addgene
A better way to share plasmids

Newsletter Techniques

September 2011: How to Create Your Perfect MCS

Does this scenario sound familiar - you desperately need to make retrovirus expressing your new favorite gene and the only restriction sites in your standard vector are also in the coding sequence of your gene? You can remove them by site directed mutagenesis, or settle for a different vector, but why not just improve the multiple cloning site (MCS) of your vector. It is a couple days of work that will pay off for years to come.

The following technique can be used to easily improve the MCS of any vector. Although we will discuss how to add new restriction sites, it is just as easy to add short tags (like HA or FLAG) using this method. For the purposes of this tutorial we will show you how easy it would be to take the very popular retroviral vector pBabe-puro (Addgene plasmid #1764) which has a relatively limited MCS (BamHI - SnaBI - EcoRI - SalI) and expand that with the addition of NdeI, PacI, AscI, and MfeI to drastically improve the MCS - and best of all - for maximal compatibility they all work in NEB buffer 4.
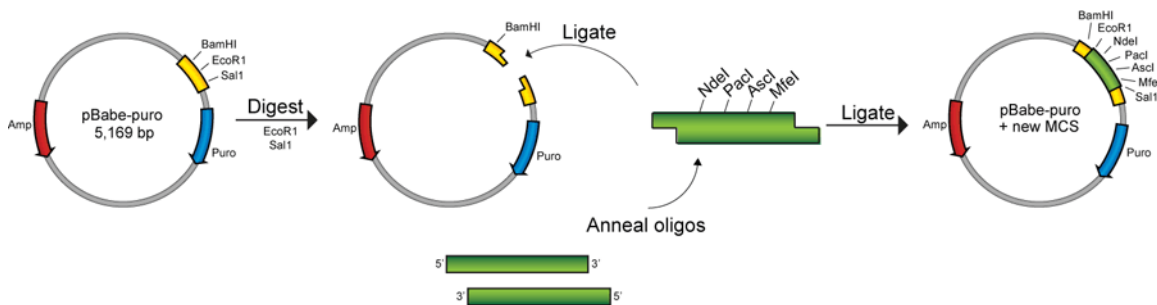
Briefly, we will design overlapping oligos that once annealed can be cloned directly into the overhangs generated by restriction digest of existing sites in the original vector.

Designing oligos: We want to add NdeI, PacI, AscI and MfeI sites so we will be generate a top oligo with each of these sites in tandem (NdeI - CATATG - PacI - TTAATTAA - AscI - GGCGCGCC - MfeI - CAATTG) and a bottom oligo as the reverse compliment so they anneal.

Top: 5'-CATATGTTAATTAAGGCGCGCCCAATTG-3' = 28bp
Bottom: 3'-GTATACAATTAATTCCGCGCGGGTTAAC-5'

We also need to make sure to include the additional bases necessary to compliment the overhangs generated when digesting the vector with EcoRI and SalI (see diagram). To do this we need to add 5'-AATTC and G-3' to the top oligo and 3'-G and CAGCT-5' to the bottom oligo making our final oligos 34bp each.

Top: 5'-AATTCCATATGTTAATTAAGGCGCGCCCAATTGG-3'
Bottom: 3'-GGTATACAATTAATTCCGCGCGGGTTAACCAGCT-5'

Order from your favorite oligo synthesis company the following oligos (same as above):

Top: 5'-AATTCCATATGTTAATTAAGGCGCGCCCAATTGG-3'
Bottom: 5'-TCGACCAATTGGGCGCGCCTTAATTAACATATGG-3'

Digest and purify vector: While waiting for your oligos to arrive, digest 1ug empty pBabe-puro with EcoRI and SalI and gel purify.

Anneal oligos: The oligos should be resuspended in annealing buffer (10mM Tris, pH7.5-8.0, 50mM NaCL, 1m EDTA) and mixed in equimolar concentrations. We recommend mixing 2ug each in a total volume of 50uL - add additional annealing buffer if necessary to get to 50uL. Efficient annealing can be achieved by one of two methods:

1- Place the mixed oligos in a 1.5mL microfuge tube
  - Place tube in 90-95 degree hot block and leave for 3-5 minutes
  - Remove the hot block from the heat source (turn off or move block to bench top) allowing for slow cooling to room temperature (~45 minutes).

2 - Place mixed oligos in a PCR tube, and place tube in a thermocycler programmed to start at 95 degrees for 2 minutes and then to gradually cool to 25 degrees over 45 minutes.

Ligation:
-Dilute 5uL of annealed oligos with 45uL nuclease-free water and quantify (should be about 8ng/ul).
-Mix the annealed oligos with cut vector in ratios between 4:3 and 6:1 (ie 100ng vector with between 75-600ng annealed oligos) in a standard ligation reaction.
-Transform 2-3uL into your favorite bacteria and plate.
-Be sure to pick multiple colonies for mini-prepping and verify insert by sequencing.

And don't forget, if you generate a useful plasmid; share it with the rest of the scientific community by depositing it at Addgene.