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Creation and Use of Your Infectious Vector

From: Stewart Lab (http://www.stewartlab.wustl.edu/html/lab_protocols.html)

CONSTRUCTION OF LONG TERM RNA*i* CONSTRUCTS

This protocol allows you to order your oligos and then clone them into pLKO.1 and pMKO.1 (These replace Lentihair and Retrohair. In addition, the cloning strategy has changed from that reported in the original paper.)

This upper case sequence is the 3' end of the hU6 promoter from -68 to +1

CATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGA
AAGGACGAAACACCGGtCCgcaggtatgcacgcgtgaattcgtcgac

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                                                                                                VpaK11AI
                                                                                                 Sau96I
                                                                                                 FmuI
                                                                                                 AvaII            SelI
                                                                                                RsrII             BstUI       SalI
                                                                                               HpaII              MluI        Tsp509I
                                                                                              BsrFI               AflIII
HincII          MaeIII                                                                       BsaWI    BspMI     Cac8I       EcoRI
TaqI
NdeI     Bst4CI                 TaqI       CviJI                                             AgeI     AciI      CviRI       ApoI    AccI
 |         |  |                  |          |                                               ||||    | |       || ||      ||      ||
**TATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGGtCCgcaggtatgcacgcgtgaattcg****
*  105

****ACGAATGGCATTGAACTTTCATAAAGCTAAAGAACCGAAATATATAGAACACCTTTCCTGCTTTGTGGCCaGGcgtccatacgtgcgcacttaagcagct
*|         |  |       .         |.         |.          .          .          ||||   | | .     || ||.    ||      ||
 1         10                  29         39                                 70     76         85       94    100
```

ORDER THE OLIGOS
(58mer for sense and antisense oligos)

- The first 21 bases in bold are the sequence for your target gene running 5' to 3'
- The second 21 bases should be the complementary sequence to the first 21
- The AS oligo is the anti-sense to the S oligo, but remember, there are overhangs that facilitate cloning so **DON'T** change the ends, just insert your sequence where indicated.

  The orientation is critical so don't change things (unless you are purposely changing the cloning strategy!)

TERMINATION SIGNAL

- The sequence TTT represents a termination signal for Pol3, so DO NOT put more than 3

T's in your stem sequence!

BLAST YOUR SEQUENCES

➢ Blast both the sense and anti-sense sequences to minimize the chance of generating off-target effects. The latest dogma (as of 12/03) suggests that if you have less than 9 contiguous nucleotides in common between you intended target and a related sequence you will not generate off-target effects. Also, if there is identity between two closely related sequences, it is better to be in the 5' region of the anti-sense sequence than the sense strand

SYNTHESIZING OLIGONUCLEOTIDES

➢ Forward oligo:

- 5' CCGG---**21 bp Sense**---CTCGAG---21 bp Antisense---TTTTTG3'

➢ Reverse oligo:

- 5' AATTCAAAAA---**21 bp Sense**---CTCGAG---21 bp Antisense---3'

ANNEAL OLIGOS

➢ Re-suspend oligos in ddH20 (1 μg/mL):

- 5 μL of S oligo
- 5 μL of AS oligo
- 5 μL of 10x NEB buffer 2
- 35 μL ddH20

➢ Incubate 4 min at 95°C

➢ Incubate 10 min at 70°C in a 1000 mL beaker and allow the water in the beaker to cool to RT **(this will take a couple of hours, but it is important that you do this slowly!)**

LIGATE

➢ Ligate into prepared vector (digested with Age1 and EcoR1). I use the Roche rapid ligation kit:

- Mix:
  - 1 μL of the prepared oligo

- 1 μL of prepared vector
- 2 μL 5x DNA dilution buffer
- 6 μl ddH20
- 10 μL 2x ligation buffer
- 1 μL ligase

- Incubate at RT for 2-15 hrs

- Transform bacteria (when using the retroviral vector **BE SURE** to use RecA minus strains or you will get many recombinants. We typically use DH5α, XL1-Blue, or XL10-Gold)

SCREEN FOR INSERTS

I use a couple of different methods depending on my background levels. Note: some of the time the background plate shows more colonies than the plate with the insert.
This doesn't mean the cloning didn't work, it more often means that the concentration of the hairpin was too high and therefore, inhibitory to the ligation reaction (especially if using the Rapid Ligation Kit from Roche). I still screen these and usually get a few positives

1. The most straightforward approach is to digest with MluI and BamHI. If you get an insert you will get a linear band (only BamHI will cut). The parental should be cut twice and give you a band around 750bp and the rest of the vector

2. PCR screening can make it easy to screen through large numbers of clones without the hassle of doing a lot of minipreps:

- Place 100μL of LB + 100 μg/mL AMP in appropriate number of wells in a 96 well plate

- Touch a toothpick to the colony and place into well (we also make a corresponding plate with each colony to expedite subsequent amplification)

- Grow 1-4 hrs

- Set up 50 μL PCR reaction
  - Mix:
    - 5 μL culture mix
    - 5 μL 10x Taq buffer
    - 0.5 μL dNTPs
    - 0.5 μL 5' U6 primer (this the human U6)

- 0.5 μL anti-sense oligo that you used to first make the hairpin (see ORDERING OLIGOS above)

- PCR program:

1 cycle

- 94°C for 5 min
  (**important** because this "blows apart" the bacteria and gives the enzyme access to the DNA)

30 cycles

- 94°C for 30 min
- 52°C for 45 min
- 72°C for 30 min

- Run a 2% gel and be sure to run a positive and negative control
  - Positive control: dilute 100 ng of vector into 100 μL LB-AMP
  - Negative control: LB-AMP alone
- Run 100bp ladder
- Use a dye that **does not** contain bromophenol blue (it will obscure the band that you are looking for)
- We get our best results by running the gel in the absence of ethidium bromide. After the run, we stain with 0.5 μg/mL ethidium bromide for 10 min followed by a 15 min de-stain in running buffer

3. Alternatively, you can digest with Nde1 and Mlu1 and look for the loss of an approximately 230bp band. You will need to run a 2% gel to see this. If your insert goes in you should only see 1 large (7.2 kb) band

4. Another approach is to cut with Nde1 to EcoR1 and run on a 2% gel. Look for a very subtle shift if you have the insert

If you chose to use Lentihair and/or Retrohair the cloning is as follows:

1. Order the oligos:

- Oligo **1-S**:

(21 bases)TTCAAGAGA**(21 bases)**TTTTTG

- Oligo **2-AS**:

AATTCAAAAA**(21 bases)**TCTCTTGAA**(21 bases)**

2. Once hybridized this must be ligated into a prepared vector (remember that you must chew back the Apa1 site which is a 3' overhang)

Sequencing of hairpins in pLKO.1:

1. Use the following primner:

   5'-CAA GGC TGT TAG AGA GAT AAT TGGA-3'

MAKING VIRUS

Day 1

1. Plate 5 x 105 293T cells in 6 cm2 dishes containing 5 mL of media. (This can be scaled up if desired)

Day 2

1. Set up (use polypropylene tubes for this; polystyrene tubes **DO NOT** work!):

   1 μg retroviral DNA encoding gene X

   1 μg packaging plasmid (such as pCLEco, pCLampho, pUMVC, pHR'CVM8.2 deltaR etc.)

   If you are using a 3 plasmid system then:

   for lenti: 1 μg packaging (pHR'8.2deltaR) at a 8:1 ratio with the envelope plasmid (pCMV-VSV-G)

   for MuLV: the packaging plasmid (pUMVC3) at a 8:1 ratio with the envelope plasmid (pCVM-VSV-G)-a total of 1μg

   DME without serum to 94μL total

   6 μL Fugene

Mix and wait 15 to 30 minutes at room temperature

Add to 293T cells without touching the sides of the dish (**DO NOT CHANGE MEDIA**)

If you are using amphotropic virus then move immediately to BL2+ in a secondary container, which has an absorbent material.
(This does not mean a couple of hours; it means **Immediately**!). The rest of this protocol is the same for all viruses---the BL2+ safety practices are in place if you are using amphotropic viruses

Day 3

1. Change the media to whatever media you wish to use when infecting target cells. 293T cells are easily detached so remember not to put the media directly onto to cells, but rather "run" it down the side of the dish. Remember that you will get the highest titer virus when your cells are "happy."

2. Plate out your target cells

Day 4

1. Remove the medium from the 293T cells and use a 0.45 u syringe filter to remove any 293T cells. **DO NOT** use the 0.2 u filter, as it is likely to shear the envelope from your virus making it noninfectious

Note: After filtering, the filter should be removed and placed in the biohazard bag in the hood and the syringe rinsed with bleach and decontaminated for a minimum of 20 min. It is useful to place a plastic beaker with bleach in the hood in advance

2. Add 8 to 10 _g/ml of polybrene (Hexadimethrine bromide) or protamine sulfate to the target cells

3. Carry out infection for 1 to 4 hours. Remove virus and replace with fresh media

Note: If you wish to do a second infection the following day, it is important to put fresh media on the cells and not let the virus remain on the cells overnight. The media contains huge amounts of envelope, both associated and unassociated with viral particles, which will bind all the cell surface receptors required for virus adsorption, resulting in their down-regulation. Hence, if you don't change the media after the initial infection, very few receptors will be available for the next round of infection. In addition, very few cells tolerate the presence of high levels of the VSV-G envelope for extended periods of time (i.e. a lot of your cells may die)

Day 5 +

1. Allow the cells to recover and begin to express the virus-encoded genes. The cells usually require 48 hours for this to occur

2. Add drug if you are scoring for the presence of a vector that carries a drug resistance marker. **Prior** to this step it is advisable that you titrate the drug to be used for selection in order to know precisely how much to add. In addition, it is necessary to bring an extra plate of uninfected cells (often referred to as "canaries") which will function as a positive control in the kill assay. Add drug to both plates. When your canaries are dead, you can remove the drug.

Testing for Horizontal Transfer (When using the 3 or 4 plasmid system you are only required to do this every couple of months on a randomly chosen virus prep. When using the two plasmid system or packaging cell lines you must do this EVERY time you make a new virus stock!!)

1. Once you have cells that have emerged from selection and are growing, you can test for horizontal transfer, i.e. for the inadvertent generation of replication-competent virus, which may have occurred during the course of your experiment. Please note that the vector-infected cells, prepared as described above, must be growing and "happy." You should essentially treat them as if they were 293T cells. When they are 50 to 75% confluent, remove media from these cells, filter it, add polybrene or protamine sulfate, and infect your new target cells

The question is often raised as to which cells to use as target cells to test for horizontal transfer resulting from the inadvertent creation of infectious retroviruses. The best choice is a cell type that can be easily infected by the specific viral vector you are using. Useful cells are those that have a relatively rapid cell cycle. At present (6/01), we have found that C3H10T1/2 cells are especially useful

2. Wait 48 hours and add selection drug to these cells **AND** to a set of canary cells. Both sets of cells should die at the same time. If the canaries die but the cells infected by the viral vector don't do so with identical kinetics, then you have good evidence that horizontal transfer has occurred. In the latter case all cells and media resulting from this experiment should be destroyed by the addition of bleach.

GFP use

Many have asked what to do if they have used green fluorescent protein (GFP) instead of a selectable drug marker in the vector. In this case, you proceed exactly as outlined above. 48 hours after infection, instead of adding drug, you lift the cells and the canaries and re-suspend them in 2% formaldehyde or paraformaldehyde and run FACS to determine if any of the cells are green

♦ You should run at least 10,000 cells to be sure. If you don't know how to run flow, speak with someone who does. Also, both formaldehyde and paraformaldehyde autofluoresce in the green channel, so it is important that you have a set of infected canary cells here as well

Selection (drug concentrations to be used with CH310T1/2 cells):

Neomycin 1 mg/mL

Hygromycin 300 μg/mL

Puromycin 2 μg/mL

Zeocin 1.2 mg/mL

Blastocidin 15 μg/mL

Last modified: 5/4/05