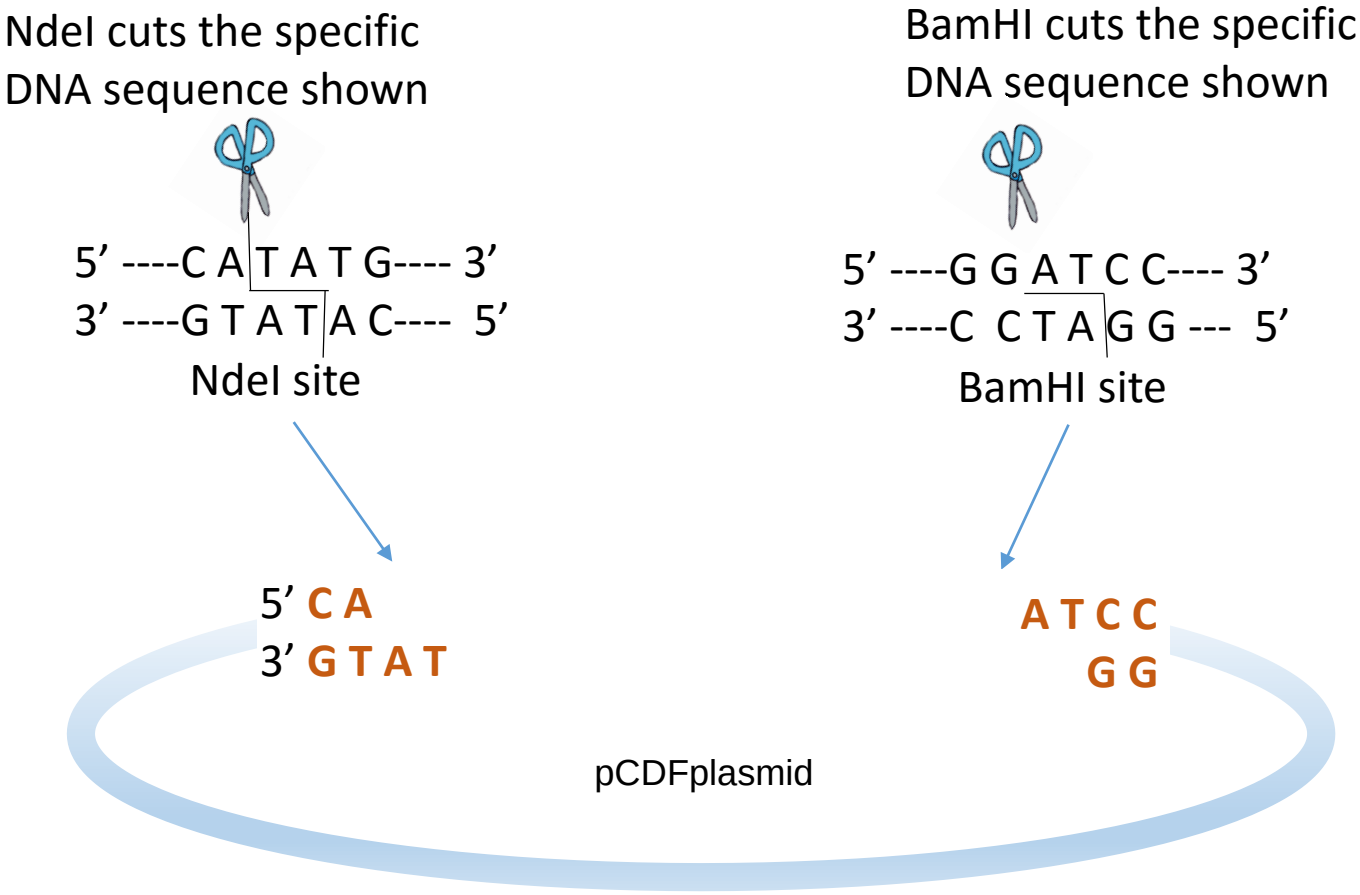
Step I - Restriction Digestion (overview-cont'd.):

SSB- Insert - The entire SSB gene has to be cut out from the pUC57 plasmid using restriction enzymes NdeI and BamHI that cut at specific restriction sites at the start and end of the SSB DNA respectively.



Step I - Restriction Digestion (Overview- cont'd.):

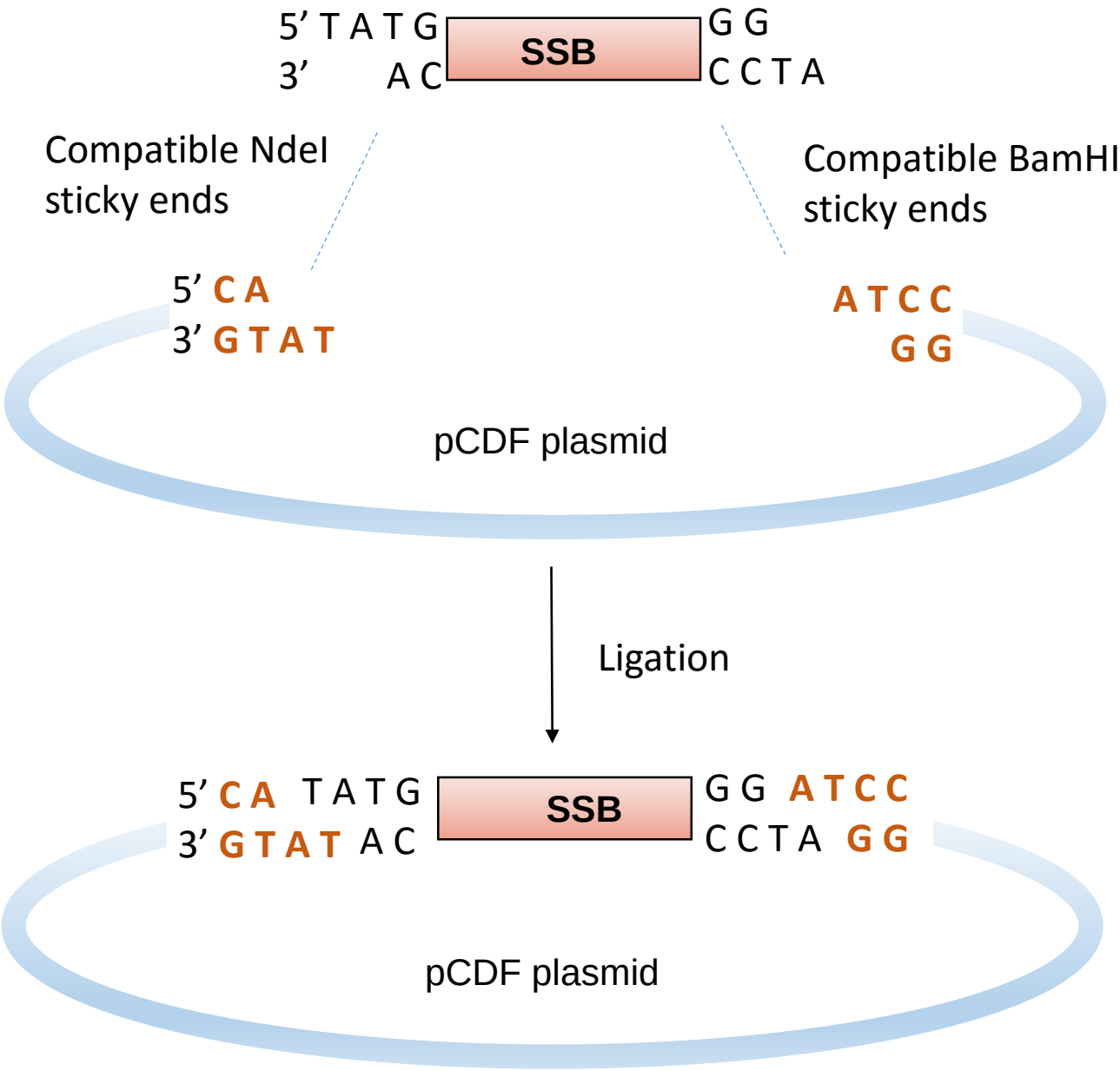
Vector – The vector-pCDF is also cut with the same enzymes NdeI and BamHI sites to generate sticky ends that will be compatible with the ends of the *SSB* gene insert and will help in inserting *SSB* into the vector.



Step I - Restriction Digestion (Overview – cont'd.):

SSB- Insert - The entire *SSB* gene has to be cut out from the pUC57 plasmid using restriction enzymes NdeI and BamHI that cut at respective restriction sites at the start and end of the SSB DNA.

Vector – The vector-pCDF is also cut with the same enzymes NdeI and BamHI sites to generate sticky ends that will be compatible with the ends of the SSB DNA insert and will help in inserting the gene into the pCDFvector.



Step 1- Restriction digestion protocol

Reaction I - restriction digestion of pCDF vector

Label a 1.5mL microcentrifuge tube as vector and your group name , and set up the following reaction:

pCDF plasmid DNA – 10 uL
*NdeI---------- 1 uL
*BamHI---------- 1 uL
CutSmart buffer 10X --- 5 uL
DI water ---------- 33 uL

50uL (total)

*Add enzymes- NdeI and BamHI last!

Reaction II - restriction digestion of pUC57 with the SSB gene.

Label a 1.5mL microcentrifuge tube as pUC57 and your group- SSB gene name and set up the following reaction:

pUC57 plasmid DNA – 20 uL
*NdeI---------- 1 uL
*BamHI---------- 1 uL
CutSmart buffer 10X --- 5 uL
DI water ---------- 23 uL

50uL (total)

*Add enzymes- NdeI and BamHI last!

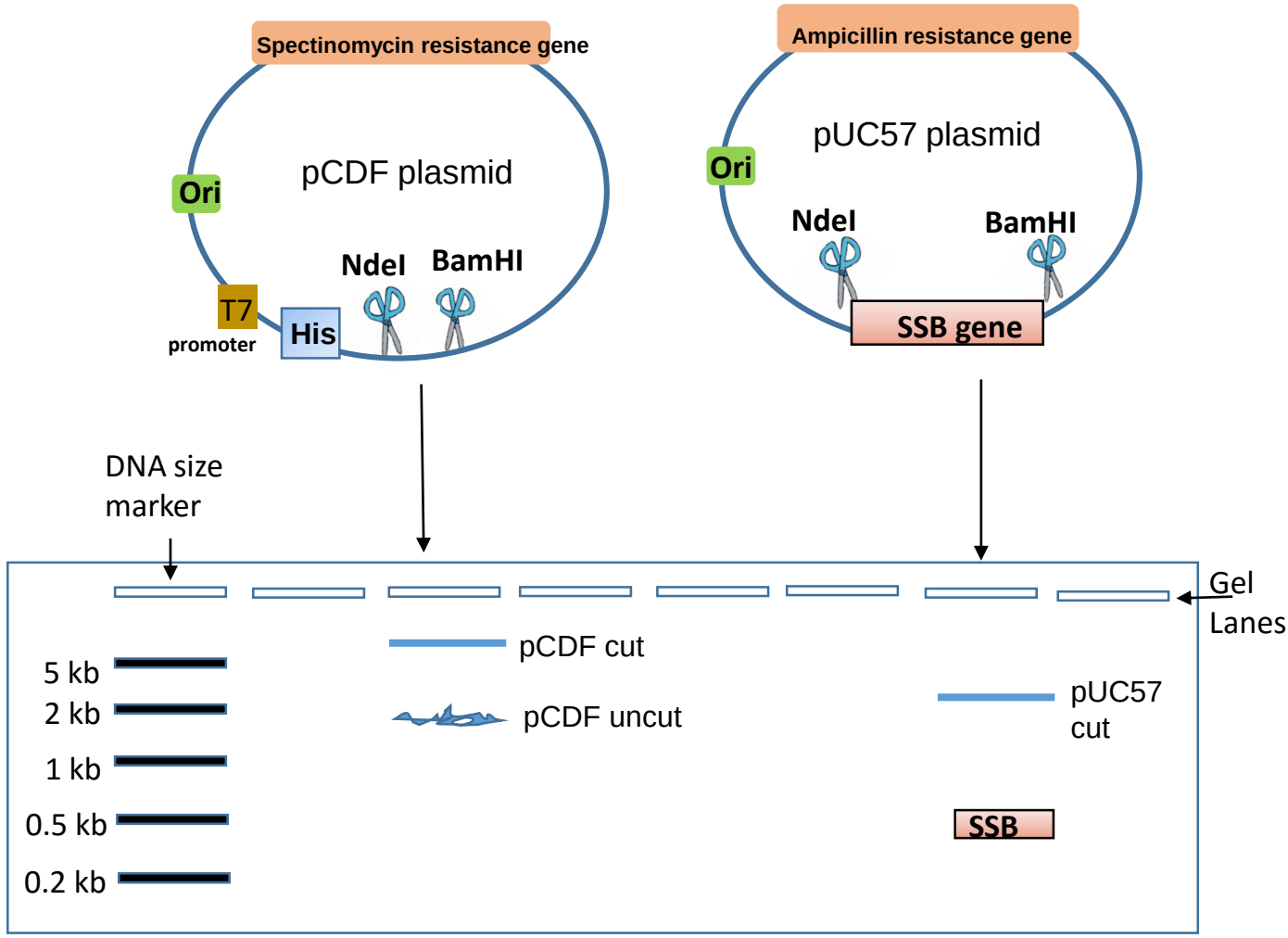
Gently mix and incubate Reaction I and Reaction II at 37°C for 1 hr!

Note- To save time, proceed to step II using the reaction tubes that were incubated before class.

Step II : Agarose gel electrophoresis (Overview)

- After restriction digestion, SSB DNA is separated from the pUC57 plasmid using agarose gel electrophoresis.
- pCDF cut plasmid is separated from any uncut plasmid using agarose gel electrophoresis.
- The *SSB* gene and the cut pCDF vector are extracted from the gel using the gel extraction kit.

pUC57 plasmid DNA (cut) size : **2.7 kb**
SSB gene size is : approximately **0.5kb**
pCDF plasmid DNA (cut) : **5.4kb**
Any uncut plasmid DNA is supercoiled –will not run at expected size and will run faster than cut plasmid DNA.



Step II - Agarose Gel Electrophoresis protocol to analyze digested products

Prepare 1% agarose gel:

1. Add 1 g of agarose to 100 mL of 1X TAE buffer in a 200mL beaker.
2. Microwave solution till agarose is dissolved. (caution –hot!)
3. Once the beaker is cool to the touch – add 10 µL of SYBR Safety Stain.
4. Mix gently and pour into the casting tray.
5. Place the comb and wait for gel to polymerize (solidify).

Prepare sample to be loaded onto the gel:

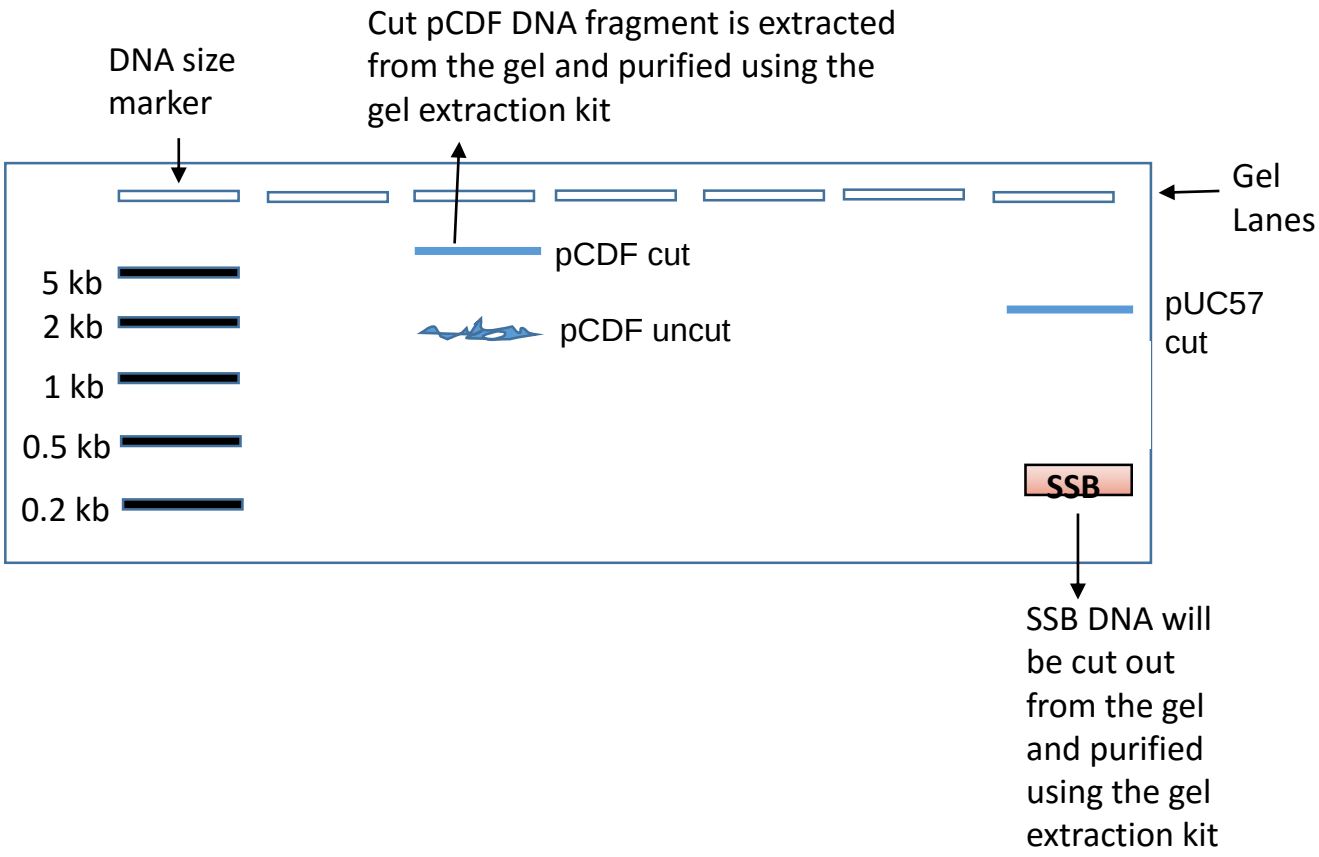
1. Remove Reaction I and Reaction II tubes that were incubated at 37°C (end of step I).
2. Add 8ul of 6X loading dye to reaction I tube.
3. Add 8ul of 6X loading dye to reaction II tube.

Loading reactions onto the gel:

1. Load 5uL of DNA marker in gel lane I
2. Leave lane 2 and 3 empty. For lanes 5 and 6- split 58 uL of reaction I (pET28a) into 29 uL and load into lanes 5 and 6.
3. Leave lanes 7 and 8 empty. For lanes 9 and 10-split 58 uL of reaction II into 29 uL and load into lanes 9 and 10.
4. Run at 120V for 45min to 1hr.

Step III : Gel extraction of SSB DNA (Overview)

The 5.4 kb cut pCDF fragment and the 0.5 kb SSB DNA fragment is cut out from the agarose gel and the DNA is purified from the agarose gel material using the gel-extraction kit.



Step III- Gel extraction protocol

1. Place agarose gel on UV. Wear protective goggles. Label a microcentrifuge tube as cut pCDF and your group name and another tube as SSB gene of your group.

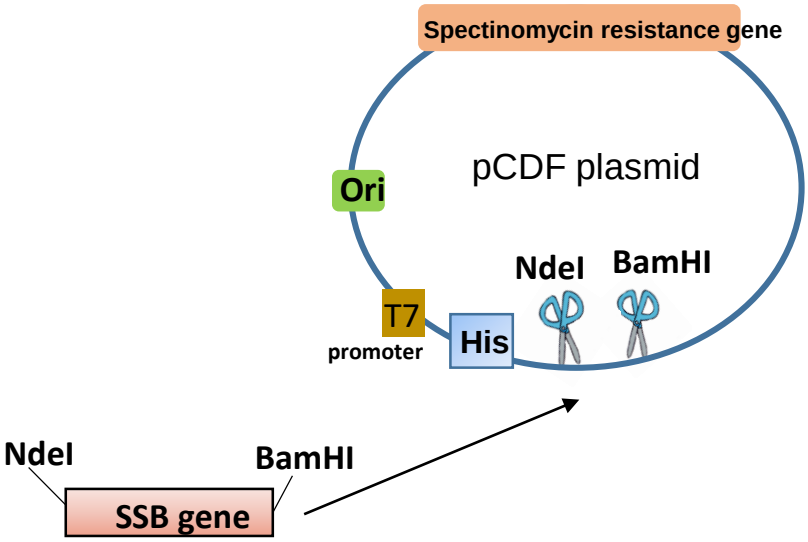
2. Cut out the 5.4kb pCDF DNA using a clean razor blade and place in the labeled tube.

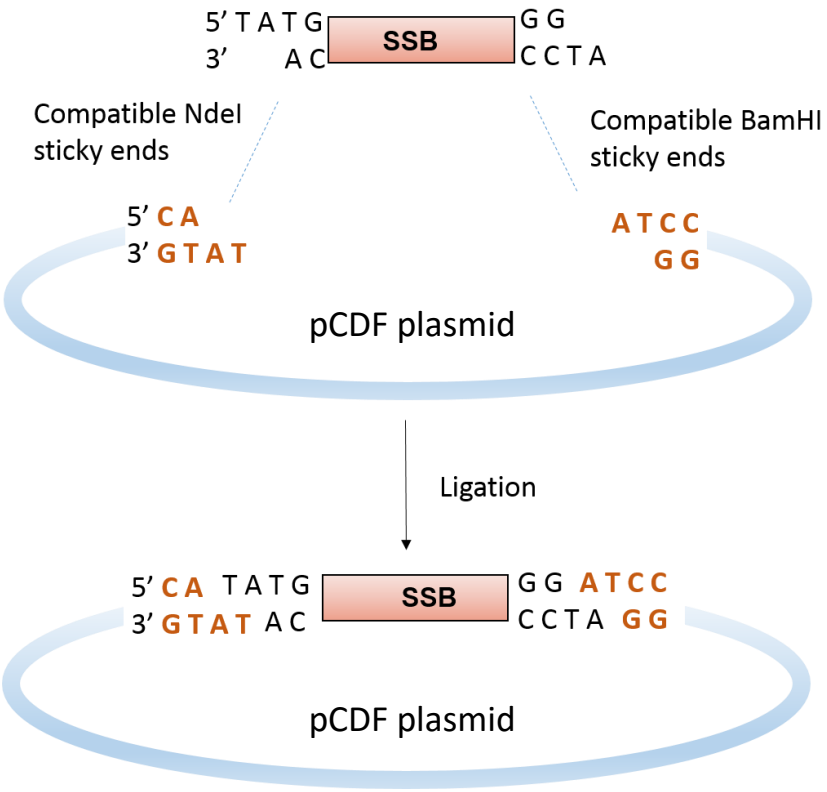
3. Cut out the 0.5 kb SSB DNA using a clean razor blade and place in the labeled tube.

4. Follow-Qiagen Gel Extraction Kit protocol – please see attached Qiagen-company product sheet.

Step IV : Ligating SSB DNA with pCDF plasmid DNA (Overview)

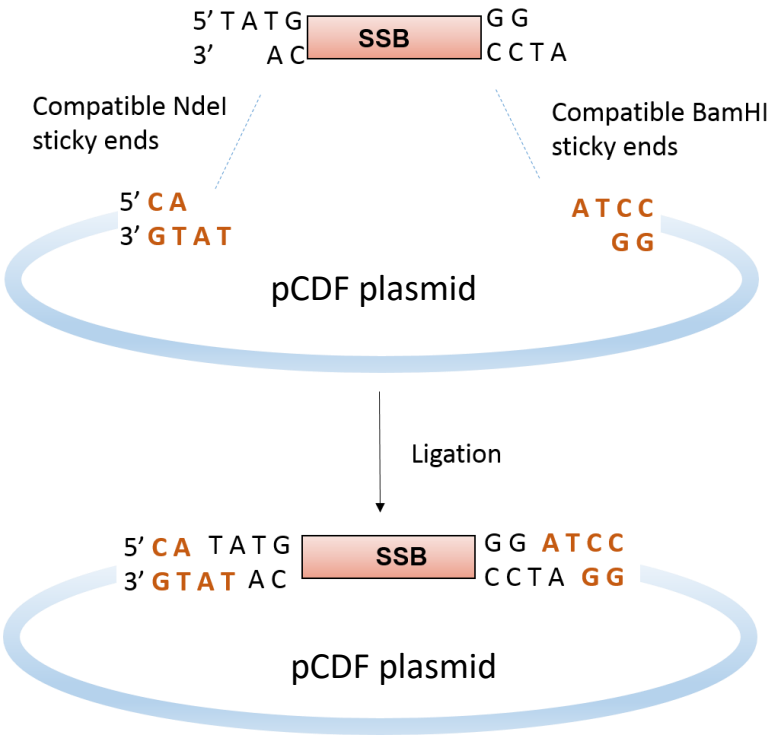
The NdeI/BamHI cut-sticky ends of SSB DNA insert is compatible with the NdeI/BamHI cut ends of pCDF vector. The SSB gene is inserted into the pCDF vector by ligating the compatible sticky ends using the enzyme ligase.

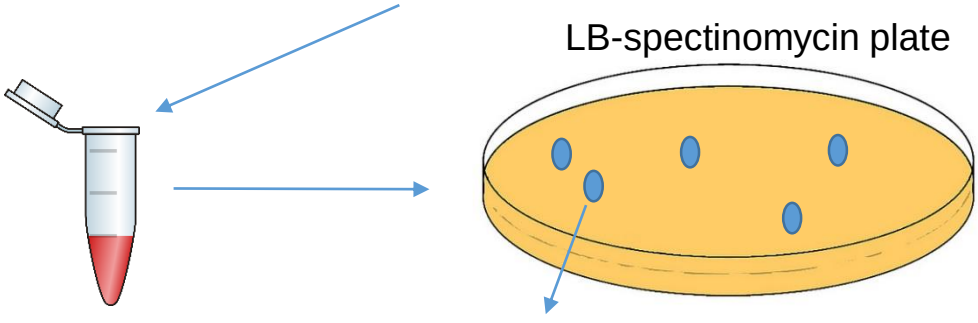




Step V : Transforming the ligated SSB DNA with pCDF plasmid DNA into DH5alpha bacterial cells (Overview)

Transformation- Helps to make more copies of the ligated DNA. For transformation, heat shock is used, which opens up the bacterial cells temporarily so that they can take in the ligated plasmid. Only the cells that take up the plasmid survive on plates with kanamycin as the plasmid carries the kanamycin resistance gene. Every time the transformed cells divide, the plasmid DNA also replicates and more plasmid DNA copies are made. After overnight culture, colonies of bacteria are observed on the LB-kan plates that contain the plasmid DNA.





Bacterial cells are mixed with ligated plasmid DNA and heat shocked.

Only the bacterial cells that take up the plasmid DNA, survive and form colonies on plates that contain LB growth media and the antibiotic-kanamycin

Step IV- Ligation protocol

1. After gel extraction, pCDF vector is ligated with the *SSB* gene insert. For ligation, in a 1.5mL microcentrifuge tube set up the following reaction:

pCDF(cut) ------ 1 uL
SSB DNA ---------- 3 uL
Ligase ---------- 1 uL
Ligation buffer (2X)- 10 uL
DI water ---------- 5 uL

20 uL (total)

2. Incubate at Room Temperature for 10min.
3. Proceed to step V-Cell transformation.

Step V - Cell transformation protocol

1. Thaw DH5α cells **only on ice**. (Note-DH5α are competent cells very sensitive to temperature)

2. Label a 1.5mL microcentrifuge tube with your gene name.

3. Add 50 uL of DH5α cells to the tube.

4. To the same tube, add 5uL of the ligation reaction (from step IV).

5. Mix gently by tapping with fingers.

6. Incubate on ice for 20min.

7. Heat shock by incubating tube at 42°C for 45 seconds.

8. Place back on ice for 2 minutes.

9. Add 250 uL LB-media.

10. Mix gently by tapping and help cells recover by shaking in incubator at 37°C for 45min to 1hr at 220rpm.

11. Next to a flame (careful!) ,plate the entire reaction on a LB-Kanamycin plate-label with your group name and incubate the plate at 37°C overnight.