RNAi CLONING

Designing Primers

1. Pick an exon sequence of the desired gene about 400-500bp in length. Keep in mind that if your sequence spans two exons you should use cDNA for the PCR amplification.
2. BLAST the sequence to make sure the sequence does not have any 21-mers.
3. Design primers about 21bp in length. Keep in mind the annealing temperature should be within a few degrees of each other.
4. Add a restriction site at the 5' end of the forward primer and another at the 3' end of the reverse primer. Remember to pick enzymes that do not cut in the gene and are present in the L4440 vector multiple cloning site. See page 4 for the L4440 Map and unique restriction sites.
5. Add four A's to the 5' end of the forward primer and another four at the 3' end of the reverse primer.
   Example: Forward: AAAACTGCA/Gactcgtggagcttcaaagcaacgc
   Reverse: agttcttgccaaattcaatcgacT/CTAGAAAAA

PCR Amplification

Reaction Mix:

25µL 2X PCR Reaction Mix
5µL DNA template (N2 genomic DNA or cDNA)
5µL Forward primer (10µM)
5µL Reverse primer (10µM)
10µL H_2O

PCR Parameters:

① 1 min. at 94°C
② 30 sec. at 94°C
③ 30 sec. at 55°C
④ 2 min. at 72°C
⑤ Go to ② 35x
⑥ 5 min. at 72°C
⑦ 4°C ∞

Note: the annealing temperature will change depending on the primers and the length of the gene being amplified.

1. Run a few µLs on a 1% agarose gel to make sure the gene amplified is of the correct size.
2. PCR Purify (QIAquick PCR Purification Kit) and resuspend in 30µL of dH_2O.

Restriction Digest

1. Make a 45µL total reaction using all 30µL of purified DNA.
   Example:
   30µL DNA
   4.5µL 10X Buffer 4
   4.5µL 10X BSA
   1µL Age I
   1µL XbaI
   4µL dH_2O
2. Do a corresponding reaction with the vector L4440.
   Example:
   3µL L4440 (~2µg total)
   4.5µL 10X Buffer 4
   4.5µL 10X BSA
   1µL Age I
   1µL XbaI
   31µL dH_2O
3. Incubate overnight at the temperature specified by restriction enzymes.
4. Add ~10µL of DNA loading dye to each tube.
5. Run all 55µL on a thick 1% agarose gel. If possible, run the uncut L4440 as a control.
6. Gel purify (QIAquick Gel Extraction Kit) and resuspend in 30-40µL of dH_2O.

Ligation

1. Determine the amount of insert and vector needed to get a ratio of 3:1 by either running equal amounts of insert and vector on a gel and doing a rough comparison or by using the below calculation:

   $$\frac{\text{length of insert (kb)}}{\text{length of vector (kb)}} \times \text{ng of vector} \times 3 = \text{ng of insert needed}$$

2. Make two 20µL reactions for each gene, one with the cut insert and vector and one with just the vector.
   Example:

Insert + Vector	**Vector-Only (Negative Control)**
0.75µL L4440 (cut)	0.75µL L4440 (cut)
6µL Insert (cut)	-
2.5µL 10X Buffer	2.5µL 10X Buffer
1µL T4 DNA Ligase	1µL T4 DNA Ligase
14.75µL dH_2O	20.75µL dH_2O

3. Incubate overnight at 15°C.

Recommended: Transformation into Top10 or DH5α Competent Cells

1. Add 4µL of each ligation to a thawed vial of competent cells.
2. Incubate for 30 minutes on ice.
3. Incubate for 30 seconds at 42°C and place directly on ice.
4. Add pre-warmed S.O.C. medium (volume varies depending on competent cell type used).

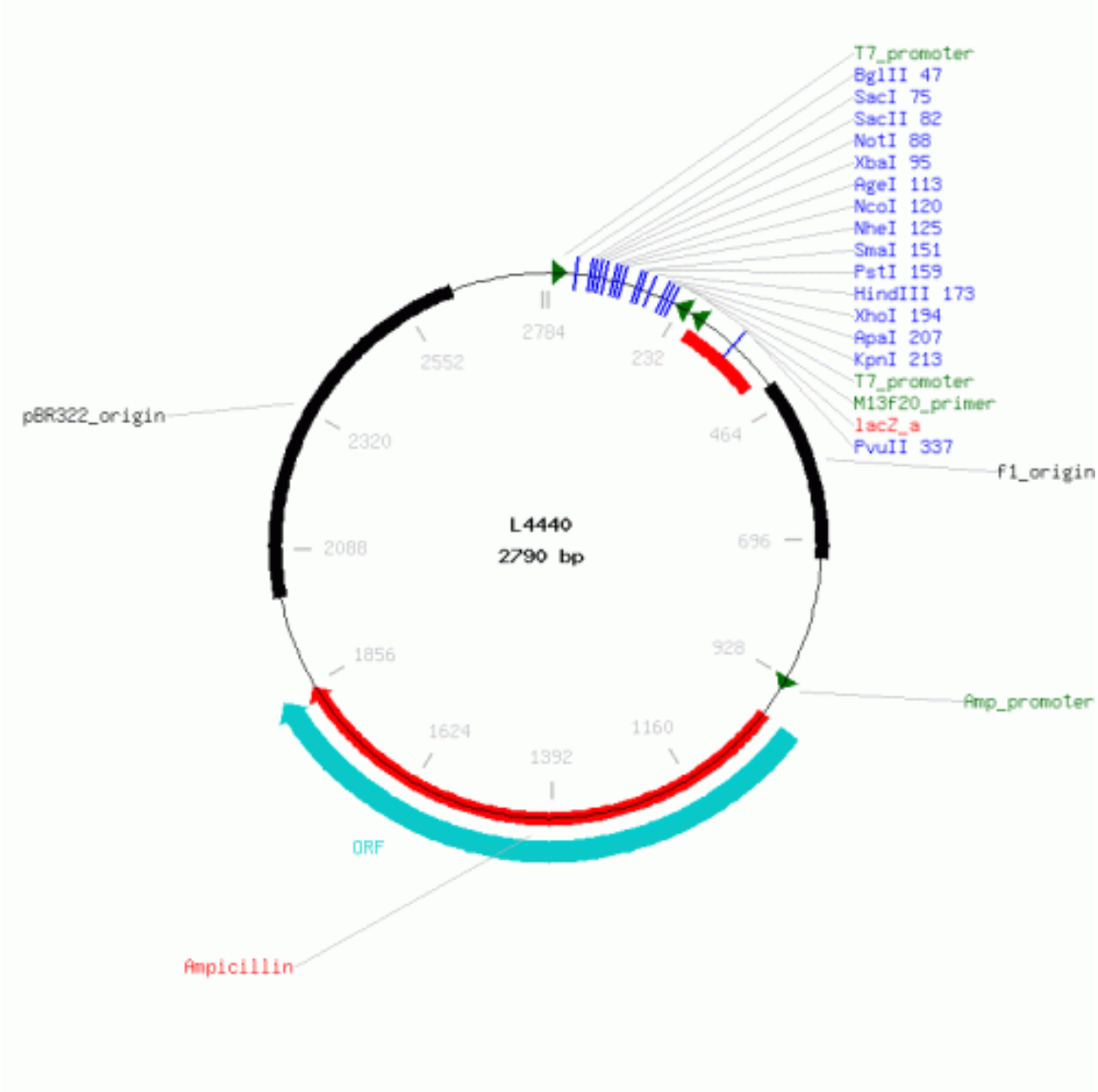
5. Shake for 1 hour at 37°C.
6. Plate cells onto an LB + Ampicillin plate.
7. Incubate overnight at 37°C.
8. If the ratio of insert + vector colonies to vector-only colonies looks good (many insert colonies and very few vector-only):
    a. Pick several insert + vector colonies.
    b. Grow overnight at 37°C in LB + Ampicillin (100µg/mL).
    c. Extract plasmid (QIAprep Spin Miniprep Kit).
    d. Send for sequencing using the standard L4440 forward and reverse primers.*
9. If there is not a good ratio of insert + vector colonies to vector-only colonies, do a single colony PCR to find the colonies that have the insert:
    a. Pick a colony with a pipet tip and mix in a PCR tube containing 50µL of H_2O.
    b. Streak the same tip on an LB + Ampicillin plate.
    c. Repeat for multiple colonies.
    d. Heat PCR tubes for 10 minutes at 95°C to lyse the cells.
    e. Set up a PCR reaction using the same primers and parameters as used to amplify the initial insert. Add 5µL of lysate in place of the DNA.
    f. Run PCR products on a gel and pick colonies that have a band of the correct size to transform into HT115 competent cells.

<u>Transformation into HT115 Competent Cells</u>

1. Add 5µL of each miniprep to a thawed vial of HT115 competent cells.
2. Incubate for 30 minutes on ice.
3. Incubate for 30 seconds at 42°C and place directly on ice.
4. Add 50µL of competent cells to 950µL of pre-warmed S.O.C. medium.
5. Shake for 1 hour at 37°C.
6. Plate about 500µL of calls onto an LB + Ampicillin plate.
7. Incubate overnight at 37°C.
8. Pick one or two colonies to grow overnight at 37°C in LB + Ampicillin (100µg/mL).
9. Make a glycerol stock of each before miniprep. Final glycerol concentration should be about 25%.
10. Send for sequencing using the standard L4440 forward and reverse primers.*

* L4440F: 5'- ACG ACT CAC TAT AGG GAG ACC -3'
L4440R: 5'- AAA CGA CGG CCA GTG AGC GC -3'
Designed by Cindy Voisine

L4440 Plasmid Map



** There are <u>not</u> restriction sites for SacI, SacII and PstI.