Gene Construction (via Restriction Cloning):

Step 1: Design and order oligos (25nmol scale, desalted)

Step 2: Resuspend each lyophilized oligo to 1 μg/μL with sterile water

Step 3: Make 40 μL of a 10 μM stock of each oligo

Step 4: Make the PCR reaction mix:

5 μL plasmid template (10 ng/uL; ~50 ng)
1 μL fwd primer (10 μM)
1 μL reverse primer (10 μM)
18 μL water
25 μL Q5 Polymerase 2X MM (NEB; or other MM)

If using a standard polymerase, make sure to add 10X buffer and dNTPs.

Step 5: Run PCR reaction (72°C extension temperature for Q5)

95°C	2 min	
95°C	30 sec	10 cycles
55°C	30 sec	
72°C	30 sec	
95°C	30 sec	20 cycles
60°C	30 sec	
72°C	30 sec	
72°C	10 min	
hold at 4°C		

Step 6: Run the PCR Cleanup Kit (Qiagen) on the PCR product

- Elute the DNA from the column with 50 μL of water

Step 7: Set up the restriction digestion of the PCR product and the vector

- Consult the NEB (New England Biolabs) catalog or website for the appropriate reaction conditions (buffer and BSA)
- Set up 20uL reactions (each enzyme individually—controls, vector dd and PCR dd)
    - Pipette each reaction gently to resuspend the enzyme and mix completely
- Incubate at 37°C for 2 hours

Enzyme 1 only	**Enzyme 2 only**	**Vector dd**
2 μL 10X Buffer	2 μL 10X Buffer	2 μL 10X Buffer
X μL vector plasmid (~1000 ng)	X μL vector plasmid (~1000 ng)	X μL vector plasmid (~2000 ng)
X μL water (20uL total rxn)	X μL water (20uL total rxn)	X μL water (20uL total rxn)
1 μL enzyme 1	1 μL enzyme 2	1 μL enzyme 1
		1 μL enzyme 2

PCR Product dd

2 μL 10X Buffer
14 μL Amp PCR product
1 μL enzyme 1
1 μL enzyme 2

Optional Step 7b: Dephosphorylate vector

- Calf Intestinal Alkaline Phosphatase (CIP) is active in CutSmart Buffer
- Add 1uL CIP to your VECTOR dd reactions -- do not add to your insert dd tubes; mix by pipetting
- Incubate at 37°C for 30-60 minutes

Step 8: Gel purify double-digested (dd) vector and insert

- Pour a 1% Agarose gel (1X TAE) - 50mL for small gel; 120 mL for large gel
- Add 10X DNA Loading buffer to each of the samples
    - Dark blue (bromophenol blue) runs at ~500 bp → use this for large DNAs (vector, etc)
    - Light blue (xylene cyanol) runs at ~3000 bp → use this for small DNAs (PCR products, etc)
- Load the gel
    - 1 μL NEB 2-log ladder + 8 μL water + 1 μL BPB dye (dark blue) → Load all 10 μL
    - ~1000 ng uncut vector
    - Load ALL of each digestion reaction
    - Load uncut PCR product if desired
- Run for 45-70 min at 100 V (for TAE buffer) until the bromophenol blue runs ~3/4 of the way down the gel
- Extract dd vector and insert from the gel and run the Qiagen Gel Extraction kit on each band (elute from column in water)

Step 9: Ligate the dd PCR product to the dd vector

- Determine the concentration of double-digested vector and insert from the gel purification by nanodrop
    - Use single digestion controls to determine the % of cleavage
- Determine the amount of insert that is needed for a 1:3 molar ratio (vector: insert)
    - This equation will tell you how much insert to use for a 1:1 molar ratio, multiply by 3 to get the amount for the 1:3 ligation

$$\frac{Xbpvector}{Xbpinsert} = \frac{50ngvector}{Xnginsert}$$

Control Ligation	**1:3 Ligation**
1 μL 10X T4 Ligase Buffer	1 μL 10X T4 Ligase Buffer
X μL dd vector (~50 ng)	X μL dd vector (~50 ng)
X μL water	X μL dd insert (~1:3 molar ratio)
1 μL T4 DNA Ligase	X μL water
	1 μL T4 DNA Ligase

- Pipette the reactions gently to mix
- Incubate ligation reactions at room temperature for 1-2 hours

Step 10: Transform ligation reactions into Z-comp cells

- Thaw appropriate molecular biology Z-comp cells (XL1-blue, TOP10, etc) on ice (1 tube contains 100 μL of cells—enough for 2 transformations)
- Add 2 μL of each ligation into 50 μL of cells, stir with pipette tip (do not pipette up and down or vortex), let sit on ice for 2-3 minutes
- Plate entire transformation onto a warmed agar plate
- Incubate overnight at 37°C

Step 11: Count colonies on plates, record number of colonies on vector-insert ligation plates vs. control ligation plates

Analyze your results if possible before sequencing: Colony PCR or Restriction Analysis
1. Colony PCR:

- **Day 1:** Determine how many colonies to do PCR on (If there are many more colonies on the ligation than the control ligation, 6-10 colonies is sufficient) – determine based on how many lanes you can run in a gel
- Circle and number the colonies on the plate
- Control(s):
    - Colony from control ligation plate
    - Plasmid that was used for vector (~5-10 ng of purified plasmid/rxn)
    - Positive control plasmid if available
- Prepare Master Mix of reaction (30 μL/reaction – I usually make one more volume than necessary):

Colony PCR

3 μL 10X Thermopol Buffer
0.3 μL 10mM dNTPs
1 μL fwd primer (10 μM)
1 μL rev primer (10 μM)
24.4 μL water
0.3 μL Taq polymerase

- Distribute 30 μL of reaction mixture into each PCR tube
- With a sterile toothpick carefully pick a colony and place the toothpick in the PCR tube
- Add plasmid DNA to any controls where it is needed
- Run PCR reaction

95°C	2 min	
95°C	30 sec	} 25 cycles
50°C	30 sec	} 25 cycles
68°C	1 min/kb	} 25 cycles
68°C	5 min	
hold at 4°C		

- Add gel running buffer (3 μL/samples) and run the completed reactions on a 1% Agarose gel (load all 30 μL)
- Pick the remaining colony into 3mL LB + Amp and grow overnight for minipreps
- **Day 2:** Miniprep and send for sequencing

2. Restriction Analysis:

- **Day 1:** Determine how many colonies to do restriction analysis on (If there are many more colonies on the ligation than the control ligation, 6-10 colonies is sufficient) – determine based on how many lanes you can run in a gel
- Pick colonies into 3mL LB + Amp and grow overnight for minipreps
- **Day 2:** Miniprep cultures
- Set up restriction analysis (10uL reactions)
    - Choose an enzyme or a pair of enzymes that give you a different product for your desired construct compared to the template you started with.
    - Examples:
        - Use an enzyme that cuts once in your template but twice in your desired construct; look for a small digested fragment
        - Use the same set of enzymes you used to do your cloning if you are inserting a different sized fragment than you started with (linker cloning)
    - Make sure to run appropriate controls:
        - Single cut on template construct
        - DD on template construct
        - DD on colony from control ligation plate
    - Make a master mix of the dd and add to 1-2 uL of miniprep DNA (make 1 more MM volume than you expect to use)

dd MM

1 μL 10X Buffer
6 μL water
0.5 μL enzyme 1
0.5 uL enzyme 2

- Incubate restriction digestions for 30-60 minutes
- Run a 1% agarose gel
    - Make sure to run uncut template vector, single cut controls, and control colony controls.
- Select positive constructs and send to sequencing