Sarah W. Stedman Nutrition and Metabolism Center

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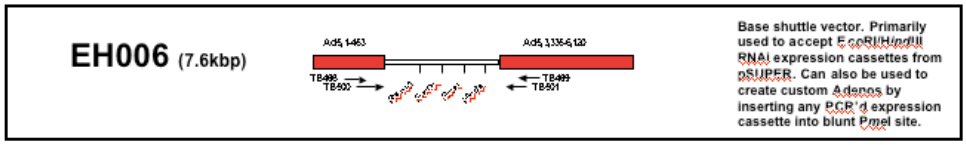
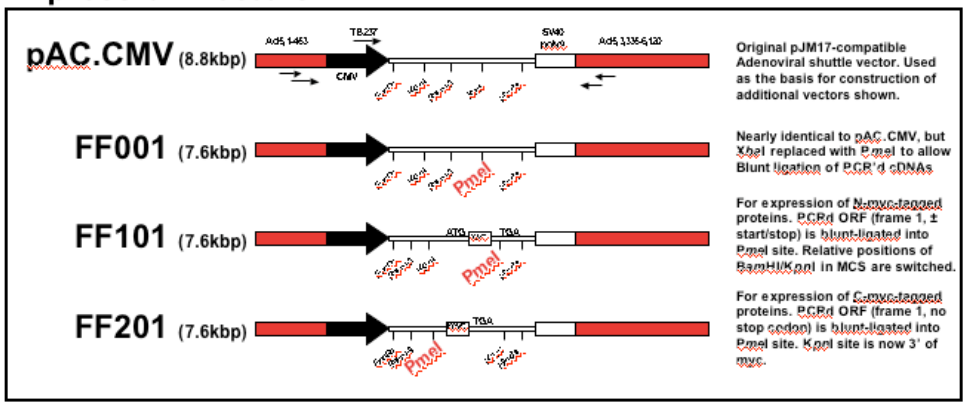
Sarah W. Stedman Center Guide to Vector- and Adenoviral-based siRNA-mediated Gene Silencing

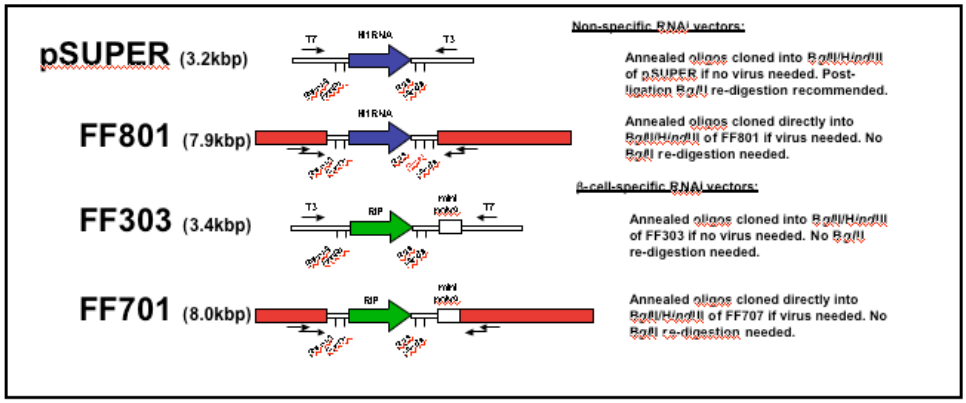
Basic Vector



Expression Vectors



RNAi Vectors



Design of RNAi oligos:

All above RNAi vectors:

```
5'- GATCCCC(N19)TTCAAGAGA(6IN)TTTTTGGAAA -3'
    3'- GGG(N19)AAGTTCTCT(6IN)AAAAACCTTTTCGA -5'
```

RIP RNAi vectors:

```
5'- GATCCG(N19)TTCAAGAGA(6IN)C -3'
    3'- GGC(N19)AAGTTCTCT(6IN)GTCGA -5'
```

Design of PCR oligos for N- or C-myc cloning:

```
sense: 5'- ACCATG…N_I -3' ; ATG = start codon
antisense: 5'- XYZ…N_I -3' ; XYZ = last codon before STOP
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Figure 1. pJM17-compatible adenoviral shuttle vectors.

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Construction of siRNA plasmids and Adenoviruses

I. A couple of important points before cloning begins:

1. **RNAi target selection isn't covered here.** Thomas Tuschl, who got siRNA to work in mammalian cells, has a web site that discusses selection of RNAi targets (http://www.rockefeller.edu/labheads/tuschl/). Click on siRNA for the user's guide. This was put together before pSUPER was published, so he's assuming you'll be doing your RNAi with DNA/RNA oligos. As such, his targets are in the form AA(N19)(TT).

   Alternatively, you may also use the tools provided by several on-line sources (oligoengine.com, idtdna.com, ambion.com, to name a few) to design select RNAi targets. The Stedman Center developed it's own algorithm that works as well as any, so if you need help with siRNA target design, contact us.

   When choosing targets for use with vector-based methods, the targets are the same, but only the N19 portion is actually used in the oligo. This is a lot clearer in the online supplementary data from Brummelkamp paper, which can be found at: http://www.sciencemag.org/cgi/content/full/1068999/DC1

2. **For each target, you will need to order 2 oligos;** a sense and an antisense oligo, each 64 nucleotides long. The general sequence of each is:

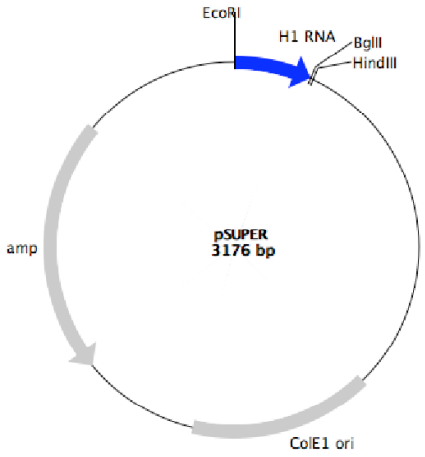
   sense: 5'-GATCCCC(N19)TTCAAGAGA($N19_{R\&C}$)TTTTTGGAAA-3'

   antisense: 5'-AGCTTTTCCAAAAA(N19) TCTCTTGAA($N19_{R\&C}$)GGG-3'

   Important!: The R&C subscript indicates the **R**everse & **C**omplement of N19

3. **When ordering the oligos, be sure to specify that the oligos must be PAGE-purified**. These are relatively long oligos (64mers): A preparation of standard, desalted oligos will very likely contain lots of truncated oligo. This will likely give you lots of heartache when you experience great difficulty isolating a plasmid clone that contains full-length, error-free target siRNA target.

II. Cloning oligos into pSUPER:



If B*gl*II/H*ind*III-linearized pSUPER is already available, skip Step 2A. If not, steps 1 and 2A may be done in parallel.

Step 1: Annealing oligos:

1. Resuspend oligos to a concentration of 5 OD/100μl (i.e. 20 µl/OD) in STE (10 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA). The total amount of oligo (total OD, nmoles, and mg) should be printed on the label. Typically, from a 100 nmole starting material, these PAGE-purified 64mers yield approximately 1.5 – 2.5 total OD.

2. From the total nmoles printed on the label, calculate the final pmoles/μl (typically 82-83), and mix 500 pmoles each oligo in an eppendorf tube. Bring the final volume to 20 μl with STE.

3. Preheat heating block to 95°C (with water in the block holes), and heat oligo mix 95°C for 5 minutes.

4. Remove block from element and allow block to gradually cool, preferably to room temperature (to approx 35°C works too), then place tube on ice. It takes approximately 1 hour for the block to cool to 35°C.

Step 2: ligating into pSUPER:

A. Linearization of pSUPER with B*gl*II/H*ind*III:

1. Digest approximately 500 ng pSUPER in a final volume of 20 μl (25 ng/μl final) with B*gl*II/H*ind*III.

Mix:

H_2O to 20 μl

500 ng pSUPER
2 μl NEB buffer #2
0.5 μl B*gl*II
0.5 μl H*ind*III

2. incubate 37°C, 2-3 hours.
3. Heat-kill reaction by incubating at 95°C for 15-20 minutes. Place reaction on ice.

B. Ligation of annealed oligos into pSUPER. Your controls should include 2 controls:

1). No ligase control: Vector, but neither insert nor ligase.
2). Ligase control: Vector + ligase, but no insert.
For both controls, final volume is brought to 20μl with H_20

1. Mix:

15 μl H_20
2 μl 10x ligase buffer
1 μl B*gl*II/H*ind*III-linearized pSUPER
1 μl annealed oligos
1 μl ligase

2. Incubate room temperature 2-3 hours or 16°C overnight

Step 3: Re-digestion with B*gl*II: *We found this step to be important. Without re-digestion with BglII, you may end up with an extremely high background.*

Because the B*gl*II and H*ind*III sites were placed immediately adjacent to each other, one enzyme mostly fails to cut at its target site once the other enzyme has cut at its target site. The result is that the overwhelming majority of the pSUPER molecules considered "B*gl*II/H*ind*III-cut" (by virtue of B*gl*II and H*ind*III being in the tube together), are, in fact, only cut with *either* B*gl*II *or* H*ind*III: The vast majority of ligation products, therefore, are simply single-cut pSUPER molecules that re-ligated to re-form pSUPER. This will produce a very high background upon transformation of bacteria, making it nearly impossible to pick a pSUPER clone that actually contains the 64mer insert.

Fortunately, ligation of the 64mer into double-digested pSUPER destroys what was once the B*gl*II site. To reduce background substantially, therefore, an aliquot of the ligation reaction is used as substrate for an additional B*gl*II digestion. Re-ligated pSUPER is then linearized, while wanted clones remain circular. Background is reduced because linear plasmids can't reproduce in bacteria, so these clones won't grow.

Optional Control: In addition to the ligation reactions that are re-digested with B*gl*II, consider running a parallel set of no-B*gl*II reactions (containing H_2O instead of B*gl*II). As you will see below (Step 5), by doing both sets, one will be able to see that B*gl*II is indeed linearizing re-ligated pSUPER.

Mix:

15 μl H2O
2 μl NEB buffer #3
2 μl ligation reaction
1 μl B*gl*II or H_20

incubate 37°C 2-4 hours.

Step 4: transforming bacteria:
If you've been doing the appropriate controls, you should have 6 transformations to do if you're trying to clone a single 64mer. If you're tackling a panel of four 64mers, you'll have a total of 12 transformations. I also suggest including pUC19 (a vial of which is included in the box of competent cells) as a "transformation control". Subcloning and Library-efficiency cells should yield ≥ 10 and 100 colonies per pg transformed, respectively.

Transform 2 μl of the B*gl*II reaction into competent bacteria. Any standard cloning strain should work fine. We typically use DH5α cells (Invitrogen; sub-cloning efficiency, approximately 50μl cells per transformation). Follow the manufacturer's recommendations closely. Plate transformants on LB plates containing 100 μg/ml carbenicillin, and place plates in the 37°C bacterial incubator.

Step 5: Picking clones:
Approximately 18 hours after placing the plated transformed bacteria in the 37°C incubator, look at the plates get a visual estimate of the relative numbers of colonies on each plate. For example, you should now see that re-digestion of the ligation reactions with B*gl*II significantly reduces the number of colonies obtained after transformation. Ideally, you would see zero colonies on the re-digested "ligase" control plate but this, in my experience, rarely happens.

Importantly, though, you should see colonies on the plate containing cells transformed with the "pSUPER + insert + B*gl*II re-digestion" reaction. Often times the number of colonies on this plate may be roughly the same as on the "no insert" control. It's been my experience, however, that despite this, many of the colonies on the "+ insert" plate are, in fact, positive for the insert and several clones from this plate should be picked anyway.

To grow these cultures, I generally use Terrific Broth (TB) containing 100 μg/ml carbenicillin (carb), though LBcarb will work as well. TB is a very rich media, and bacteria grow to very high cell densities, leading to higher DNA yield than that obtained by using LBcarb (often as much as 15 μg from a 2 mL culture).

Inoculate 6-8 liquid cultures from the "+ insert" plate for subsequent mini-prep anaylsis. Using sterile technique, add 2 ml TBcarb to each of 6-8 labeled 15 ml polypropylene culture tubes. Touch the tip of a pipet tip (on the end of a pipettor) to a selected colony,

then touch the TBcarb with this tip, pipetting up and down a few times. Place the tubes in a 37°C shaking incubator set at 225-250 rpm.

Step 6: Screening for positive clones #1 - Miniprepping DNA:
To miniprep plasmid DNA, a commercial miniprep kit is recommended, since the resulting plasmid prep is cleaner than one obtained using "homebrew" reagents. As a result, one can send positives clones for sequencing directly, without having to re-prep them, or clean them up.

Although the QIAGEN miniprep kit works fine, we've been using the Bio-Rad miniprep kit lately. Follow the manufacturer's instructions closely. Cultures may be miniprepped after as little as 6 hours post-inoculation. Thus, if you do the transformations in the early afternoon, cultures may be inoculated first thing the next morning and mini-prepped by early afternoon: Note that 6-hour cultures obviously wouldn't be as grown as they could be, but they must be at least visibly turbid. You don't need a fully-grown culture to get enough DNA for your purposes.

Step 7: Screening for positive clones #2 - Restriction analysis:
At this point, miniprepped DNA must be analyzed by restriction analysis for the presence of the 64mer insert.

The entire pSUPER polIII expression cassette (containing the promoter, cloning site, and T5 terminator) is located within a contiguous piece of DNA flanked by an E*co*RI site at the 5' end and a H*ind*III site at the 3' end. Digestion of pSUPER with E*co*RI + H*ind*III will therefore excise a 227 base pair (bp) fragment, while similar treatment of a pSUPER-derived clone containing the 64mer insert will excise a 291bp fragment. To screen for positive clones, therefore, digest 3 μl of miniprepped DNA with E*co*RI + H*ind*III, then run the reaction on a 1.5-2.0% agarose gel. I strongly suggest also digesting an aliquot of pSUPER with E*co*RI + H*ind*III and running it along site your miniprepped samples on the gel. The E*co*RI/H*ind*III fragment from clones containing the 64mer insert will run slightly higher on the gel, due to their slightly larger size.

Mix:
14 μl H_20 (to 20 μl final)
2 μl 10 NEB E*co*RI buffer*
3μl Miniprepped DNA (or approx 1 μg pSUPER as a control)
0.5 μl E*co*RI
0.5 μl H*ind*III

*Be sure to use E*co*RIbuffer, and not NEB buffer #2: E*co*RI and H*ind*III are active in NEB #2, but E*co*RI displays "star" activity (i.e. nonspecificity) in NEB#2.

- Incubate 37°C, 1 hour.
- Remove samples from 37°C, add 2 μl 10x loading dye.
- Use 123bp ladder as a DNA MW marker.

- Run samples on 1.5-2.0% 1X TBE* agarose gel at 100-110 volts until tracking dye (bromophenol blue) has run approximately halfway or two-thirds the way.
- Visualize on gel-doc station in common area. Digestion of positive clones will produce a 291bp E*co*RI/H*ind*III fragment, whereas negative clones (i.e. pSUPER) will yield a 227bp fragment.

> ***Cautionary note:** If you typically use TBE at 0.5X, it's been my experience that reactions containing 1x E*co*RI buffer run very strangely on 0.5X TBE agarose gels

Step 8: Screening for positive clones #3 - Sequencing:
At this point, you should have been able to see that at least one of your clones is positive for your insert. In fact, for a very good reason, at least 3 of you clones will hopefully be positive for the insert: Even though oligo manufacturers invest considerable time and expense doing QC, a 64mer is a relatively long oligo, and, despite being PAGE-purified, errors still occur, and it is possible that one or more (or all!) clones harbor an error in the cloned siRNA sequence. As a result, the sequence of the cloned 64mer in positive clones MUST be confirmed by sequencing. You may easily find that at least one of your clones contains an error. I suggest sending 3-5 positive clones for sequencing. If you get the identical error in all clones, do the following:

- Recheck the sequence of the oligo that you designed. Compare it to the target sequence of the cDNA. It's possible you made an error here.
- Re-check the oligo sequence on the spec sheet that came with the oligo. It's possible one of the oligo's was actually made with this error as part of the ordered sequence. This would be unusual, since you should have re-checked and confirmed your oligo sequence just before the final "order" button was pushed.
- If none of these pans out, it's likely there was a systematic error that introduced a mutation into one of your oligos. If this is the case, assume both oligos are bad. Call the manufacturer and explain the problem, and ask to have the oligos re-made. In this case, you start again at Step 1.

For sequencing pSUPER clones, you will typically need 0.5-1.0 μg DNA. To quantitate your miniprepped DNA, make a 1:25 dilution of your DNA in H_2O, and read the OD_{260} on the spectrophotometer. If you're using the "DNA quantitation" program on our spec, it will also read OD_{280}. The 260:280 ratio is then calculated as a measure of DNA purity: A relatively clean prep should have a 260:280 ratio close to 2.0. To get the concentration of your DNA (in ng/μl), multiply the concentration reading the spec calculates by your dilution factor.

The amount of plasmid and oligo needed for each sequencing reaction depends on the sequencing facility you use. In our case, for each reaction, we mix 0.5 μg pSUPER clone and 10 ng oligo brought up to a final volume of 12 μl with water. I suggest using the T7(short) and T3 oligos (10ng/μl stocks). If the pSUPER you're using was the pSUPER described in Brummelkamp, *et al.* (Science (2002), **296**, 550), and/or you purchased it

from oligoengine.com, the T7 and T3 oligos are in the forward and reverse direction, respectively. It's been my experience that sequence obtained with the T3 (reverse) oligo is often better than that obtained with the T7 oligo. Therefore, if you are going to sequence with either the T7 or the T3 oligo, choose the T3.

III. Cloning the siRNA expression cassette into the adenoviral shuttle vector:

Step 9: Purify expression cassette from pSUPER clone:
Once you've identified which clones contain error-free 64mer inserts, you'll have to excise the entire siRNA expression cassette with E*co*RI/H*ind*III, resolve it away from the pSUPER vector by running it on a gel, purify this fragment from the gel, and ligate it into the adenoviral shuttle vector.

- Mix:
0.5-1.0 μg miniprepped pSUPER/siRNA clone DNA
2 μl 10x NEB E*co*RI buffer*
0.5 μl E*co*RI
0.5 μl H*ind*III
H_2O to 20 μl

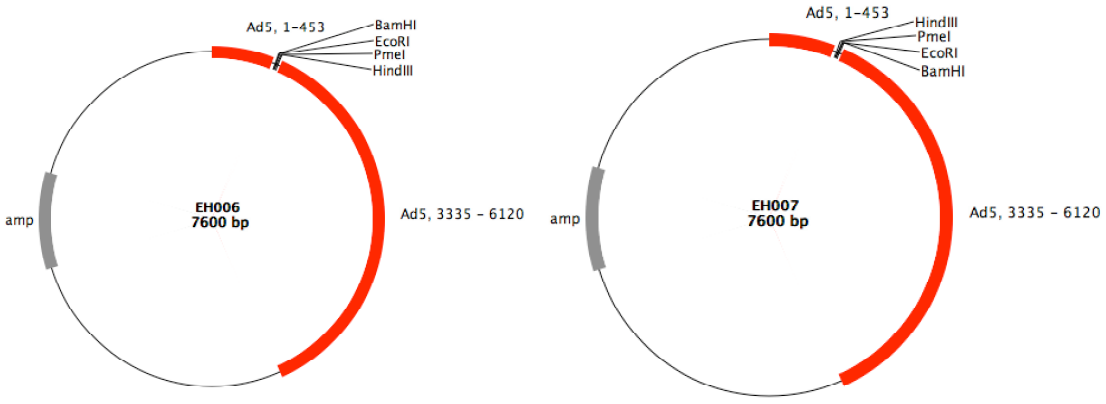
* use E*co*RI buffer here. See note in Step 7.

- Incubate ≥2 hours, 37°C
- Remove reaction from 37°C and add 2 μl 10x loading dye (see comment about 1x E*co*RI buffer in Step 7).
- Run reaction(s) on 1.2-1.5% agarose gel at 100-110 volts until tracking dye has run approximately halfway or two-thirds down the gel.
- Transfer the gel to a UV lightbox. Turn the UV light on, and, if possible, turn the UV intensity down until you can just see the band (UV damages DNA).
- Cut the band out of the gel with a clean razor, and transfer to a clean eppendorf tube.
- To purify the DNA from the gel slice, we use a commercial kit from QIAGEN. Follow their instructions:
    - Add 300 μl QX1 per 100 mg gel slice
    - Vortex resin ≥30 seconds, and add 10μl.
    - Incubate 50°C 10 minutes with mixing every 2 – 5 minutes or so
    - Spin full speed 30 seconds. Pour off supernatant
    - Wash once with 500 μl QX1 (vortex, spin, pour off supernatant), and twice with 500 μl wash buffer.
    - After 2nd wash, spin briefly and remove residual liquid with pipettor. Allow tubes to air dry (IMPORTANT: tubes must be fully dried – residual ethanol will inhibit elution of DNA form resin)
    - Add 20 μl elution Buffer. Mix by pipetting up and down, and allow tubes to incubate room temp for 10 minutes.

- Spin tubes full speed for 1 minute. Remove supernatant (containing purified DNA) to clean eppendorf.

Step 10: Ligating siRNA expression cassette into adenoviral shuttle vector (EH006): Plasmid EH006 is a pAC.CMV-derived adenoviral shuttle vector designed to accept pSUPER/siRNA expression cassettes as either E*co*RI/H*ind*III or B*am*HI/H*ind*III fragments. EH006 differs from EH007 in that the former will orient the expression cassette in the (+) orientation relative to the adenoviral genome (i.e. expression goes "left-to-right). It has been noted that recombinant adenoviruses express cDNAs better when the expression cassette is cloned in the (+) orientation.

As of this writing, we are experimenting with siRNA expression cassettes in both orientation. In contrast to adenoviral *expression* systems, our early results suggest that expression of siRNAs from EH007-derived viruses are just as effective as those expressed from EH006-derived viruses. Of one wishes to clone in the (-) orientation as well, plasmid EH007 should be used in the same way as plasmid EH006.



Preparation of E*co*RI/H*ind*III-linearized EH006 is as described for pSUPER in Step 2A.

Mix:
6 μl H_2O
1μl 10x NEB ligase buffer
1 μl E*co*RI/H*ind*III-linearized EH006, diluted 1:5 in H_2O
1 μl gel-pure E*co*RI/H*ind*III fragment*
1μl ligase*
*Controls that should be included are: E*co*RI/H*ind*III-linearized EH006, with 2 μl H_2O added in place of ligase and insert (no-ligase control), or 1 μl H_2O added in place of insert (+ligase control).

Ligate 2 hours room temperature, or 16°C overnight.

Step 11: Transforming bacteria:

(see Step 4): Transform 2 μl of the ligation reaction into competent bacteria. Plate on LBcarb plates, and place plates in 37°C bacterial incubator for at least 16 hours.

Step 12: Screening clones: Two methods for screening are discussed here: The first method, described for screening pSUPER clones involves miniprepping plasmid DNA, followed by digestion with E*co*RI/H*ind*III. However, clones may also be screened by PCR.

A. **Miniprepping and restriction analysis** (see Step 5): Approximately 18 hours after transforming, look at the plates to get a visual estimate of the relative numbers of colonies on each plate (see Step 5). If so, pick 6-8 colonies and inoculate 2 ml TBcarb (or LBcarb; see step 5) cultures for miniprep analysis.

Miniprep all 2 ml of the culture (see Step 6). Elute DNA from miniprep columns with 50 μl elution buffer. You may notice that recovery of pAC-based adeno shuttle vectors tends to be lower than pSUPER clones.

Because the insert was cut out of your pSUPER clone, the phosphate groups on the 5' ends of each DNA strand are intact, making it theoretically possible that one or more of your positive EH006/EH007 clones actually contains 2 copies of the insert, joined head-to-head, and ligated into the shuttle vector at either the E*co*RI or H*ind*III site. Although this is unlikely to happen, taking the extra step to confirm this didn't occur is worthwhile before proceeding to make virus.

To screen for single-copy insertion, positive clones should digested with E*co*RI and H*ind*III separately. If digestion of a positive clone with E*co*RI or H*ind*III alone linearizes your vector, your insert is present in a single copy. If, however, one of these digestions yields a 600bp (approximate) fragment (twice the size of a single inset), 2 copies of the insert managed to ligate in. These clones should be discarded.

Screen for positive clones by digestion with E*co*RI/H*ind*III (see Step 7). Include your pSUPER/siRNA clone as a positive control, and (optional) pSUPER as a negative control). Clones found to be positive for insert should also be screened for the presence of a single copy of the insert by separate E*co*RI and H*ind*III digestions (see note above).

Mix:
15 μl H_2O
2 μl 10x E*co*RI buffer
2 μl miniprepped DNA
0.5 μl E*co*RI
0.5 μl H*ind*III

- Incubate 37°C, 1-2 hours, add 2 μl 10x loading dye (see comment about 1x E*co*RI buffer in Step 7).
- Use 123bp ladder as DNA MW marker.
- Run reaction(s) on 1.5-2.0% agarose gel until tracking dye has run approximately halfway or two-thirds down the gel.

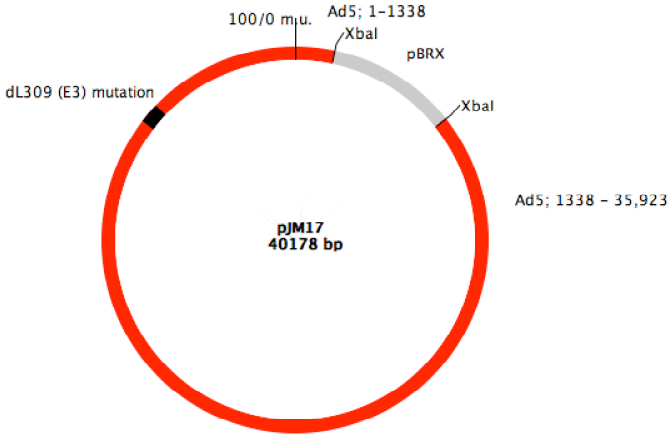
- Photograph the gel on a gel-doc station.
- Select clones positive for the presence of the 291bp insert. Importantly, though, look at the size of the vector band. You should notice the EH006 vector band runs higher that the vector band from your pSUPER/siRNA control, since EH006 is approx 8.5kkbp, while pSUPER is approximately 3kbp.

B. PCR analysis:
You may also screen clones by PCR. In this case, I use oligos TB498 (sense, 5'-TCGCCCAGGTGTTTTTCTC) and TB499 (antisense, 5'-TCACATCCAGCATCACAGG), which bind to the adenoviral sequences that flank the E*co*RI/H*ind*III insert. The predicted product size for positive (with a single insert) is 490bp. Clones that yield a 200bp product are negative for insert, whereas clones yielding a 781bp product contain 2 copies of insert (see note about multiple insert copies in step 14 above).

If you've screened by PCR, you may also purify the PCR product (I use QIAGEN's PCR purification kit with good results) and send it for sequencing. For sequencing, I use oligos TB500 (sense, 5'-TTCTCAGGTGTTTTCCGC) and TB501 (antisense, 5'-TATGTTTACCGCCACACTCGCAGG). Each oligo is kept as a 100μM stock. For TB500 and TB501, this corresponds to 545 & 730 ng/μl, respectively. For sequencing, dilute to 10 ng/μl, and use 3 μl per reaction. See Step 8 for the details of sequencing plasmids.

IV. Co-transfection of 293 cells:

Recombinant adenovirus is currently made by co-transfecting HEK293 cells with the adenoviral shuttle vector and the plasmid pJM17. Plasmid pJM17 is a large plasmid (approx 40kbp), and can be a bit tricky to work with. Avoid vortexing and excessive pipeting, as this will shear the DNA. Preparation of pJM17 isn't trivial, and isn't covered here. Likewise, propagation and purification of the virus isn't covered here, either, but will be in future revisions.



The 293 cells should be relatively low passage, and should have been kept in a state of active growing, i.e. cells that have been left confluent for several days prior to being split for transfection probably won't work well.

- Cells should be plated in a 6-well plate and be 50-70% confluent.
- For each transfection, 2 well of cells is used.
- Aspirate medium, and replace with 2 ml DMEM + 10% FCS
- Prepare transfection solution in the following order:
    1. 200 μl DMEM (no FCS)
    2. 2.4μl FuGene*
    3. 0.8 μg pJM17*
    4. 0.8 μg adeno shuttle vector*

    *if you have enough DNA, 6μl FuGene, 4μg total DNA will give a higher level of transfection (as determined by reporter transfection), though, at this point, it's not clear this will increase your chances of getting virus.

- Let solution sit for 5-10 minutes, but less than 45 minutes.
- Add transfection solution dropwise to cells (approximately 100 μl per well) and mix by swirling.
- Place plate(s) back in incubator.
- After 12-24 hours, add 4 ml DMEM + 10%FCS.

One final note: Prior to using the virus for experiments, I suggest that the relevant portion of the recombinant genome be PCR-amplified and sequenced using the primers above. When using a lysate as a template for PCR, I found that the lysate must be diluted at least 1,000-fold prior to use. Dilutions can go as high as 10,000-fold, but a 2,500-fold dilution should work for most lysates.