Using LTRi to control your gene of interest:

1. Cut the transgene module plasmid with BsmI and blunt it (i.e. using Klenow, NEB) then cut with PstI (NEB). This removes the EGFP gene but keeps the target sequence and the PolyA intact.

2. PCR amplify the CODING SEQUENCE ONLY of your gene (start codon to stop codon of your gene). When designing your primers, include PstI in your forward primer (if your gene has PstI in it then use a different enzyme site that ligates to PstI – see "compatible cohesive ends" on the NEB website for suggestions). Your reverse primer will not need any restriction sites added to it since this side is a blunt ligation. (Because you are doing a blunt ligation, note that you should use a polymerase, such as Pfu, that does not add an extra "A" to end of your PCR product.)

For example, if we are amplifying the Cre coding sequence, these are the primers we would use:

Forward oligo:

5' – taa*ctgcag*atgtccaatttactgacc – 3'

Where the PstI site is in italics and atg is the start codon for the Cre coding sequence.

For the reverse oligo, you don't need to add the BsmI site since you will be doing a blunt ligation. I use the reverse complement of the 3' end of the gene for the reverse oligo. The 3' end of the cre gene, with a tag and stop codon, is cgcctgctggaagatggcgattag, so I would order the following oligo for my PCR:

5'- ctaatcgccatcttccagcaggcg -3'

Once the PCR is done and gel isolated (and cleaned of course) then I only digest it with PstI. Your vector will have one blunt end and one sticky end for PstI.

3. After digesting your PCR amplicon with PstI, ligate it into the transgene module that has been cut with BsmI, blunted, and cut with PstI. You now have a plasmid that has an RSV promoter driving your gene (with an intron containing Lac operator sites and the synthetic RNAi target).

4. Depending on your gene and how it affects the unique restriction sites in the transgene module, you can either cut out the promoter and gene, or PCR amplify it. I generally do a double digest using BspHI and PciI (both ligate to PciI in the LTRi vector) to pull out the portion that needs to be moved into the LTRi vector, unless these sites are in the gene.

Ligate the insert into LTRi that has been digested with PciI. Because BspHI and PciI are compatible, you will need to screen your ligation products for the correct insert orientation. The ordering of the modules doesn't matter. The only thing that I have been warned about (but never experienced) was to not put two strong promoters in the same direction. I guess the concern is that a strong promoter can blow through a stop codon. This is why all of the modules go back and forth between being clockwise and counterclockwise (this doesn't include the ampR or the origin of replication).

IMPORTANT NOTE!

There is a portion of this module that can recombine in E. coli, rendering it nonfunctional. There is a 1/18 chance that this can happen. I highly recommend that you sequence over this portion to make sure that this has not happened after you have cloned your gene into it. The portion that recombines is in the intron and if this happens then your gene is no longer in frame and it will not be expressed. Indeed, the chances are low that this can happen but it will save you lots of time of trouble-shooting if you wait the extra couple of days for the sequencing to confirm that everything is okay.

I have tried transforming $RecA^-$ cells, but the cells don't grow very well for me and I never seemed to get enough DNA from them to do any experiments. Since the chances of recombination are low, I use DH5 cells and sequence 2 preps. They are usually both correct.

Region to be concerned about:

987-1073 in the Transgene module plasmid (and the corresponding sequence in the LTRi switch – both introns!).

Sequencing primers that I use for the transgene module (you don't need to use all of them, just get the region mentioned above):

Seq1 (forward starting just before promoter): GGCGATTAAGTTGGGTAACGCC
*you need a long read to get what you need out of this primer

Seq2 (reverse starting at end of intron): GCCGCAAGCTTGGTTGGAATCTAAAATAC

Seq3 (forward starting at end of RSV promoter): AGATATTGTATTTAAGTGCC

Seq4 (forward starting in U6 promoter): CGTCACGTGGTGCGTTTTGCCT

The portion that needs to be sequenced is very difficult to sequence. You should see if your sequencing facility has a "difficult template" protocol and have them use that for your runs. I use Genewiz (genewiz.com) and they have such a protocol that has worked very well for me.

To confirm the two introns in the switch once it's all put together, I use the following primers:

Seq5 (reverse starting in TetR): CACTTTACTTTTATCTAATCTAGAC

Seq4 (forward starting in U6 promoter): CGTCACGTGGTGCGTTTTGCCT

I recommend designing primers that start at the beginning of your gene and read in the reverse direction to confirm the region in question.